University of Southampton

Dietary fatty acids affect inflammatory mediator production by murine and human macrophages and lymphocytes

Fiona Anne Wallace

B.Sc. *Hons* (Nutritional Science)
M.Med.Sci (Human Nutrition)

Doctor of Philosophy

Institute of Human Nutrition

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE
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DIETARY FATTY ACIDS AFFECT INFLAMMATORY MEDIATOR PRODUCTION BY MURINE AND HUMAN MACROPHAGES AND LYMPHOCYTES

Fiona Anne Wallace

Numerous studies over the past 30 years have shown that the type of fat eaten in the diet can have a profound effect on human health and disease. In particular, fatty acids of the n-3 family have been shown to be incorporated into cell membranes, to inhibit platelet aggregation and to decrease the concentration of triglycerides in the bloodstream. The effects of n-3 fatty acids on other systems of the body remain unclear. Some studies have shown that dietary n-3 fatty acids can affect the production of inflammatory mediators such as eicosanoids, cytokines and nitric oxide by macrophages and lymphocytes. This in turn could affect the function of a variety of cells in the immune system. Studies investigating these effects using rodent and human immune cells have often given conflicting results, which may in part be due to methodological differences. The purpose of the experiments presented in this thesis is to clarify the effects of dietary n-3 fatty acids on the production of inflammatory mediators by cells of the murine and human immune systems. The mouse studies involved feeding diets containing different types of fatty acids for 8-12 weeks and investigated lymphocyte proliferation in response to mitogenic stimulation, the ability of macrophages to kill target cell lines and the production of inflammatory mediators, including cytokines, by stimulated macrophages. These studies found that the type of fatty acid eaten in the diet affects the fatty acid composition of mouse macrophages and lymphocytes, and this in turn had an effect on the ability of these cells to produce some inflammatory mediators, including prostaglandin E_2, leukotriene B_4, tumour necrosis factor-α, interleukin-1β, and interleukin-6. The exact effects of dietary fatty acid manipulation on inflammatory mediator production by macrophages were dependent on the activation state of the macrophage under investigation. In addition, dietary fatty acid manipulation had an effect on the ability of macrophages to kill target tumour cells. Dietary fatty acid manipulation altered the fatty acid composition of mouse lymphocytes and affected the ability of lymphocytes to produce the Th-1 cytokines interleukin-2 and interferon-γ. The ability to produce the Th-2 cytokine interleukin-4 was not affected. Proliferation of mouse lymphocytes in response to mitogens was decreased following unsaturated fatty acid feeding. The studies in humans involved supplementing the diet of healthy men aged 18-40 years for three months with increasing doses of n-3 fatty acid-rich fish oil (3, 6, or 9 g/fish oil per day), linseed oil, or a placebo oil containing a mix of fatty acids found in the typical UK diet. Supplementation of the diet with fish oil caused significant increases in the proportions of n-3 fatty acids present in plasma phospholipids. There were also some changes in the proportions of n-3 fatty acids present in circulating mononuclear cells, although the changes were not as marked as those seen in the plasma. Supplementation of the diet with 6 and 9 g fish oil per day for three months caused a significant decrease in the ability of mononuclear cells to produce interleukin-6 and prostaglandin-E_2 upon stimulation. In conclusion, supplementation of the rodent and human diet with n-3 fatty acids can affect the proportions of fatty acids present in immune cells and the ability of these cells to produce some inflammatory mediators. The precise effects however depend on the dose of n-3 fatty acid given and the activity of the immune cells under investigation. These results help to clarify some of the contradictions in the literature, and the research presented in this thesis provides information crucial to underpin the use of fatty acids as a nutritional means of modulating the immune response.

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List of Abbreviations

AA	arachidonic acid; 20:4n-6
ALNA	α-linolenic acid; 18:3n-3
AP	autologous plasma
AP-1	activator protein-1
ATP	adenosine triphosphate
B220	CD45R, B-cell marker
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CD3	cluster of differentiation molecule 3
CD4	cluster of differentiation molecule 4
CD8	cluster of differentiation molecule 8
CO	coconut oil
COMA	committee on medical aspects
Con-A	concanavalin A
COX	cyclooxygenase
DAG	diacylglycerol
DHA	docosahexaenoic acid; 22:6n-3
DNA	deoxyribonucleic acid
DPA	docosapentanoic acid; 22:5n-3
DGLA	dihomo-gamma-linolenic acid; 20:3n-6
ELISA	enzyme linked immunosorbent assay
EPA	eicosapentaenoic acid; 20:5n-3
FATP4	fatty acid transport protein 4
FO	fish oil
^3H	tritium
HDL	high density lipoprotein
HETE	hydroxyeicosatetranoic acid
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal
H-PETE	hydroperoxyeicosatetraenoic acid
IDL	intermediate density lipoprotein
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IFN-γ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LF	low fat
LOX	lipoxygenase
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
LTB_5	leukotriene B_5
LTC_4	leukotriene C_4

LX	lipoxin
MAFF	ministry of agriculture, fisheries and food
MAC-1	CD11b macrophage marker
MHC	major histocompatability complex
Mϕ	macrophage
mRNA	messenger RNA
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acid
NF	nuclear factor
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor κ B
NK	natural killer cell
•NO	nitric oxide
OO	olive oil
p	probability value
PA	phosphatidic acid
PAF	platelet activating factor
PBMC	peripheral blood mononuclear cell
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PI	phosphatidyl inositol
PGE_2	prostaglandin E_2
PGE_3	prostaglandin E_3
PGI_2	prostacyclin I_2
PGJ_2	prostaglandin J_2
PGE_2	prostaglandin E_2
PHA	phytohaemogglutinin
PIP_2	inositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipid
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PPAR	peroxisome proliferator activated receptor
PS	phosphatidyl serine
PUFA	polyunsaturated fatty acid
r	regression coefficient
RPMI	rosewell park memorial institute
Sd	standard deviation of the mean
Se	standard error of the mean
SH2	src homology 2 domain
SI	stimulation index
SNP	sodium nitroprusside
SO	safflower oil
SOCS-3	suppressor of cytokine signalling-3.
STAT	signal transducer and activator of transcription
TAG	triacylglycerol
TCR	T cell receptor

TNF-α	tumor necrosis factor alpha
TNFR	TNF receptor
TX	thromboxane
TXA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule- 1
VLDL	very low density lipoprotein

Chapter 1: Introduction

"A human being is primarily a bag for putting food into; the other functions and faculties may be more godlike, but in point of time they come afterwards. A man dies and is buried, ...but the food he has eaten lives after him in the sound or rotten bones of his children. I think it could plausibly be argued that changes in diet are more important than changes of dynasty or even religion .... Yet it is curious how seldom the all-importance of food is recognized."

George Orwell. The Road to Wigan Pier (1937)

Or.... on the other hand.....

"Its all pie and mash init?" Burger King Employee (2000) Waterloo Station Burger King, London, UK

1.1 Dietary Fatty Acids

We are what we eat. On crude analysis, all living organisms are primarily composed of protein, carbohydrate, water and fat, nutrients that we obtain through our meals. Our growth and development from a fertilized egg in *utero* is in part dependent on the nutrients supplied through our mother's diet. We continue to grow throughout adulthood in response to the nutrients we consume. Nutrition is essential for life and it has been recognized from antiquity that food and individual food components can have a number of beneficial, and also detrimental, effects on the body. Individual dietary components can (and do) modulate the activities of a number of biological systems. The studies reported in this thesis investigate the effects of one macronutrient, dietary fat, on one biological system, the immune system. In particular, the effects of fatty acids of the n-3 family on the production of inflammatory mediators produced by, and affecting, the cells of the immune system are examined in detail. These studies illustrate how a dietary component can profoundly influence the function of a system crucial for health.

1.1.1 Dietary Fat

Man derives energy from each of the major macronutrients, protein, fat and carbohydrate. Of these, fat has the highest energy content with 37.7 kJ/g as opposed to 16.7 kJ/g for protein and carbohydrate (McNeill, 1993). The typical UK diet derives around 40% of calories from fat, with between 100-200 g fat, mainly in the form of triacylglycerols (TAGs - three fatty acids esterified to a glycerol backbone), consumed per day (OECD, 1991). While fatty acids are an important dietary component and are essential to life, most experts agree that the current level of fat intake is too high and should be decreased to approximately 30-35% of calories (British Nutrition Foundation, 1992; COMA, 1994). Fats found in foods consumed by man include TAGs, phospholipids (PLs), glycolipids, sterols and sterol derivatives, short and long chain fatty acids and their derivatives (Calder, 1996a). The most abundant fatty acids have straight chains of an even number of carbon atoms; the chain lengths vary from 4 carbon atoms in milk to over 30 in some fish oils (British Nutrition Foundation, 1992). Fatty acids eaten in the diet can be used within the body in many ways: as an energy source; as a storage form of energy; as structural components of cell membranes; as precursors to active cellular mediators such as eicosanoids. Fats also aid in the absorption of fat soluble vitamins such as vitamin A (and its precursors), and the vitamins E, K and D. Fat also imparts flavour (Brauss, 1998) and provides texture to foods, making it more palatable. Animal fat, which is usually high in saturated fatty acids, makes up between 60-80% of daily fat intake (COMA, 1994). The intake of various types of fatty acids in the typical UK diet is shown in table 1.1. Over-consumption of saturated fatty acids leads to elevated levels of TAGs and cholesterol in the bloodstream and has been linked to the development of numerous disorders including coronary heart disease (COMA, 1994), obesity (COMA, 1994), type II diabetes (Berry, 1997) and some cancers (Willett, 1990). In contrast, the consumption of polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) from vegetable and fish oils may lower blood lipid levels and may afford protection against these disorders (British Nutrition Foundation, 1992). In particular, fish oil consumption has been shown to lower TAG concentrations and to afford protection against a number of diseases common in Western societies (Nettleton, 1995).

Table 1.1 Intake of fatty acids in the UK population.

FAT	*Intake (g/day)*	*% Daily Energy*
Total fat	94.8	40
Saturated fatty acids	42.0	16
MUFA	31.4	15
PUFA	15.8	7
n-6 PUFA	13.8	5.1
n-3 PUFA	1.9	0.7

Adapted from Gregory *et al.* (1990) and British Nutrition Foundation (1999).

1.1.2 Fatty Acid Nomenclature

Fatty acids are composed of the elements carbon, hydrogen and oxygen arranged in long hydrocarbon chains with a methyl group at one end and a carboxylic acid group at the other end. The fatty acids which are eaten in the diet are either saturated or unsaturated, and the latter may be of the n-3, n-6, n-7 or n-9 families (see figure 1). The nomenclature n-x relates to the position of the first double bond in the fatty acid chain counted from the methyl end. The first double bond counted from the methyl group of the polyunsaturated fatty acid chain may be located in the third position, as for n-3 fatty acids, in the sixth position as for the n-6 fatty acids, in the seventh position of the n-7 family, or in the ninth position for the n-9 family. A list of the most common fatty acids is presented in table 1.2.

Figure 1.1 Some fatty acids occurring in food and body lipids. The zig-zag lines represent hydrocarbon chains with a carbon atom at the intersection of lines. Adapted from Gurr (1992)

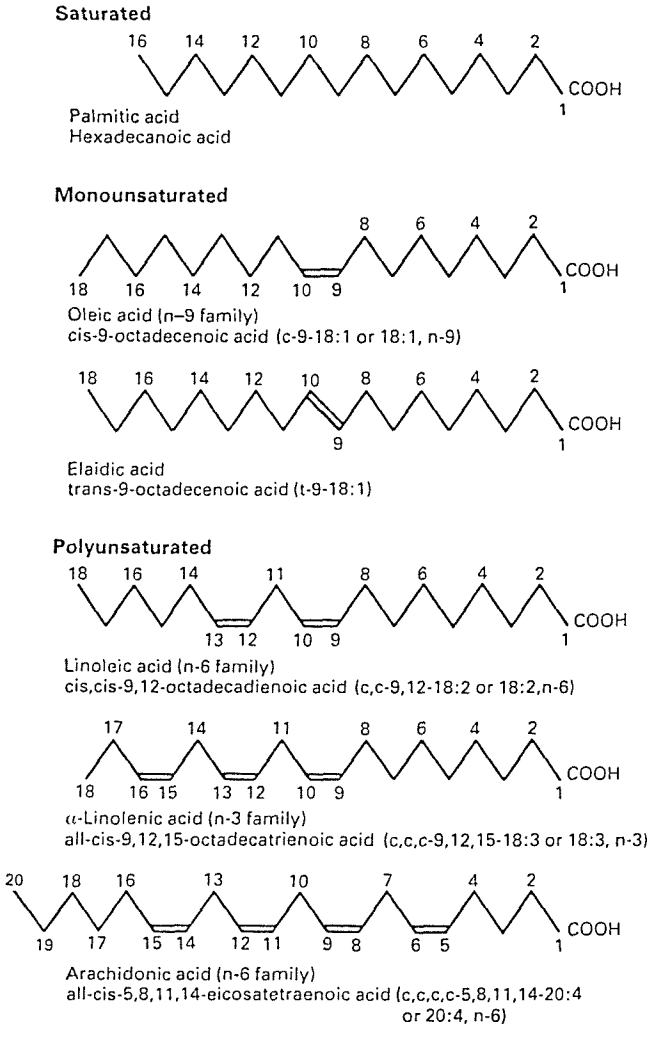


Table 1.2 The most commonly found fatty acids and their nomenclature. All double bonds are in the cis configuration unless otherwise stated.

Systemic name	*Common name*	*Shorthand*	*Family*	*Comments*
Octanoic	Caprylic	8:0	Medium chain	Rapid source of calories (energy)
Decanoic	Capric	10:0		Rapid source of calories (energy)
Dodecanoic	Lauric	12:0	Saturated	Hyperlipidemic; hypercholeseterolemic; prothrombic
Tetradecanoic	Myristic	14:0		Hyperlipidemic; hypercholeseterolemic; prothrombic
Hexadecanoic	Palmitic	16:0		Hyperlipidemic; hypercholeseterolemic; prothrombic
Octadecanoic	Stearic	18:0		Neutral or hypolipidemic; precursor of oleic acid
9-Hexadecanoic	Palmitoleic	16:1n-7	Monounsaturated	
9-Octadecanoic	Oleic	18:1n-9		Hypolipidemic; precursor of 20:3n-9 in essential fatty acid deficiency
Trans 9-Octadecanoic	Elaidic	18:1n-9		Analagous to 18:0
11-Eicosaenoic	Gadoleic	20:1n-9		
13-Docasaenoic	Erucic	22:1n-9		Impaired fatty acid oxidation in hearts of rats
15-Tetracosaenoic	Nervonic	24:1n-9		
9,12-Octadecanoic	Linoleic (LA)	18:2n-6	n-6 polyunsaturated	Essential fatty acid (intake should exceed 2-3g/day); precursor of arachidonic acid; hypolipidemic compared to saturated fatty acids.
9,12,15-Octadecatroenoic	α-linolenic (ALA)	18:3n-3	n-3 polyunsaturated	Hypolipidemic; membrane fluidity; precursor of EPA and DHA; decreases eicosanoid synthesis.
6,9,12-Octadecatrienoic	γ-linolenic (GLA)	18:3n-6	n-6 polyunsaturated	Precursor to AA and DGLA
11,14,17-Eicosatrienoic	Mead	20:3n-9	n-9 polyunsaturated	Elevated in essential fatty acid deficiency
8,11,14,17-Eicosatetraenoic	Dihomo-γ-linolenic (DGLA)	20:3n-6	n-6 polyunsaturated	Precursor of the PGE_1 series of eicosanoids
5,8,11,14-Eicosatetraenoic	Arachidonic (AA)	20:4n-6	n-6 polyunsaturated	Precursor of eicosanoids (2-series Prostagandins, 4-series Leukotrienes)
5,8,11,14,17-Eicosapentaenoic	Eicosapentaenoic (EPA)	20:5n-3	n-3 polyunsaturated	Hypolipidemic; precursor of eicosanoids (3-series prostaglandins, 5-series leukotrienes;) decreases eicosanoid synthesis in some cells
4,7,10,13,16-Docosapentaenoic	Docosapentaenoic (DPA)	22:5n-3	n-3 polyunsaturated	Precursor of DHA
4,7,10,13,16,19-Docosahexaenoic	Docosahexaenoic (DHA)	22:6n-3	n-3 polyunsaturated	Hypolipidemic; essential for vision and neural membranes; decreases AA synthesis; decreases eicosanoid synthesis in some cells

1.1.3 n-3 Fatty Acids in the Diet

Certain fatty acids, particularly those of the saturated, n-9 and n-7 families can be made *de novo* from protein and carbohydrate (British Nutrition Foundation, 1999). The pathway of *de novo* fatty acid synthesis in the mammalian body terminates with the production of palmitic acid (16:0) (Stryer, 1995). By a separate elongation mechanism, palmitic acid can then be elongated to stearic acid (18:0) (Stryer, 1995). This in turn can be desaturated to oleic acid (18:1n-9), the main component of olive oil. Mammals cannot synthesize fatty acids of the n-6 or n-3 families however, because cells derived from species located high up in the evolutionary hierarchy cannot introduce double bonds in to the carbon chain of fatty acids closer to the methyl group than carbon atom number 7 or 9 (British Nutrition Foundation, 1999). Therefore n-6 and n-3 fatty acids must be obtained from the diet. Chloroplasts in phytoplankton and plants can synthesise the essential n-3 and n-6 fatty acids and convert them into each other, and for this reason, n-3 and n-6 essential fatty acids are best obtained either from foods of vegetable or marine origin (see figure 1.2). In general, the content of the most common very long chain n-3 fatty acids is highest in the marine animals containing the highest amount of fat, such as seals and whales (Bang & Dyerberg, 1972). Arachidonic acid is found in the highest concentration in foods from animal sources (British Nutrition Foundation, 1992). Estimates of arachidonic acid intake by humans are few but it appears that habitual intake in western populations is between 50 and 300 mg/day (Mann *et al.*, 1995). The habitual intake of EPA and DHA in the UK population is approximately 250 mg/day (British Nutrition Foundation, 1999). Table 1.1.3 gives the n-3 fatty acid content of some commonly eaten foods.

Figure 1.2 The dietary sources of n-3 fatty acids according to the food chain in water and on land. Note that the only rich source of the very polyunsaturated n-3 fatty acids is from the phytoplankton and the sea life that directly or indirectly feeds on phytoplankton.

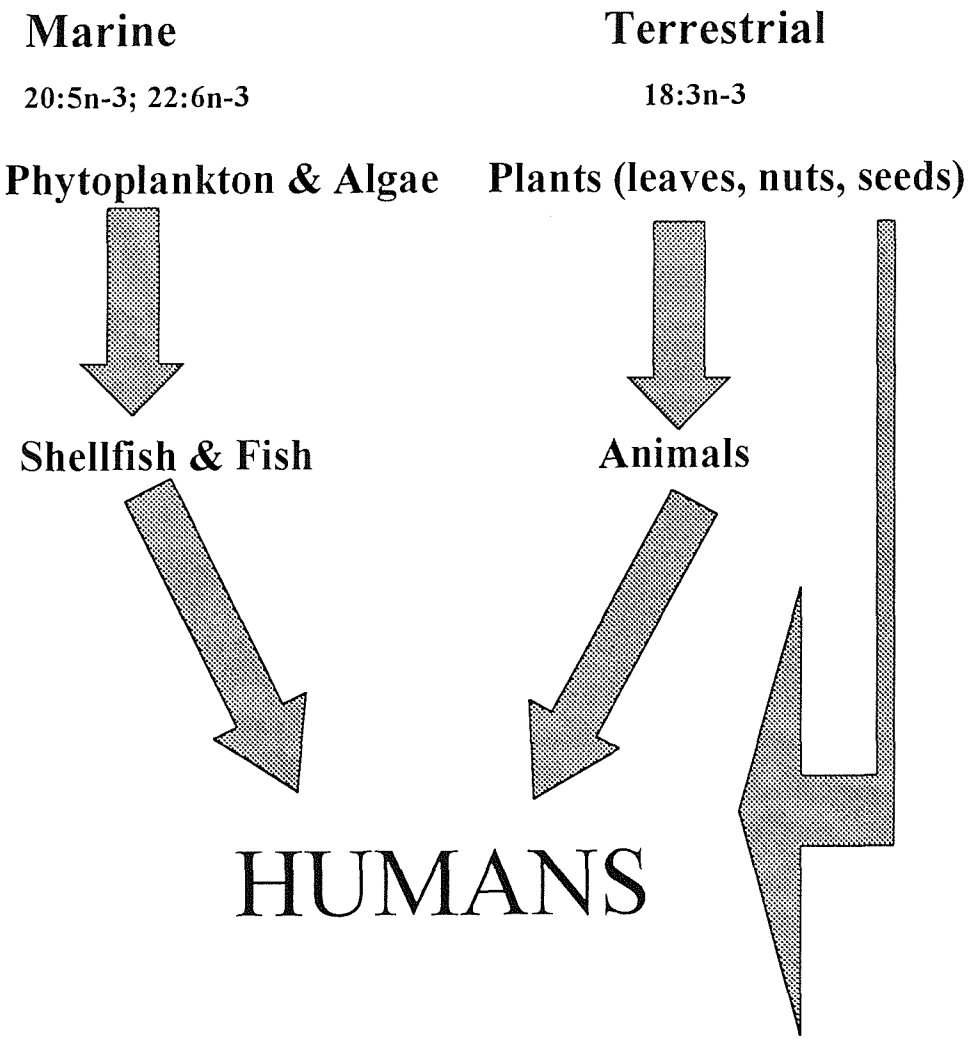


Table 1.3. The n-3 fatty acid content of some commonly eaten foods. Fatty acids shown are g/100g total fatty acids.

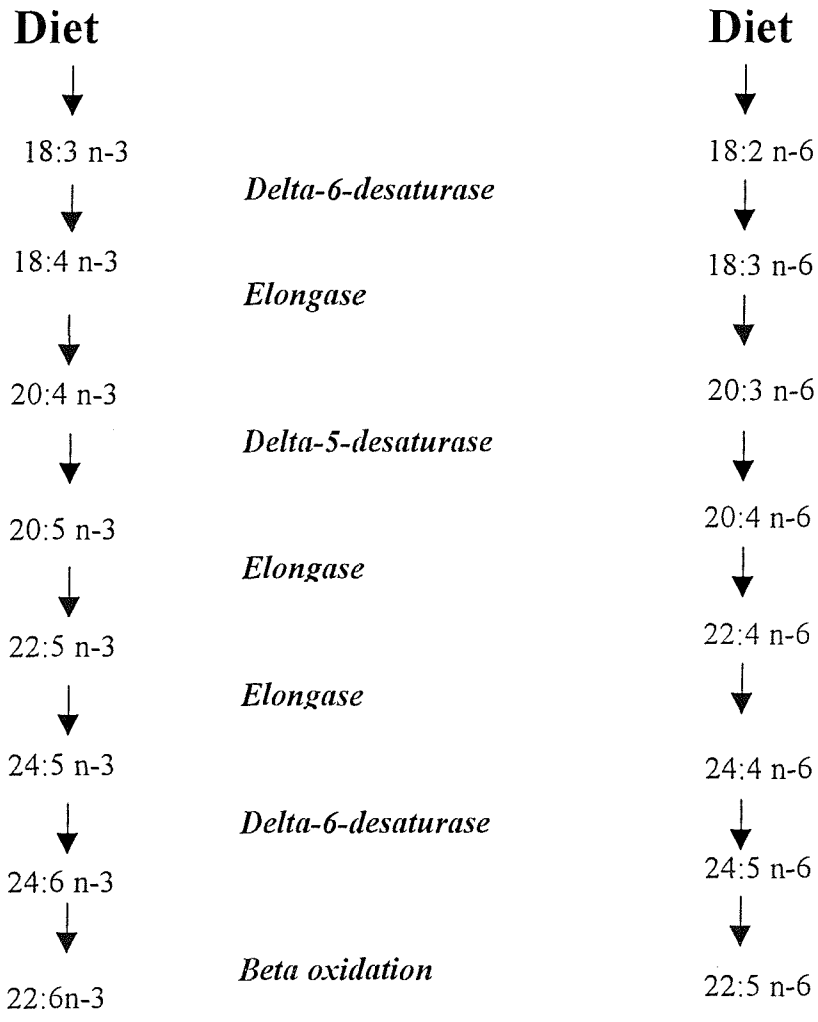
Food	*ALNA*	*EPA*	*DHA*	*EPA + DHA*
Fats and oils				
Perilla oil	63.6	0	0	0
Linseed oil	53.3	0	0	0
Canola oil	11.1	0	0	0
Wheat germ oil	6.9	0	0	0
Soybean oil	6.8	0	0	0
Margarine, hard, soybean hydrogenated	3.0	0	0	0
Lamb fat	2.3	0	0	0
Butter	1.2	0	0	0
Chicken fat	0.02	0.01	0.03	0.04
Beef fat	0.6	0	0	0
Nuts and seeds				
Walnuts, English	6.8	0	0	0
Walnut, black	3.3	0	0	0
Hickory nuts	1.0	0	0	0
Vegetables				
Soybeans, green, raw	3.2	0	0	0
Radish seeds, sprouted	0.7	0	0	0
Beans, navy, pinto, cooked	0.3	0	0	0
Fish and shellfish				
Atlantic mackerel	0.1	0.9	1.6	2.5
Atlantic salmon, farmed	0.1	0.6	1.2	1.8
Pacific herring	0.3	0.9	1.0	1.9
Atlantic herring	0.1	0.7	0.9	1.6
Lake trout	0.4	0.5	1.1	1.6
Bluefin tuna	0	0.4	1.2	1.6
Atlantic sturgeon	Trace	1.0	0.5	1.5
Anchovy, European	0	0.5	0.9	1.4
Lake whitefish	0.2	0.3	1.0	1.3
Sprat	0	0.5	0.8	1.3
Coho salmon farmed	0.1	0.4	0.8	1.2
Sockeye salmon	0.1	0.5	0.7	1.2
Sardines, canned, drained	0.5	0.4	0.6	1.1
Pink salmon	Trace	0.4	0.6	1.0

Adapted from Nettleton (1995)

1.1.4 Elongation of Dietary Fatty Acids

Mammalian cells have the capacity to desaturate and elongate both the n-6 linoleic acid and n-3 α-linolenic acid (ALNA) to other long chain fatty acids that are subsequently either incorporated into membrane phospholipids or are metabolised to eicosanoids (Mohrhauer *et al.*, 1967; Holman, 1998). Each of the fatty acid families share the same elongation and desaturation enzymes (Brenner & Peluffo, 1969; Figure 1.3). Δ6 desaturase is regarded as the rate limiting step of the desaturation pathway (Bernert & Sprecher, 1975; Sprecher *et al.*, 1999). The Δ6 desaturase enzyme has a preference for the n-3 PUFA (Km for alpha-linolenic acid in rats is between 29 and 33 μM: Hrelia *et al.*, 1990) over the n-6 PUFA (Km for linoleic acid in rats is between 43 and 92 μM; Hrelia *et al.*, 1990). Since all fatty acid families compete for the same Δ6 desaturase enzyme, it is possible for a particular fatty acid to influence the production of fatty acids from other families (Mohrhauer & Holman, 1963; Whelan, 1996). The diet of man however usually contains a much higher proportion of n-6 fatty acids such as LA (18:2n-6) than n-3 fatty acids such as ALNA (18:3 n-3) (see table 1.1), so that it is the metabolism of the former which predominates. This may explain the relatively high levels of arachidonic acid found in most human cell membranes. Arachidonic acid is an important precursor of some of the prostaglandins involved in regulating many biological processes. A decrease in the n-6 PUFA level in the diet favours the desaturation of alpha-linolenic acid (Whelan, 1996). This results in the incorporation of eicosapentaenoic acid rather than arachidonic acid into the neutral and phospholipids of tissues.

Figure 1.3 Metabolic pathways for the desaturation and elongation of n-3 and n-6 fatty acids.



1.1.5 Digestion and Absorption of Fatty Acids

1.1.5.1 Digestion

Fatty acids are consumed in the diet mainly as components of TAGs. TAG is the most concentrated form of energy available to biological tissues (Gibbons *et al.*, 2000). Figure 1.4 outlines the processes involved in the digestion of TAGs and absorption of fatty acids. The TAGs which form the bulk of the fats in the diet must be broken down into partial glycerides and fatty acids by a pancreatic lipase in the small intestine before they can be absorbed (Kinsella, 1990; Gurr, 1993). In most adults, the processes of TAG digestion and absorption are very efficient and over 95 percent of the 100 g or so of dietary fat consumed each day in the British diet is absorbed (Gurr, 1993). Dietary TAGs are partially hydrolysed by lingual lipase, and subsequently the formation of a fat emulsion is aided by the mechanical action of the stomach (Lai & Ney, 1998). This emulsion then enters the small intestine and is modified by mixing with bile salts produced in the liver, which further emulsifies the mixture, and with pancreatic enzymes such as pancreatic lipase. Digestion takes place in the duodenum, and is catalysed by pancreatic lipase. The main products of TAG digestion are 2 monoacylglycerols and non-esterified fatty acids. Phospholipid digestion yields lysophospholipid and fatty acids released from the 2 position of the phospholipids by pancreatic phospholipases (Gurr, 1993). Cholesterol esters in the dietary fat must be hydrolysed by a pancreatic cholesterol esterase before absorption can begin (Gurr, 1993; Natarajan, 1999; Saltiel, 2000). Figure 1. 4 outlines digestion and absorption of fatty acids.

1.1.5.2 Absorption and Re-esterification

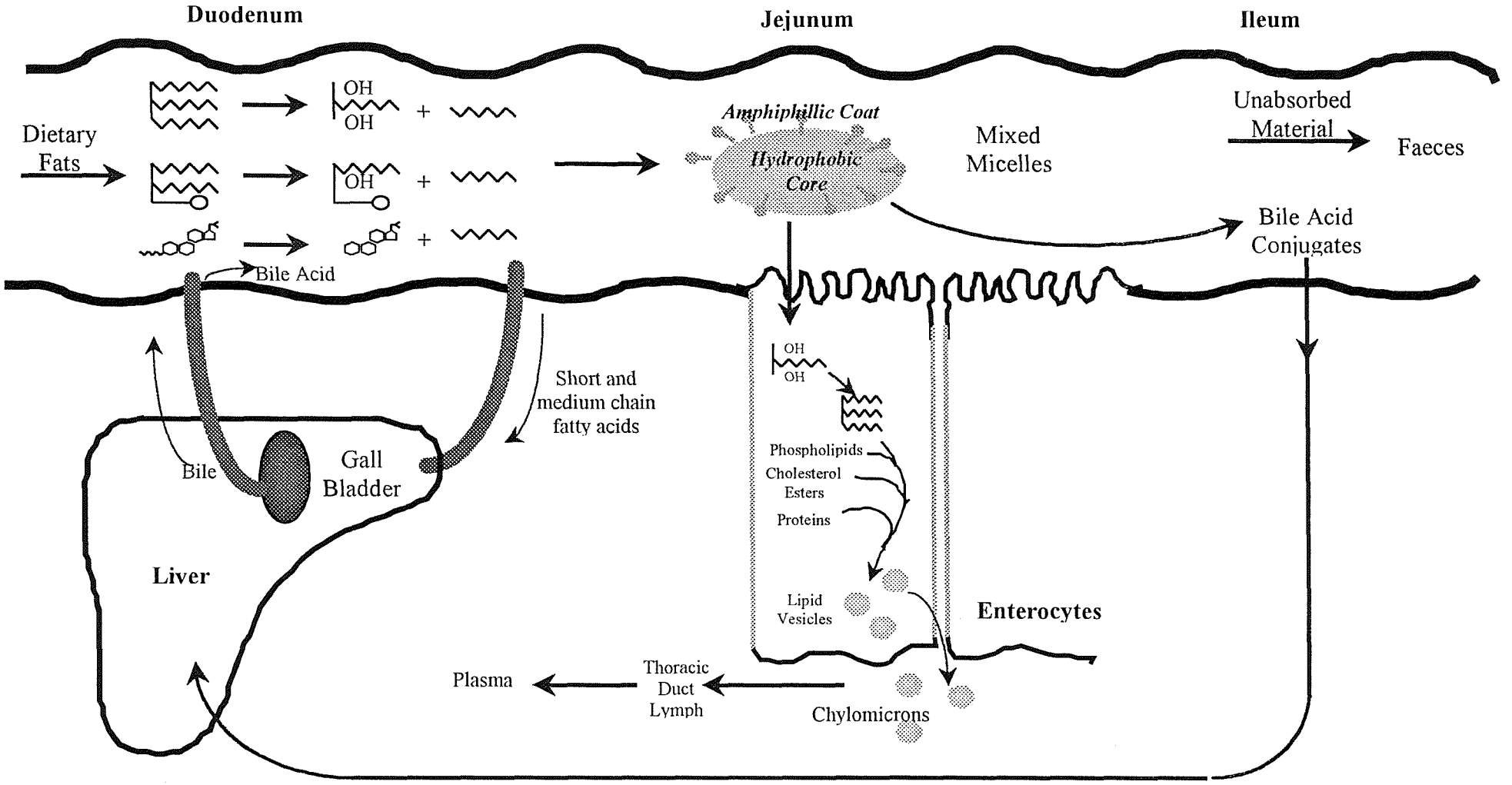
Lipid absorption occurs mainly in the jejunum, where the absorbed fatty acids can be re-esterified into TAGs and secreted into chylomicrons or utilized for oxidation and phospholipid synthesis (Holehouse *et al.*, 1998). Some digestion products pass from the mixed micelles into the enterocyte membrane by passive diffusion (Carey *et al.*, 1983). A diffusion gradient is maintained by the presence of fatty acid binding proteins that rapidly bind the fatty acids entering the cell. It has recently been determined that fatty acid transport protein 4 (FATP4) is the principal fatty acid transporter in enterocytes (Stahl *et al.*, 1999). Intestinal fatty acid binding proteins have been suggested to have many functions in the intestinal epithelium. These functions include

enhancing the uptake and intracellular transport of fatty acids, targeting individual fatty acids to specific metabolic pathways, and to act as a cofactor for enzymes using fatty acid substrates (Holehouse *et al.*, 1998). The diffusion gradient is also maintained by the rapid re-esterification of fatty acids to the monoacylglycerols, which along with free fatty acids, are one of the main digestion products crossing the intestinal mucosa, into TAGs. About 4 g/day of short chain fatty acids are absorbed, mainly from dairy products (Gurr, 1993); these pass directly into the hepatic portal vein and are metabolized by the liver.

1.1.5.3 Re-esterification Inside the Enterocyte

The first step in re-esterification is the activation of fatty acids to their acyl-CoA thioesters. The preferred substrates are the long-chain n-3 fatty acids (British Nutrition Foundation, 1992). In man and other animals, esterification takes place mainly on the 2-monoacylglycerols, which together with the free fatty acids are the major forms of absorbed lipids. Re-synthesis of TAGs therefore, occurs mainly through the so-called monoacylglycerol pathway (Gurr, 1993). TAGs resynthesised in the enterocytes are esterified with fatty acids having chain lengths greater than 12 carbon atoms (Elliot *et al.*, 1999). Short (C4-C6) and medium (C8-C10) chain fatty acids are absorbed directly into the portal blood and carried to the liver where they are rapidly oxidized (Figure 1.4). They do not, therefore, contribute to plasma lipids, nor are they deposited in adipose tissue in significant quantities. Phospholipids (mostly phosphatidylcholine) eaten in the diet and taken up into the enterocyte as lysophosphatidyl choline (lyso-PC) are also re-esterified. A fatty acid is esterified into position 2 to form phosphatidylcholine by an acyl transferase located in the vilus tip of the intestinal brush border (British Nutrition Foundation, 1992). The absorption of cholesterol is slower and less complete than that of the other lipids, about half of the absorbed sterol being lost during desquamation of cells. Most of the cholesterol that is absorbed is esterified either by reversal of cholesterol esterase or via acyl-coA:cholesterol acyltransferase. The latter enzyme is induced by high concentrations of cholesterol in the digesta (British Nutrition Foundation, 1992).

Phospholipids and some apolipoproteins which provide the coat which stabilizes the chylomicron are synthesized in the rough endoplasmic reticulum in the enterocyte (Gurr, 1993). These gradually increase in size and are pinched off from the endoplasmic reticulum to form lipid vesicles and fuse with Golgi apparatus. The Golgi apparatus also provides carbohydrate moieties for the apolipoproteins and acts as a vehicle for the transport of the fully formed chylomicrons to the lateral surface of the enterocyte. The final phase of export from the cells involves fusion with the membrane and secretion into the extracellular space by a process known as exocytosis.



1.4 Fat digestion and absorption As digestion progresses, the lipolytic products form into mixed micelles which are large molecular aggregates consisting of: monoacylglycerols, fatty acids longer than 12 carbon units, bile salts and phospholipids. The mixed micelles are able to draw into the hydrophobic core the less water-soluble molecules such as cholesterol, the carotenoids, tocopherols and some undigested TAGs.

1.1.5.4 Lipoproteins

Lipoproteins are particles with a highly hydrophobic core and a relatively hydrophilic outer surface. A typical lipoprotein particle consists of a core of TAG and cholesterol ester, with an outer surface layer of phospholipid and free cholesterol (Gurr, 1993). An overview of lipoprotein metabolism is given in figure 1.5. Each particle has associated with it one or more proteins, the apolipoproteins, which have hydrophobic domains, which extend into the core and anchor the protein to the particle, and hydrophilic domains which are exposed at the surface.

Chylomicrons are the major carriers of fat from the diet, whereas very low-density lipoproteins (VLDLs) are involved in transporting endogenously synthesised TAGs from the liver (Segrest & Albers, 1996; Kris-Etherton & Yu, 1997; White *et al.*, 1998). After export from the liver, VLDLs are hydrolysed and this subsequently generates a class of lipoprotein called LDL. LDL is the major carrier of cholesterol, in human beings (about 70 % of the cholesterol in human plasma is carried on LDL) (Gurr, 1993; Harris, 1996). LDL particles are 18-22 nm in diameter and comprise 20% protein and 80% lipids of which cholesterol, mostly in the form of cholesterol ester, contributes 50-80% mostly in the form of cholesterol ester (Gurr, 1993). The transfer of cholesterol from the bloodstream can occur by passive endocytosis or by a specific receptor-mediated uptake process (Brown *et al.*, 1981) in which the receptor recognises the apoB component of the LDL and binds to it. The LDL-receptor complex is taken into the cell and the LDL degraded by lysosomal enzymes (Frayn, 1996). The resulting free cholesterol interacts with the endoplasmic reticulum membranes in which are located the enzymes of cholesterol biosynthesis, and inhibits hydroxymethylglutaryl-CoA (HMGCoA) reductase, the rate limiting enzyme in this sequence (Gurr, 1993). In this way, endogenous cholesterol biosynthesis is regulated by the amount available from the diet.

The remaining class of lipoproteins is the high density lipoproteins (HDL). Their role is to carry cholesterol from peripheral cells to the liver, where it is degraded or repackaged, a process known as 'reverse cholesterol transport'. Protein (mainly apo A-

I and II, and some C, D and E) constitutes 50% of the mass of HDL while approximately 22% is phospholipid, 20% cholesterol ester and only 8% TAG (Frayn, 1996; Bruce *et al.*, 1998).

The concentration and proportions of lipoproteins in the plasma, especially the chylomicrons and VLDL, respond to the influx of digestion products from lipids and carbohydrates after the consumption of a meal. The average long term concentrations of VLDL, LDL and HDL may also to some extent be determined by habitual intakes of fats and carbohydrates (Kris Etherton & Yu, 1997). The mechanisms by which these changes occur are not entirely clear, but may result from reductions in the rate at which the apoB receptors in the liver remove LDL. This may be brought about by some saturated fatty acids and this effect may be reversed by some unsaturated fatty acids (Gurr, 1993; British Nutrition Foundation, 1999).

1.1.5.5 Chylomicrons

Chylomicrons are a form of lipoprotein (see figure 1.5). They are large spherical particles 75-600 nm in diameter, which are secreted into lymphatic vessels and pass via the thoracic duct into the jugular vein. In the bloodstream the chylomicrons acquire additional proteins called apo C and E which allow them to be recognised and taken up by a receptor on target tissues (Karpe & Hamsten, 1995; Frayn, 1996). The first tissues encountered by the chylomicrons once they enter the bloodstream are the lungs. The chylomicrons pass through the lungs and the ventricles of the heart with little modification, and then rapidly enter the capillaries of the muscles, mammary glands and adipose tissues, where they interact with the enzyme lipoprotein lipase (LPL). This enzyme catalyses the hydrolytic breakdown of TAGs to release the fatty acids, which are then taken up into cells of the target tissue. After a meal an elevated insulin concentration directs most of the chylomicron breakdown to adipose tissue by activating the adipose tissue LPL and inactivating skeletal muscle LPL (Frayn, 1996). The hormonal balance during a fast activates muscle LPL. About half of the chylomicron TAGs are hydrolysed in 2-3 minutes (Frayn, 1996), but the particles are not completely degraded. Remnant particles containing proportionately less TAG and more cholesterol are poor substrates for LPL and are taken up by the liver, where the cholesterol is used for membranes, new lipoprotein biosynthesis, or converted into bile

acids (Gurr, 1993). If the diet contains an appreciable amount of fat, endogenous fatty acid and TAG biosynthesis are suppressed, although, since the process of lipid turnover occurs continually in all tissues, a low level of biosynthetic activity is always present (Gurr, 1993). During chylomicron assembly after absorption, the preference by phospholipid biosynthetic enzymes for 20:4n-6 (AA) leads to its incorporation into the phospholipid surface rather than into the triglyceride core. This AA remains in the chylomicron remnants and eventually is taken up by the liver. From the hepatocyte, AA is either further metabolized, incorporated into membranes, re-incorporated into lipoproteins for excretion or oxidized for energy (German *et al.*, 1996). Parts of the chylomicrons are used to make HDL which, with the help of LCAT, remove excess cholesterol from membranes and other lipoprotein particles, converting it into cholesterol esters (see figure 1.5) (Natarajan *et al.*, 1999). This process involves the interconversion of two forms of HDL: HDL2 and HDL3 and cholesterol esters are taken into the liver for further processing. The endogenous pathway is concerned with the transport and metabolism of fats made in the body itself. The products are VLDL, which are metabolised in a manner analogous to the chylomicrons. Their remnants are usually called intermediate density lipoproteins (IDL) and are further metabolised to LDL that is taken up by the LDL receptor (Gurr, 1993).

1.1.5.6 Apolipoproteins

Apolipoproteins are proteins that confer specificity on the lipoproteins and determine the way in which they are metabolised, and to a large extent, the tissues in which they are metabolised. The apolipoproteins recognise and interact with specific receptors on cell surfaces, following which the receptor-lipoprotein complex is taken into the cell by a process of endocytosis. There are several apolipoproteins, identified by a series of letters A-E with subclasses of lipoproteins being present in some cases (Frayn, 1996; see table 1.4). While many of these proteins are involved in receptor recognition, some are also involved in the functioning of enzymes of lipoprotein metabolism; thus

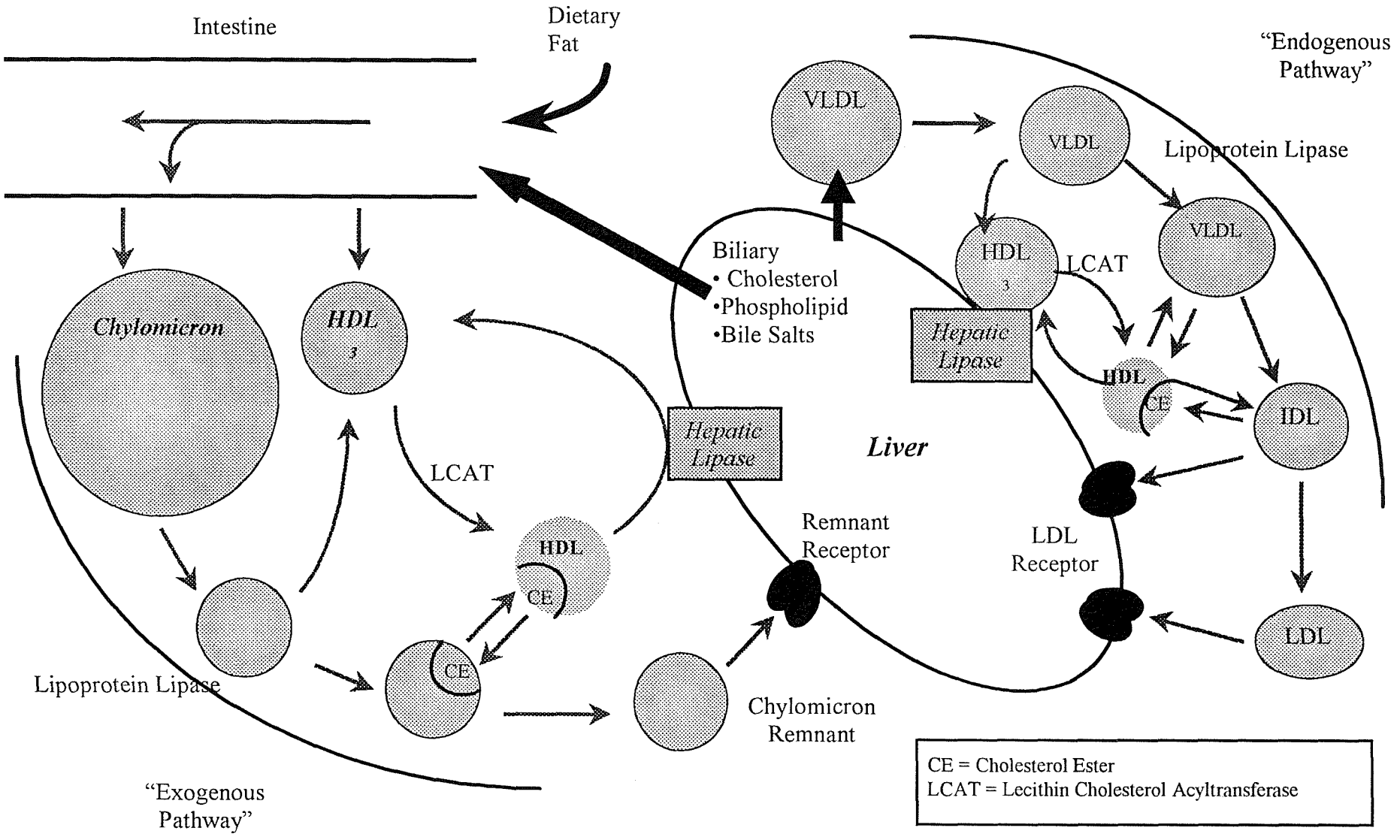


Figure 1.5 The metabolism of lipoproteins.

apo CII is needed for the activation of lipoprotein lipase and apo A-I for an enzyme involved in cholesterol esterification (Gurr, 1993; Frayn, 1996; Le *et al.*, 2000).

Table 1.4 Composition and characterisation of the major lipoprotein classes

Fraction	*Density range (g/ml)*	*Diameter (nm)*	*Major lipids*	*Major apo-lipoproteins*	*% Protein*	*% TAG*	*% Cholesterol*	*% PL*
Chylo-microns	<0.950	80-100	Dietary TAG	B48, A1, A2, C, E	1	90	5	4
VLDL	0.950-1.006	30-80	Endogenous TAG (From liver)	B100, C, E	10	65	13	13
LDL	1.019-1.063	20-22	Cholesterol and cholesterol ester	B100	20	10	45	23
HDL	1.063-1.090	9-15	Cholesterol ester and PL	A1, A2, C, E	50	2	18	30

Adapted from Frayn, 1996. Apolipoprotein C refers to the presence of apolipoproteins C1, C2 and C3 which are usually found together.

1.1.6 Fatty acid transport into cells

The mechanisms involved in the transport of fatty acids into cells are still under a great deal of investigation. The various lipoproteins can be taken up into cells through receptor-mediated processes (Hamilton, 1998). On the other hand transport of non-esterified fatty acids (bound to albumin in the circulation) into cells has been viewed either as a simple diffusion process regulated mainly by lipid physical chemistry or a more complex process involving specific transporters (Hamilton, 1998; McArthur *et al.*, 1999). Unesterified fatty acids are in constant flux and need to enter and leave cells rapidly. The transport of exogenous fatty acids into cells involves three distinct steps, and begins with the fatty acids present in an unbound form outside the cell. First, the fatty acid must adsorb to the outer side of the plasma membrane (adsorption). When fatty acids are present in the bloodstream bound to albumin, they must desorb from the albumin to enter the plasma membrane. Second, they must cross the membrane with

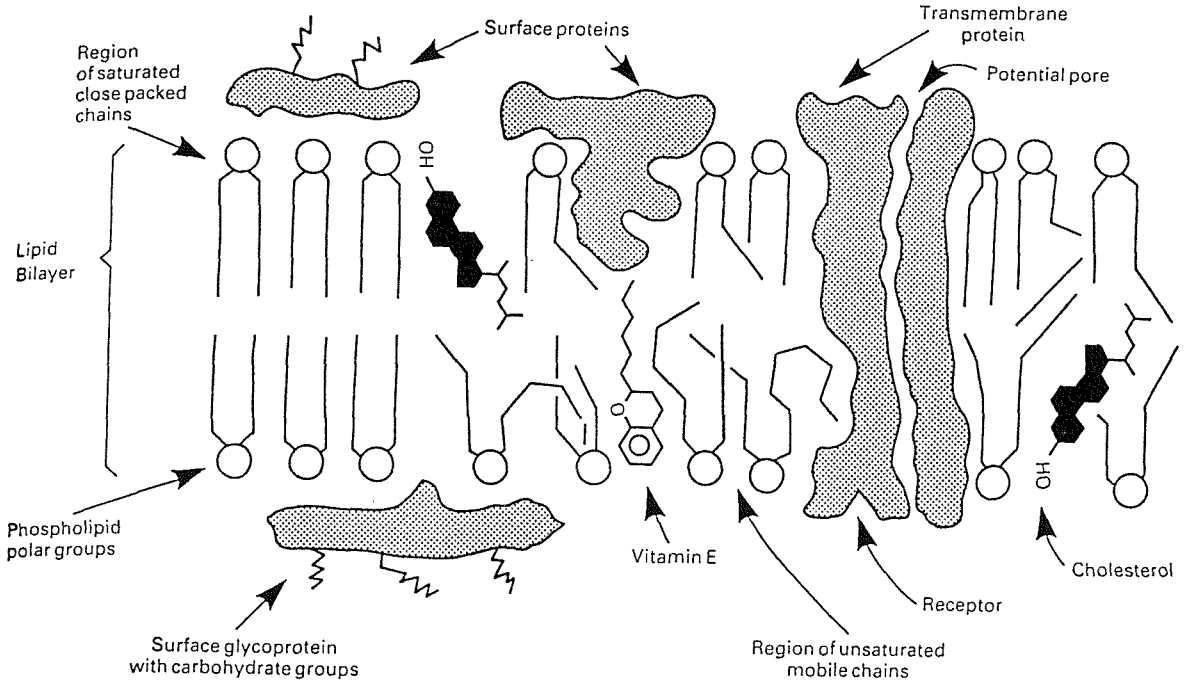
accompanying re-orientation of the carboxyl head groups to the cytosolic face of the membrane (transmembrane movement). Third, they must leave the cytosolic side of the plasma membrane to be utilised in intracellular locations (desorption). It is possible that these three steps might be catalysed by proteins in biological membranes, and many hypothetical schemes of fatty acid uptake into cells have been postulated (Spector, 1986). It is also possible that transport across the membrane might occur through passive diffusion through the lipid bilayer (Hamilton, 1998), this might be especially relevent for the absorption of NEFAs. It is also possible that further mechanisms may exist and that different fatty acids may be taken up into cells in various ways depending on their affinity for the albumin molecule (Hamilton, 1998). At the present time the exact mechanisms of how fatty acids get transported into and out of cells are not entirely certain. What is clear is that fatty acids eaten in the diet affect the fatty acid composition of cellular membranes (Murphy, 1990; Clandinin, 1991, Calder *et al.*, 1994; Calder, 1996a), and that this may lead to altered cellular function.

1.1.7 Structural Fats

Structural fats are those that contribute to the architecture of cells, mainly as constituents of cell membranes. The cellular membranes of animals are composed primarily of phosphoglycerides, cholesterol and proteins, but these may vary greatly in their specificity, composition and molar ratios (Alexander & Smythe, 1998). In animal membranes phosphoglyceride, mixed acid esters of glycerol with two fatty acid residues and one phosphoric acid residue, are the major structural lipids (Gurr, 1993). The general term to describe lipids containing phosphorous is phospholipid. Phospholipids are amphiphilic molecules, as they contain a polar head group such as choline, along with non-polar fatty acid hydrocarbon chains. In biological membranes, the phospholipid molecules associate together into a bilayer that forms a continuum throughout the membrane, with the fatty acid chains pointing inwards towards each other and the polar headgroups on the surfaces (Murphy, 1990). Protein molecules, which may have a mainly structural function or serve as enzymes or receptor molecules, are inserted into the phospholipid bilayer, interacting by non-covalent forces with both the polar and non-polar regions of the lipids. They may be located at external or internal sides of the membrane or project through from one side to the other

(see figure 1.6). Lipid molecules are mobile along the plane of the membrane, and there is limited movement of protein molecules across the membrane.

Figure 1.6 Membrane structure. A schematic representation of what a biological membrane might look like. The small spheres are the polar head groups of phospholipid molecules which form a double layer (lipid bilayer) throughout the membrane with fatty acid chains (represented by the tails) pointing inwards towards each other. Cholesterol and vitamin E are inserted between the fatty acid chains. Protein molecules are inserted at intervals into the bilayer, sometimes mainly at one face or another, sometimes extending through the membrane. Sugar groups may be attached to the surface proteins. From Gurr, 1993.



1.1.8 Membrane Fluidity

Fatty acids have important roles in membrane structure, and there are several ways by which they can influence the functions of membrane proteins and perhaps some intracellular proteins. It is primarily the physical properties of the fatty acyl chains of the phospholipids that determine the fluidity of the overall membrane (Anel *et al.*, 1990). The fatty acid chains are in constant motion, and the degree of molecular motion within the membrane, is influenced by the nature of the fatty acid chains, interactions

between the fatty acid chains and cholesterol, and interactions between proteins and lipids (Clandinin *et al.*, 1991). The chemistry of the individual fatty acids influences their shape, which in turn determines the space they occupy in the bilayer. Most membranes have a high proportion of unsaturated fatty acids and this seems to be an essential requisite for the proper functioning of cell membranes (Murphy, 1990; Clandinin *et al.*, 1991; Calder 1995c). Membrane fluidity, although usually expressed as a single physical parameter has two components. The first is the order of the membrane that is determined primarily by the packing of the fatty acyl chains. Fatty acyl chains that are all *trans* in configuration lead to a membrane with overall less fluidity because they will pack in a more organised manner. The second component of membrane fluidity refers to the rotational or lateral motion of the fatty acyl chains; the introduction of a double bond into the fatty acyl chain, as in the case of many long chain PUFA, results in an inflexible bend in the membrane structure (Calder, 1996). The chain can therefore not pack as tightly as saturated fatty acid chains and this results in an increase in the average surface area per fatty acyl chain. By inserting *cis* bonds the rate and range of acyl chain motion can be increased (Gurr, 1993). Membrane fluidity also depends on the number of double bonds in the chain, the position along the hydrocarbon chain, and the length of chain (Clandinin *et al.*, 1991; Murphy, 1990). By altering the type of fatty acid eaten in the diet, it is possible to influence the fatty acid composition of cell membranes and perhaps therefore to affect membrane fluidity. Cholesterol is also a key regulator of membrane fluidity (British Nutrition Foundation, 1999). Cholesterol inserts into lipid bilayers with its long axis perpendicular to the plane of the membrane with the hydroxyl group hydrogen bonding with the carbonyl oxygen atom of a phospholipid head group while the hydrocarbon tail penetrates the non-polar core of the bilayer (see figure 1.6). Cholesterol blocks large motions of fatty–acyl chains and so decreases membrane fluidity (Gurr, 1993).

1.1.9 Phospholipids

The fatty acid composition of phospholipids in cell membranes is usually characteristic for the cell type (Gibney & Hunter, 1993) but may change with progress through the cell cycle, with age, in response to stimuli or to changes in the environment or the diet (Stubbs & Smith, 1984). The AA or EPA present in cell membranes is esterified mainly to the 2 position of phospholipids such as phosphatidylcholine (PC),

phosphatidylethynolamine (PE) and phosphatidylinositol (PI) (Calder *et al.*, 1994; Nettleton, 1995). In general, different cell membranes have different phospholipid compositions (Gibney & Hunter, 1993). For example, in the plasma membrane that surrounds the cell, phospholipids are sited asymmetrically in the two bilayers. The phospholipids PI and phosphatidylserine (PS) are located on the cytoplasmic face whereas PC is found predominantly on the serosal face (British Nutrition Foundation, 1992). AA and EPA can be liberated from membrane phospholipids either through sequential action of phospholipase C (PLC) and diacylglycerol lipase or by the direct action of phospholipase A_2 (PLA_2) (Serhan *et al.*, 1996) (see figure 1.7). AA may derive from PC or PI (or other phospholipids). Most of the AA released from human peripheral blood and rat spleen lymphocytes in response to stimulation by the mitogen phytohaemagglutinin (PHA) comes from PI, not PC, (Resch *et al.*, 1984). Cytosolic PLA_2 becomes catalyticaly active in the presence of the free Ca^{2+} concentrations found in stimulated cells and preferentially cleaves AA containing phospholipids. A variety of stimuli including cytokines and growth factors activate PLA_2 to hydrolyse cellular phospholipids (Hancock, 1997). Once liberated these fatty acids can be metabolised by either the cyclooxygenase (COX) or lipoxygenase (LOX) pathways to produce eicosanoids (Pruzanaski & Vadas, 1991; Hancock, 1997; British Nutrition Foundation, 1999).

1.1.9.1 Phosphatidylinositol

An example of membrane lipid metabolism of central importance in cellular physiology is the inositol lipid cycle. The inositol lipid is central to the control of cell metabolism as its regulation underlies such processes as cell division and growth. PI has a well defined role in locating and probably directing various enzymes to the external surface of the lipid bilayer, where they can then act extracellularly (Stryer, 1995). The inositol may occur in PI as unmodified inositol, inositol-4-phosphate, and inositol-4, 5-bisphosphate (PIP_2) (Stryer, 1995; Hancock, 1997). Stimulation of a target cell can induce the hydrolysis of the inositol moiety of the phospholipid by phospholipase C (see figure 1.7). The inositol moiety released can cause change in the behavior of the cell. The form of inositol most closely associated with hormone-induced signals is inositol-1,4,5- trisphosphate (IP_3). Increased concentrations of IP_3 in the cell can induce the release of calcium into the cytoplasm from the endoplasmic

reticulum (Hancock, 1997). The calcium ions can then induce further signaling pathways. Hydrolysis of IP_3 from membrane PIP_2 yields the diacylglycerol (DAG) 1-acyl-2-arachidonyl-glycerol, that can act as an activator of protein kinase C. The DAG released from membrane phospholipids by enzymatic hydrolysis is in fact not a single chemical but a family of related compounds differing in fatty acid compositions. The main role of DAG is as an activator of PKC (Hancock, 1997). DAG can also be generated from phosphatidic acid (PA), resulting from membrane cleavage of PC by phopholipase D.

The calcium released as a result of IP_3 formation can favor the formation of complexes of this ion with the calcium dependent regulatory protein calmodulin. Calcium-calmodulin complexes can activate several enzymes, including kinases and phosphatases such as calcineurin (Abbas *et al.* 1994). Calcineurin is a key enzyme needed for the transcription of the IL-2 gene, and activation of calcineurin results in the nuclear translocation of transcription factors, such as nuclear factor of activated T cells (NFAT). Certain lipid mediators such as PGE_2 have been shown to inhibit IL-2 synthesis by blocking the activity of calcineurin (Paliogianni *et al.*, 1993), which would in turn, inhibit lymphocyte proliferation.

1.1.8.2 Platelet Activating Factor

Platelet activating Factor (PAF) is synthesised by acetylation of 1-alky-glycerophosphocholine which is derived from a membrane phospholipids by PLA_2 mediated release of a fatty acid (see figure 1.7). If the fatty acid is AA then PLA_2 can be considered responsible for the generation of the precursors of several lipid mediators in one reaction. PAF causes bronchoconstriction and endothelial cell retraction and activates inflammatory leukocytes (Abbas *et al.*, 1999).

1.1.10 Eicosanoids

The eicosanoids are a family of oxygenated derivatives of DGLA (20:3n-6), AA (20:4n-6) and EPA (20:5n-3). They include the prostaglandins (PGs) and thromboxanes (TXs), which are together termed to prostanoids, and leukotrienes (LTs), lipoxins (LXs) and other less stable molecules (Hwang, 1989; Kinsella *et al.*, 1990; Morita *et al.*, 1999). PGs and LTs are metabolites produced by enzymatic cyclooxygenation and lipoxygenation, respectively. These compounds have a short half-life and act locally to the cell from which they are produced (Clandinin *et al.*, 1991). An outline of eicosanoid metabolism is given in figure 1.8.

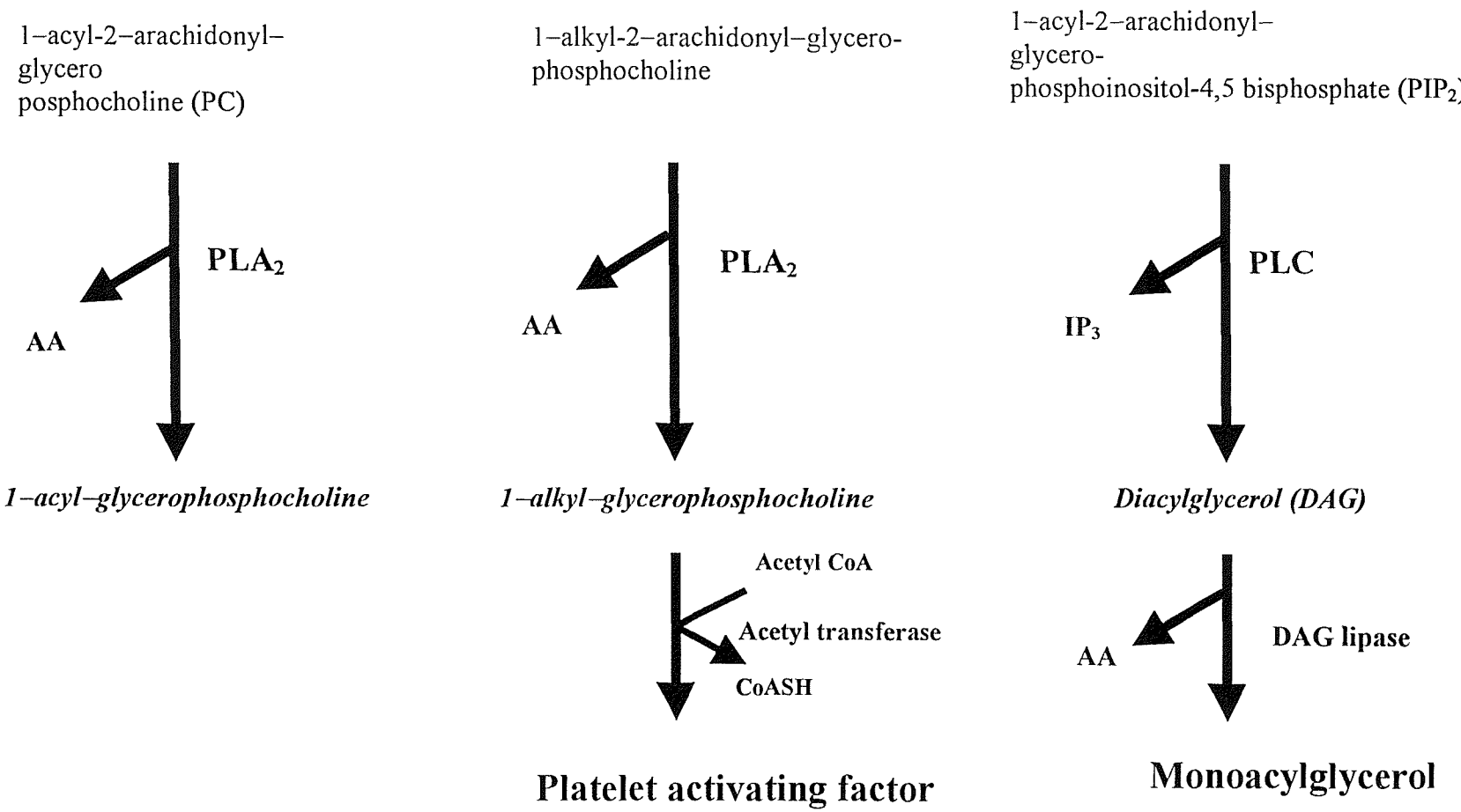
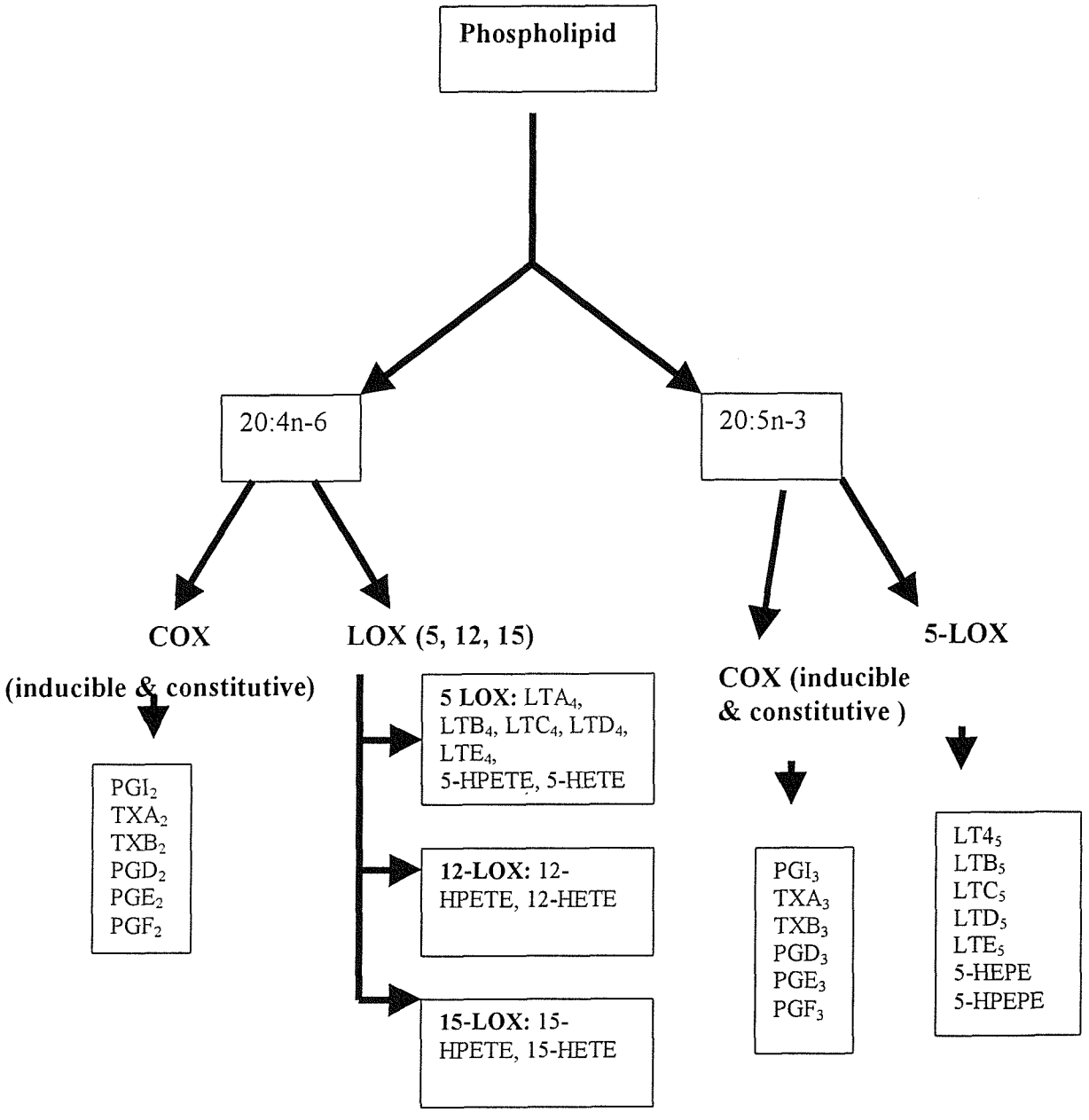


Figure 1.7 The reactions by which arachidonic acid can be released from membrane phospholipids.

Figure 1.8 The generation of eicosanoids from arachidonic acid and eicosapentaenoic acid



1.1.9.1 Prostanoids

Prostanoids are a group of metabolically related compounds that are formed in every tissue. They are derived by the action of cyclooxygenase (COX) on EPA, AA and DGLA. Because of their extreme potency and their short circulation time, prostanoids are considered local hormones. All prostanoids contain 20 carbon atoms, arranged in a (bi)cyclic structure, carrying two side chains, one of which is terminated by a carboxyl group (Gurr, 1993). The other side chain contains a hydroxyl group at carbon number 15 and is terminated by a methyl group. Prostanoids can be considered cyclic hydroxy fatty acids. Prostanoids are classified into prostaglandins, thromboxanes, or prostacyclins depending on the configuration of the cyclic part of their molecule. Prostaglandins and prostacyclins contain a ring of 5 carbon atoms, while thromboxanes contain a 6 carbon ring. Prostaglandins have the ability to contract smooth muscle, to inhibit or stimulate the adhesion of blood platelets and to cause constriction or dilation of blood vessels with related influence on blood pressure (British Nutrition Foundation, 1992). All prostanoids are produced locally near to their sites of action, are released in minute quantities, act rapidly and are quickly destroyed by degradative enzymes.

1.1.9.2 Leukotrienes

Leukotrienes are derived through the action of lipoxygenase (LOX) on EPA or AA. Leukotrienes are classified by their chemical structure into five families, A to E, and have a subscript number to indicate the number of double bonds they contain. Some of the leukotrienes (LTC_4, LTD_4 and LTE_4) have cysteine, a sulphur-containing amino acid, linked to the molecule and may have one or two other amino acids as well, LTA_4 and LTB_4 do not have attached amino acids. There are three lipoxygenase enzymes, the 5, 12, and 15 LOX enzymes (see figure 1.1.9). LOX enzymes are less widespread than COX enzymes and have been found mainly in cells produced in the bone marrow such as platelets, neutrophils, eosinophils, monocytes and macrophages (Nettleton, 1995). The 5 LOX enzyme catalyses the transformation of AA to 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE). The same enzyme further catalyses the transformation of 5-HPETE to an unstable epoxide intermediate, LTA_4. LTA_4 is converted to LTB_4 by enzymatic hydrolysis (von Schacky *et al.*, 1993). LTB_4 is a

dihydroxy fatty acid which regulates the functions of certain leukocytes (neutrophils, eosinophils, macrophages, lymphocytes) (Lewis *et al.*, 1990). The cysteinyl LTs tend to produce effects different from LTB_4. Both types of LT are found in, or are formed by, monocytes. The LOX pathways of arachidonic acid metabolism are a source of interest because of the roles of the metabolites in a number of diseases. These diseases include psoriasis, gout, arthritis, inflammatory bowel disease and asthma (Calder & Miles, 2000). Some of the eicosanoids most important to physiological processes are described in table 1.5.

Table 1.5 Some properties of some of the more biologically active eicosanoids.

Factor	*Biological properties*	*Reference*
Thromboxane A_2 (TXA_2)	Platelet aggregation; vasoconstriction; increase of intracellular calcium	VonSchacky *et al.*, 1988
Leukotriene B_4 (LTB_4)	Neutrophil chemoattractant; increase of intracellular calcium	Strasser *et al.*, 1985
Prostacyclin I_2 (PGI_2)	Prevents platelet aggregation; vasodilation; increases cAMP	Fischer & Weber, 1984
Prostaglandin E_2 (PGE_2)	Influences cytokine synthesis; inhibits macrophages; promotes Ig class switching; inhibits lymphocyte responses	Kinsella *et al.*, 1990

1.1.9.3 n-3 PUFA and eicosanoids

The n-3 PUFAs, EPA and DHA, competitively inhibit the oxygenation of AA by COX. In addition, EPA (but not DHA) is able to act as a substrate for COX and LOX. Thus, fatty acids eaten in the diet can affect eicosanoid synthesis. Consumption of diets high in n-3 fatty acids have been shown to lower PGE_2, TXA_2 and LTB_4 production (British Nutrition Foundation, 1992).

1.2 The Immune System

1.2.1 The Immune System

The immune system is one of the most complex organ systems in the body. It is unique in that it does not exist as a connected group of organs, such as the digestive system, but as a network of cells which move around the body. These cells include accessory cells such as macrophages that present antigens to T lymphocytes, B lymphocytes that synthesise protective antibodies and T lymphocytes that kill tumour cells or virally-infected cells. The system is normally classified into two components: the natural immune system and the specific immune system, with certain immune cell types being considered to belong to each one. It is the interplay between the cells of each of these arms of the immune system that provides the host with protection against a range of environmental and internal insults.

1.2.2 Natural Immunity

Natural or innate immunity is the mechanism of defence which exists prior to exposure to infectious pathogens and which is not enhanced by such exposures. Natural immunity is provided by a combination of mechanisms which include the protective surface phenomena in which protection is given by the physicochemical barrier of the skin, the maintenance of acidic environments in the digestive or genitourinary tracts and secretion of lysozyme and antibacterial substances at the sero-mucosal surfaces. Natural immunity is also provided by the non-specific cellular responses that include the spontaneous destruction of invading pathogenic organisms by the phagocytic leukocytes (e.g. macrophages and neutrophils) by the process of phagocytosis. Some aspects of natural immunity are described in table 1.6.

1.2.3 Specific Immunity

Specific or acquired immunity is initiated in response to foreign antigens that are recognised by specific receptors on lymphocytes and serves as a re-enforcement of natural immunity. This part of the immune system can be subdivided into two sub-classes, humoral and cell-mediated immunity, based on the components of the immune system that mediate the response. Humoral immunity is mediated by blood-borne macromolecules (such as antibodies produced by B cells) that are involved in the

identification and disposal of antigens, such as infectious microbes or viruses and in allograft rejection. The primary effector cells in cell-mediated immunity are the T lymphocytes that recognise specific antigens. Exposure to an antigen enhances the ability of the immune system to respond to that antigen. Response to a subsequent exposure is more rapidly induced and is greater in magnitude than the first response, a property known as immunological memory. Some aspects of specific immunity are described in table 1.6.

Table 1.6 Characteristics of natural and specific immunity.

	Natural	*Specific*
Physicochemical barriers	Skin, mucous membranes	Antibodies in mucosal secretions
Circulatory molecules	Complement	Antibodies
Cellular components	Phagocytes (macrophages, neutrophils) and NK cells	Lymphocytes
Soluble mediators	Macrophage-derived cytokines (TNF-α, IL-1β, IL-6) and eicosanoids	Lymphocyte-derived cytokines (IFN-γ, IL-2, IL-4, IL-10)

Although natural and specific immune systems may occur separately, a single invading pathogen often results in the activation of both responses concurrently, thus producing an integrated, intercommunicating system of defence. For example, antibodies produced by B cells can bind to infected cells and are recognised by macrophages, a cell type mostly associated with the natural immune response. The macrophage will then phagocytose the infected cell and destroy it (Auger, 1989). Antibodies also activate a cascade of plasma proteins called complement, which participate in a number of processes including the lysis and opsonization of microbes. The intricate communication system between the two arms of the immune system occurs with the help of various cytokines and adhesion molecules that allow the components of innate and acquired immunity to interact. They send each other signals regulate each other's activities and act together towards the final goal of eliminating the invading micro-organism and its products.

1.2.4 Cells of the Immune System

1.2.4.1 Phagocytes

There are two major classes of phagocytes, the circulating phagocytes, which include monocytes and granulocytes (neutrophils, eosinophils and basophils), and the fixed phagocytes, which are mainly macrophages (Auger, 1989) which can reside in the peritoneum, liver, spleen, lymph nodes, bone marrow, brain and lungs. Phagocytes are involved in phagocytosis and killing of micro-organisms, removal of debris and dead cells, regulatory interactions in haemopoesis, synthesis of biologically active compounds including eicosanoids, and co-operative and effector functions in the immune response.

1.2.4.2 Macrophages and Monocytes

The mononuclear phagocyte system comprises of peripheral blood monocytes, their bone marrow precursors and tissue macrophages (Auger, 1989). The mononuclear phagocytes constitute one of the largest cellular populations of the immune system. They are a highly mobile blood-borne class of cells of a common lineage whose primary function is phagocytosis. Monocytes enter the blood from the bone marrow and migrate to the tissues where they undergo further differentiation into a variety of histological forms. It is the natural physiology of these cells to lie dormant in the tissues until they contact appropriate signals (Adams & Hamiliton, 1992; Abbas *et al.* 1994). The major function of the mononuclear phagocyte system is to trap micro-organisms and foreign substances that are in the bloodstream and in various tissues and to eliminate them via phagocytosis. The system also functions in the destruction of aged and imperfect cells such as erythrocytes.

All the cells in the mononuclear phagocyte system originate in the bone marrow and, after maturation and subsequent activation, can achieve varied morphological forms. The first cell type that enters the peripheral blood after leaving the marrow is incompletely differentiated and is called a monocyte. Monocytes are 10 to 15 μm in diameter, they have a kidney shaped nucleus and a finely granular cytoplasam containing lysosomes, phagocytic vacuoles and cytoskeletal filaments. Human monocytes circulating in the blood have a half-life of about three days (Whitelaw,

1972). Once settled in a particular tissue, the monocyte differentiates into macrophages (Mϕ) which are present in a variety of forms such as:

- Kupffer cells in the liver
- Alveolar macrophages in the lung
- Splenic macrophages in the white pulp
- Peritoneal macrophages free floating in peritoneal fluid
- Microglial cells in the central nervous tissue

These cells have various functions that are summarised in table 1.7. The most important of these are the ability to kill invading organisms by phagocytosis and the ability to present antigen to T-lymphocytes. The number of tissue macrophages greatly exceeds the number of circulating monocytes, perhaps by a factor of 400 (Auger, 1989).

Table 1.7 Summary of macrophage functions

Killing of micro-organisms	*Stimulation of acute inflammation*	*Antigen presentation*	*Killing of tumour cells*
Phagocytosis	Generation of short lived inflammatory mediators i.e. eicosanoids, cytokines, reactive oxygen & nitrogen species	Expression of class I and class II molecules on their surface	Phagocytosis
Generation of reactive oxygen species	Recognise antibody- coated cells through Fc receptors		Generation of reactive oxygen & nitrogen species
Generation of nitric oxide	Recognise complement		Generation of cytokines e.g.. TNF

Macrophages can have different levels of activity depending on their state of activation and differentiation. For this reason macrophages are generally sub-classified by their activation state into three different sub-classes, resident, inflammatory and activated, each differing in morphological state and functional activities (Abbas *et al.*, 1999).

1.2.4.3 Resident Macrophages

Resident macrophages are tissues macrophages that have not been exposed to agents such as viral antigens or inflammatory cytokines. Due to their resting state they are small in size and possess very little functional activity; they have very low rates of secretion of lysozyme and reactive oxygen metabolites such as hydrogen peroxide or superoxide.

1.2.4.4 Elicited Macrophages

Elicited macrophages are macrophages that have been exposed to inflammatory, but not immunological, stimuli such as Brewer's thioglycollate broth. Inflammatory macrophages display similar but not identical patterns of response as those indicated for activated macrophages. They have increased size, rate of spreading and rate of phagocytosis but do not show increased anti-microbial and anti-tumour activity.

1.2.4.5 Activated Macrophages

Activated macrophages are macrophages which have been exposed to immunological stimuli such as bacterial lipopolysaccharide (LPS – also known as endotoxin) or cytokines released from activated lymphocytes. As a result of activation, these macrophages undergo a series of morphological and functional changes, which include an increase in size, ruffling of the plasma membrane, an increase in lysosome content in the cytoplasm, an increased rate of phagocytosis and an increase in production of reactive oxygen species and cytokines. This increase in production of cytokines and oxygen species endows macrophages with increased anti-microbial and anti-tumour activity (Johnston, 1988).

1.2.5 Activation of Macrophages

Macrophage activation can be defined as acquisition of competence to complete a complex function (Adams & Hamilton, 1984). Macrophage activation may occur by a number of means, by cytokines produced from activated lymphocytes (e.g. IFN-γ), by the products of complement activation, or by direct interaction with agents such as LPS (for an extensive review on macrophage activation see Adams & Hamilton, 1992). Macrophages in the different stages of activation are marked by profound changes in expression of membrane and secreted proteins and in some stages changes in specific

protein expression correlate precisely with changes in the ability to execute a complex function (see table 1.8). For example, the ability of macrophages to present antigen to T-lymphocytes is dependent upon cell surface expression of immune-associated molecules such as the class II major histocompatability complex (MHC). Such molecules are minimally expressed in resident macrophages, highly expressed in elicited macrophages and moderately expressed in activated macrophages (Adams & Hamilton, 1992). Corresponding macrophage competence to present antigen to T-cells is minimal in the first two stages of development (monocytes and resident macrophages), maximal in the elicited (primed) state, and diminished in the fully activated state. An overview of some of the functional and physical changes taking place in macrophages in different activation states is presented in table 1.8. Signalling events occurring within the macrophages that lead to enhanced inflammatory mediator gene expression and production are shown in figure 1.9.

Table 1.8 An overview of some of the functional and physical changes taking place in macrophages in different activation states

	Resident Macrophage	*Elicited Macrophage*	*Activated Macrophage*
Ia (mouse), HLA (humans) expression	-	++++	++
LFA-1 expression	-	++++	++++
Secretion of TNFα	-	-	++++
Secretion of reactive oxygen species	-	+++	+++
Bind tumour cells	-	++++	++++
Elevated chemotaxis and phagocytosis	+	+	+
Proliferation	+	-	-
Present antigen	-	++	+

The functional properties of macrophages must be tightly regulated to facilitate appropriate responses to complex conditions at the inflammatory site. Erwig *et al.*, (1998) have found that it is the initial cytokine exposure which determines the

functions of uncommitted macrophages and that once encountered this renders them unresponsive to other cytokines. In such a way the cytokine milieu has a profound effect on the functioning of the immune system and the propagation of macrophage responses.

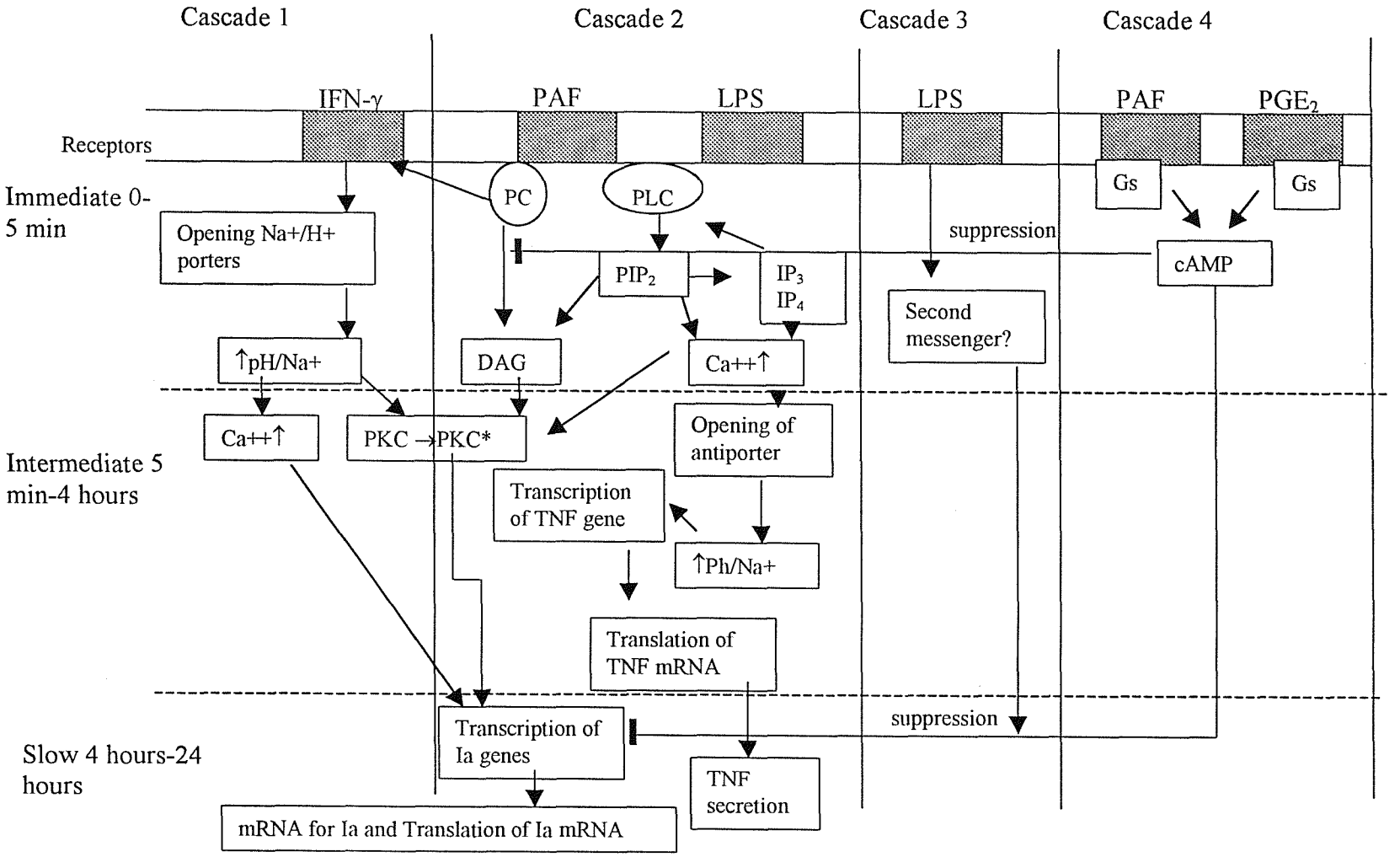


Figure 1.9 Some of the signalling events ocurring in the activation of macrophages- activation of tumour cytolysis and activation for presentation of antigen. The first two cascades (1 and 2) are inductive, cascade 3 is ampiphathic, whereas cascade 4 is suppressive. The cascades begin at the top of the figure with application of extracellular ligands which include IFN-γ, LPS, PAF and PGE_2. These respectively act on membrane receptors to initiate a variety of intracellular events. The relative time frames for each of these events is shown in the margin. Adapted from Adams & Hamilton, 1992.

1.2.6 Lymphocytes

Lymphocytes are the primary cell type associated with the specificity of the cellular immune response, as they are the cells that specifically recognise and respond to foreign antigen. They circulate in the bloodstream and are found in the lymphoid organs such as the thymus, spleen and lymph nodes. They have a large nucleus with dense chromatin surrounded by a thin rim of cytoplasm containing mitochondria, ribosomes, and lysosomes but no specialised organelles. Lymphocytes originate from stem cells resident in the bone marrow. Some immature lymphocytes enter the circulation and mature in the thymus (T lymphocytes), while other lymphocytes continue to mature in the bone marrow (B-lymphocytes) (Abbas *et al.*, 1994).

All T lymphocytes express the T cell receptor on their surface. The cell surface expression and the function of the T cell receptor (a heterodimer which may consist of α and β or γ and δ subunits) is dependent upon a group of associated proteins, the CD3 complex. A portion of the CD3 molecule is associated with a kinase inside the cell that sends an intracellular message when antigen is bound to the T cell. An outline of some of the signalling events in these cells is shown in figure 1.10. There are two subpopulations of T cells, each of which may have the same specificity for an antigenic determinant, although each subpopulation may perform different functions. These subpopulations are known as T helper cells and T cytotoxic cells. The functionally distinct lymphocyte populations express different membrane proteins, called CD proteins (which stands for cluster of differentiation, and refers to a molecule recognised by a cluster of monoclonal antibodies). These CD molecules can act as phenotypic markers of different lymphocyte populations: T helper cells express CD4 on their surface, whereas T cytotoxic cells express CD8.

1.2.7 Th-1/Th-2

Activation of T and B cells by antigen results in completely different effector functions. B cell activation results in the release of an antigen-specific receptor (i.e. an antibody) which binds to free antigen. T-cell activation in contrast does not result in the release of the antigen-specific receptor; it results in the secretion of a number of cytokines. Certain subsets of CD4+ T helper (Th) cells however may differ in their cytokine secretion patterns and effector functions (Mossman *et al.*, 1986), which

directs the adaptive immune system to respond to a challenge in different ways. These subsets are termed Th-1 and Th-2 cells (table 1.9)

Table 1.9 Characteristics of Th-1 and Th-2 cells

	Th-1	*Th-2*
Cell type	CD4+	CD4+
Cytokines produced	IL-2, IFN-γ	IL-4, 5, 10
Target for elimination	Bacteria, viruses, fungi	Parasites
Predominant beneficial immune reactions	Cell-mediated immunity, graft rejection	Elimination of parasites
Deleterious immune reactions	Chronic inflammation	Allergy

Antigenic stimulation of naïve CD4+ T cells in the presence of IL-12 and IFN-γ is thought to drive T cells toward the Th-1 subset. The IL-12 and IFN-γ that drive this T-cell differentiation step are most likely derived from viral or bacterial stimulation of natural killer (NK) cells and macrophages. Every cytokine has a specific cell surface receptor, expressed on a variety of different cell types. Thus, the cytokines released as the result of primed CD4+ T cell activation affect the function of many other cell types. The predominant cytokine synthesised after the activation of naïve or unprimed CD4+ cells is IL-2. Further stimulation of primed CD4+ cells results in the production of a large range of cytokines. The principle Th-1 cytokines have two key functions. They stimulate macrophages, enhancing their microbial actions, and also stimulate the production of IgG antibodies by B cells that bind to Fcγ receptors and complement proteins. These antibodies are primarily involved in the opsonization and phagocytosis of microbes. This would suggest that the primary function of Th-1 cells is to direct phagocyte-mediated defence against bacterial and viral infections. Th-1 dominant responses are often associated with inflammation and tissue injury because IFN-γ recruits and activates inflammatory leukocytes. In addition, some Th-1 cytokines promote the differentiation of CD8+ cells into active cytotoxic T cells (Robey & Allison, 1995).

Inappropriate Th-1 cytokine production is associated with chronic inflammatory diseases such as thyrotoxicosis, pernicious anaemia, auto-immune atrophic gastritis, Addison's disease, insulin-dependent diabetes mellitus, multiple sclerosis, ulcerative colitis, auto-immune haemolytic anaemia, rheumatoid arthritis, and systemic lupus erythematosus (SLE).

A second subset of CD4+ cells, Th-2 cells develop if antigenic stimulation occurs in the presence of IL-4 (Abbas *et al.*, 1994). Mast cells may be the source of this IL-4 in the early phases of an immune response before T cells are activated (Abbas *et al.*, 1994). Th-2 cells synthesise IL-4, IL-5, IL-10 but not IL-2 or IFN-γ. A number of Th-2 associated cytokines actually have anti-inflammatory actions. IL-4 and IL-13 antagonise the macrophage activating action of IFN-γ, while IL-10 suppresses numerous macrophage responses. Consequently the net result of Th-2 reactions is to suppress both acute and chronic inflammation. It is therefore possible that Th-2 cells are predominantly operating not as effectors but as regulators of the immune response. Th-2 cells are often associated with allergy, asthma and parasitic infections (Kelso, 1998). This is due to the fact that IL-4 increases IgE production by mast cells, and IL-5 activates eosinophils, which are important in orchestrating the body's defence against parasitic worms.

Both Th-1 and Th-2 cells synthesise several cytokines in common, including IL-3. In addition to Th-1 and Th-2, a third set of T cells Th-0 has been described that can make IL-2, IFN-γ and IL-4. Th-0 may be the precursors of Th-1 and Th-2 subsets (Kelso, 1998). Since different cytokines affect distinct target cells, the result of activating Th-1 rather than Th-2 cells, or vice versa, is to activate different types of immune responses. Cytokines produced by one subset of CD4+ cells inhibit the function of the other subset. For example IFN-γ produced by Th-1 cells inhibits the generation of Th-2 cells and IL-10 produced by Th-2 cells inhibits the generation of Th-1 type cells. (see figure 1.10).

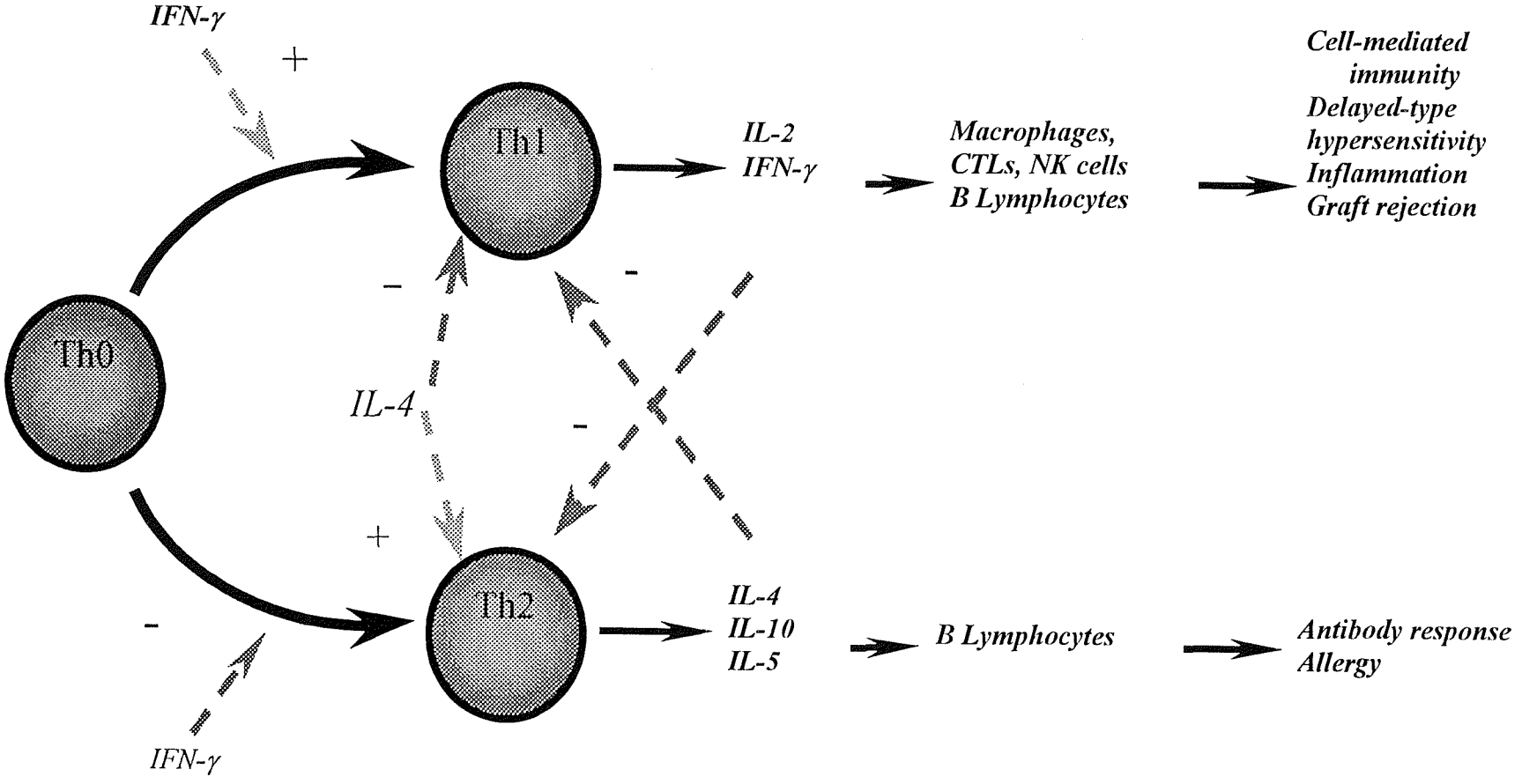


Figure 1.10 The Th-1/Th-2 paradigm

1.2.8 Lymphocyte Proliferation

The increase in lymphocyte number is termed proliferation. T lymphocyte proliferation requires activation via the T cell receptor that specifically recognises antigens presented to it by either class I or class II major histocompatability complex on antigen presenting cells. *In vitro* the activation of T cells can be initiated by mitogens such as concanavalin A (Con-A) or PHA or by antibodies that recognise the T cell receptor or by some cytokines such as IL-2.

Lymphocyte activation after the binding of antigen to the T cell receptor:CD3 complex results in lymphocyte growth, division and the transcription of the IL-2 gene and production of IL-2 (Robey & Allison, 1995). The signalling events occurring after binding are shown in figure 1.11. This increases the population of memory cells which ensures a speedier response to antigen the next time it is encountered. The proliferation of lymphocytes is a frequently used technique for investigating immune function *in vitro* and is most commonly determined by measuring the incorporation of radioactively labelled thymidine into the DNA of dividing cells. The rate of DNA synthesis is proportional to the rate of lymphocyte proliferation (Abbas *et al.*, 1999).

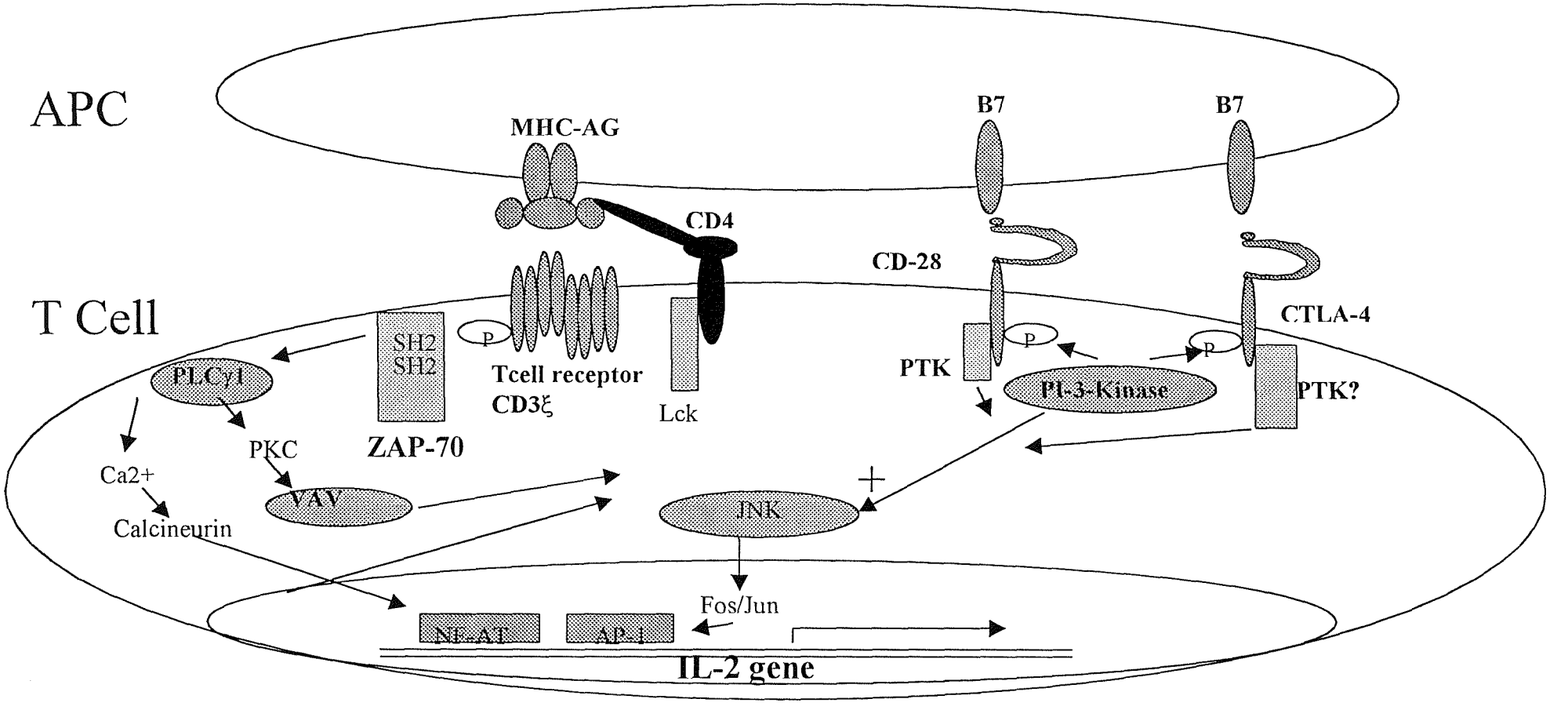


Figure 1.11 Signalling events during T cell activation. Signalling events occuring as a result of T-cell activation by the T-cel recptor (TCR/CD3) complex, CD28 and CTLA-4 after ligation with their respective ligands: major histocompatibility complex-antigen (MHC-Ag) and B7. TCR ligation is followed by phosphorylation (P) of CD3ξ, perhaps by the src family kinase Lck. CD3ξ then binds and activates the syk-family kinase Zap-70. Subsequently phospholipase Cγ-1(PLCγ-1) is phosphorylated and activated, leading to the generation of second messengers that elevate intracellular Ca2+ and protein kinase C (PKC) leading to a complex cascade of downstream events. Early events in CD28-mediated signal transduction may include binding and activation of phosphoinositide 3-kinase (PI 3-kinase), and perhaps a protein tyrosine kinase (PTK). TCR and CD28 signals appear to be integrated at the level of the mitogen activted protein tyrosine kinase (MAPK) JNK. The JNK protein tyrosine kinases phosphorylate the trancription factor c-Jun and thereby activate AP-1 transcription factor, which is crucial for the activation of the gene encoding IL-2. Not much is known about CTLA-4 mediated signaling but PI 3 kinase and PTK's might be involved. Abbreviations : APC: antigen presenting cell; NF-AT: nuclear factor of activated Tcells; SH2: src-homology 2 domain. Adapted from Robey & Allison (1995)

1.2.9 Cytokines

Cytokines are small proteins that are produced by a variety of cell types and act as soluble mediators and regulators of immune responses (Abbas *et al.,* 1994, Dinarello, 1994; Grimble & Tappia, 1998). They are crucial components of the immune system and enable the body to fight infection and to recover from major trauma. Cytokines act on nearly every tissue and organ system (Mizel, 1989) and perform their actions in a paracrine or autocrine fashion by binding to specific membrane-bound receptors. The typical cytokine is a glycosolated monomeric peptide of about 150 amino acids. Some are homodimers (e.g. IL-5), trimers (e.g. TNF-α) or heterodimers (e.g. IL-12). The cytokines are not however, members of a single gene superfamily. Remarkably few similarities have been noted in their primary nucleotide or amino acid sequences and their genes are for the most part scattered throughout the genome (Kelso, 1998).

Different cell types can produce the same cytokine or react to the same cytokine so one cytokine can have a range of differential effects on various different target cells. Furthermore, two different cytokines can have the same effect (Mizel, 1989). Cytokines are able to positively or negatively influence the production or function of each other. For example TNF-α can increase the production of IL-10 which will act to inhibit the production of TNF-α and other pro-inflammatory cytokines (Grimble, 1996). This makes the cytokine network very complex. The major functions of selected cytokines are listed in table 1.10 and a diagram of some cytokine interactions is shown in figure 1.12.

Table 1.10 Macrophage and lymphocyte derived cytokines

Cytokine	*Produced by*	*Major Functions*
Interleukin 1 (IL-1)	Monocytes, Mϕ and many other cell types	Produces fever; stimulates acute phase protein synthesis; promotes proliferation of Th-2 cells; influences IL-2 secretion
Interleukin 2 (IL-2)	Th-0 and Th-1 cells	T cell growth factor
Interleukin 4 (IL-4)	Th-2 cells and mast cells	Growth factor for B cells and Th-2 cells; promotes IgE and IgG synthesis; Inhibits Th-1 cells.
Interleukin 6 (IL-6)	T cells, Mϕ and many others	Induces acute phase protein synthesis, T cell activation and IL-2 production; stimulates B cell Ig production; haemopoietic progenitor cell growth.
Interleukin 10 (IL-10)	Th-2 cells and Mϕ	Inhibits production of Th-1 cells and macrophage function
Interleukin 12 (IL-12)	B cells and macrophages	Activates NK cells and promotes generation of Th-1 cells
Interferon gamma (IFN-γ)	Th-1 cells	Activates NK cells, and macrophages; inhibits Th-2 cells; induces expression of MHC class II on many cells
Tumour necrosis factor alpha (TNF-α)	Monocytes and Mϕ	Involved in inflammatory responses; activates endothelial cells and other cells of the immune and non-immune systems; induces fever and septic shock

1.2.10 Macrophage Derived Cytokines

1.2.10.1 Tumour Necrosis Factor -α

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine and a member of a family of secreted and cell surface proteins that mediate immune and inflammatory responses (Abbas *et al.*, 1994). The term tumour necrosis factor arose due to its ability to increase tumour destruction in tumour-bearing animals injected with LPS (Carswell *et al.*, 1975). Human TNF-α is a 17 kDa cytokine consisting of 157 amino acids with a

76 amino acid pre-sequence (Barbara *et al.*, 1996). It can exist in a soluble form or an unprocessed membrane bound form of 233 amino acids. The multiple activities of TNF-α are mediated through two distinct, high affinity receptors TNFR 55 and TNFR 75. The primary sources of TNF-α in the immune system are activated macrophages and monocytes. TNF-α activates neutrophils, monocytes and macrophages to initiate bacterial and tumour cell killing, increases adhesion molecule expression the surface of neutrophils and endothelial cells, stimulates T and B cell function, up-regulates MHC antigens and initiates the production of other pro-inflammatory cytokines such as IL-1β and IL-6. TNF-α mediates both the acquired immune responses and the innate immune responses and helps to link these two arms of the immune system. TNF-α along with IL-1β and IL-6 also modulates the production of acute phase proteins. Production of TNF-α is desirable in response to infection, but overproduction can be dangerous and TNF-α overproduction is implicated in endotoxic shock, adult respiratory distress syndrome and other inflammatory conditions (Grimble, 1990, 1996; Barbara *et al.*, 1996).

1.2.10.2 Interleukin -1

IL-1 is the second cytokine produced in response to inflammatory stimuli, including LPS and TNF, the latter of which it shares many properties with. IL-1 is a 17 kDa protein (Dinarello, 1994). It is primarily produced by monocytes and macrophages, but it can be produced by other cell types such as B cells and endothelial cells. There are two types of IL-1 which are termed IL-1α and β. IL-1α is primarily an intracellular cytokine, while IL-1β is secreted by the cell. At low IL-1 concentrations, the principle biological effects are as a mediator of local inflammation. Specifically IL-1β acts on mononuclear phagocytes and vascular endothelium to increase further synthesis of IL-1β and induce synthesis of IL-6. When secreted in larger amounts IL-1β enters the bloodstream and exerts endocrine effects. IL-1β stimulates T and B cell proliferation and induces fever and the acute phase response. In this way the biological effects of IL-1β are similar to TNF-α and both cytokines can cause fever, induce acute phase plasma proteins (such as serum amyloid A protein) by the liver and initiate metabolic wasting (Dinarello, 1988). IL-1 also premotes the synthesis of IL-2, important in T cell proliferation

1.2.10.3 Interleukin -6

IL-6 is a 26 kDa protein composed of 184 amino acids in its active state. It is a cytokine with pleiotropic activities that plays a central role in host defence. It is produced by activated monocytes and macrophages in response to IL-1 and TNF, with which it shares many of the same activities (Grimble, 1990), and by T-cells, B-cells, granulocytes, mast cells, and certain tumour cells – e.g. bladder carcinoma cells. Normal cells do not produce IL-6 unless appropriately stimulated (Grimble & Tappia, 1998). IL-6 can exert growth-inducing, growth-inhibitory, and differentiation-inducing activities, depending on the target cells. These activities include the secretion of immunoglobulins by B cells, growth promotion of various B cells, elicitation of the acute phase response, and differentiation and/or activation of T cells and macrophages. IL-6 is a major inducer of PLA_2 gene expression in human hepatoma cells (Crowl *et al.*, 1991).

1.2.10.4 Interleukin -10

IL-10 is a 35 kDa protein produced as a result of immune activation of macrophages and Th-0 and Th-2 lymphocytes (Moore *et al.*, 1993). It blocks the activation of Th-1 cells, monocytes and natural killer cells. It is often considered to be an 'anti-inflammatory cytokine' because it suppresses the production of IL-1β, TNF-α and IL-6 (Howard *et al.*, 1993). Donnelly *et al.*, (1999) have recently suggested that the ability of IL-10 to inhibit the expression of TNF-α and IL-1β genes is associated with its ability to rapidly induce synthesis of the protein SOCS-3 (suppressor of cytokine signalling-3).

1.2.10.5 Interleukin -12

Interleukin-12 is a 70-kDa heterodimer (p70) composed of two subunits p35 and p40. The major producers of IL-12 are monocytes and macrophages. IL-12 is a cytokine that possesses both proinflammatory and immunoregulatory activity. IL-12 induces IFN-γ and plays a central role in the development of Th-1 type immune responses. A number of pathogens such as measles virus and HIV have been shown to subvert the development of cell mediated immunity by actively inhibiting the production of IL-12 (Meyaard *et al.*, 1997). Several molecules made by macrophages themselves, such as

IL-10 and PGE_2, have been shown to inhibit IL-12 production when added to macrophages (Meyaard *et al.*, 1997). These molecules are often made in response to the same stimuli that induce IL-12 (Sutterwalla & Mosser, 1999). The inhibition of IL-12 occurs primarily at the level of transcription, and both the IL-12 p40 and p35 subunits appear to be affected (Sutterwalla & Mosser, 1999).

1.2.11 Lymphocyte-derived cytokines

1.2.11.1 Interleukin -2

Interleukin-2 is a 14-17 kDa glycoprotein. It is produced solely by activated T lymphocytes, and is considered a Th-1 cytokine. IL-2 is a mitogenic cytokine which induces T cell proliferation and provides a means by which antigen-triggered cells can be clonally expanded *in vitro*. IL-2 is the major autocrine growth factor for T cells and the quantity of IL-2 synthesised by activated CD4+ T cells is an important determinant in the magnitude of T cell responses. IL-2 can cause T cells to produce IFN-γ which in turn can augment T cell expression of interleukin 2 receptors (Abbas *et al.*, 1999). IL-2 also stimulates the growth of NK cells and enhances their cytolytic function.

1.2.11.2 Interferon-γ

Interferon-γ (IFN-γ) is a Th-1 cytokine following antigen/MHC specific stimulation. It enhances MHC class II expression on antigen presenting cells and can induce class II expression on some facultative antigen presenting cells (i.e. dendritic cells and macrophages) which do not normally carry class II. It acts synergistically with other B cell stimulating factors, causing B cell proliferation and differentiation as well as direct macrophage activation. It also synergises with TNF and enhances the susceptibility of target cells to cytotoxic T cells (Abbas *et al.*, 1994).

1.2.11.3 Interleukin-4

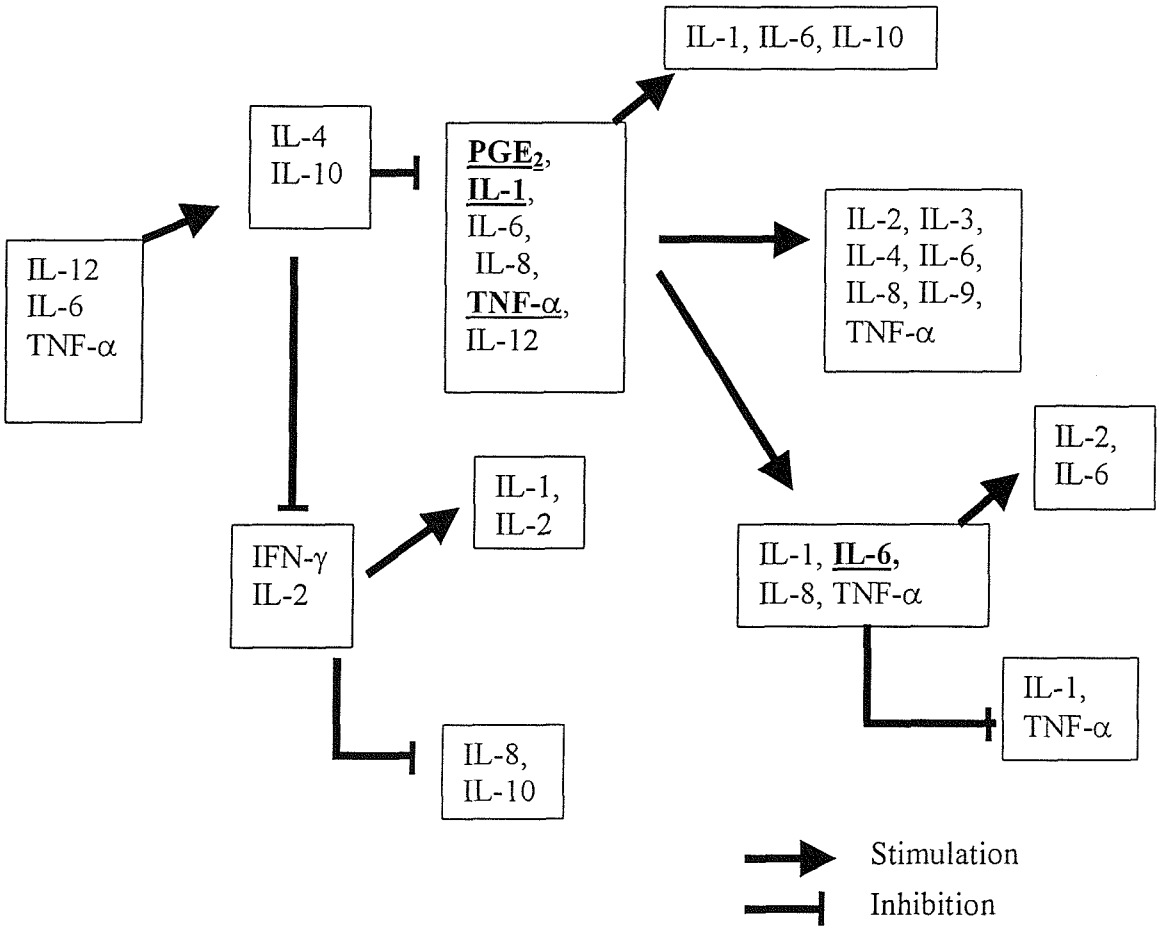
IL-4 is a pleotropic Th-2 cytokine sometimes called B cell stimulating factor (BSF-1), which has many biological effects on B cells, T cells and many non-lymphoid cells including monocytes, endothelial cells and fibroblasts. The main function of IL-4 is as a regulator of allergic reactions. It induces secretion of IgG_1 and IgE by mouse B cells

and IgG_4 and IgE by human B cells (Abbas *et al.*, 1994; Paludan, 1998). IL-4 inhibits macrophage activation and blocks most of the macrophage activating effects of IFN-γ including increased production of cytokines such as IL-1, nitric oxide (•NO) and prostaglandins (Pauludan, 1998). These effects are shared with those of IL-10 that is also produced by Th-2 cells. This is one of the main reasons why activation of Th-2 cells is often associated with a suppression of macrophage-mediated immune reactions. IL-4 stimulates the expression of certain adhesion molecules, notably vascular cell adhesion molecule-1 (VCAM-1), on endothelial cells, resulting in increased binding of lymphocytes, monocytes, and especially eosinophils. IL-4 is also important in regulating 12/15 LOX activity in monocytes (Huang *et al.*, 1999).

1.2.11.4 Cytokine and Eicosanoid Interactions

Many cytokines and eicosanoids can interact to augment or suppress the production of each other. For example, PGE_2 can augment secretion of IL-6 and IL-10 but it cannot induce IL-10 and IL-6 secretion alone (Yeh & Schuster, 1999). IL-10 can in turn down regulate production of IL-6 and PGE_2 (Kumar & Das, 1994; Niho *et al.*, 1998; Fiorentino *et al.*, 1991). IFN-γ can act with IL-2 to induce cytotoxicity of natural killer cells. IFN-γ, TNF-α and IL-1β can activate mononuclear phagocytes and through B lymphocyte stimulation augment lysis of cancer cells. IL-2, IL-6, and IL-12 induce natural killer cell and lymphokine activated killer cell cytotoxicity (Yeh & Schuster, 1999). The inhibitory effect of PGE_2 on IL-12 biosynthesis occurs by both direct and indirect mechanisms. The inhibition of IL-12 production by PGE_2 correlates with the ability of PGE_2 to increase intracellular cAMP (Tineke *et al.*, 1995). The increase in intracellular cAMP induced by PGE_2 increases the production of IL-10, which inhibits IL-12 production. However the inhibitory effect of PGE_2 is not dependent on IL-10 because neutralising anti-1L-10 antibodies did not abrogate the inhibitory effect of PGE_2 (Tineke *et al.*, 1995). IL-4 inhibits IL-6 production by monocytes, fibroblasts and synoviocytes (Gibbons *et al.*, 1990). The interactions between cytokines are very complex and for a more detailed review see Yeh & Schuster, (1999). An outline of some cytokine interactions is shown in figure 1.12

Figure 1.12 Some of the inhibitory and stimulatory interactions between the different cytokines and PGE_2. Mediators in bold and underlined are responsible for subseqent inhibitions or stimulations. (Adapted from Yeh & Schuster, 1999).



1.2.12 Nuclear Factor Kappa-B

Nuclear factor kappa B (NFκB) is a multi-protein complex that can activate a great variety of genes involved in early defense reactions of higher organisms (Rothwarf & Karin, 1999). NFκB is composed of two proteins of molecular weights 50 and 65 kDa. Binding sites for NFκB are present in the regulatory regions of certain cytokine genes including TNF-α, IL-6, IL-8, IL-12 and some IFN genes, the IL-2 receptor gene, class I and II hisotocompatability genes, several acute phase response genes, and of enzymes involved in mediator generation such as inducible nitric oxide synthase (Kopp & Ghosh, 1995). NFκB pre-exists in the cytoplasm of most cells in an inactive form complexed to IκB, its inhibitory subunit. Stimulation of the cell by a number of agents such as phorbol ester, LPS and TNF results in the dissociation of the IkB-NFkB complex. Subsequently the NFkB heterodimer migrates to the nucleus where it binds to its binding site and activates transcription of cytokines (for a review see Mercurio & Manning, 1999).

1.2.13 Nitric Oxide

Cytokines and eicosanoids are not the only molecules produced in response to inflammatory stimuli. Reactive species such as superoxide and nitric oxide (•NO) are also produced. Such species have many effects in biological systems, from attacking invading pathogens to causing lipid peroxidation. Nitric oxide is an extremely short-lived compound. It exists for about six to ten seconds and then is converted by oxygen and water to nitrates and nitrites (Moncada & Higgs, 1993). Nitric oxide has a myriad of functions in the body. For example, the synthesis of nitric oxide by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure (Snyder & Bredt, 1992). In addition, •NO is produced in large quantities during host defence and immunologic reactions (Snyder & Bredt, 1992). Because it has cytotoxic properties and is generated by activated macrophages, it has a role in non-specific immunity and also appears to regulate lymphocyte functions (Snyder & Bredt, 1992). Furthermore, it is involved in the pathogenesis of conditions such as septic shock and inflammation (Moncada & Higgs, 1993). The generation of

nitric oxide is mediated via the enzyme nitric oxide synthase (see figure 1.13). Nitric oxide synthase has three forms, an inducible form (iNOS), a constitutive form (cNOS) (Xie & Nathan, 1994), and a form that exists only in mitochondria (Giulivi *et al.*, 1998). iNOS enzymes have been shown to be regulated by eicosanoids, cytokines and protein kinase C. (Rosen *et al.*, 1995; Prabhakar *et al.*, 1993).

Figure 1.13 The pathway of synthesis and degradation of •NO. The generation of •NO is mediated via the enzyme nitric oxide synthase using the substrate L–arginine. Other reactive species can also be formed when nitric oxide is available. The peroxynitrite anion is a highly reactive species that can subsequently be protonated to form the highly reactive hydroxyl radical that can damage cells

L–ARGININE

↓ nitric oxide synthase

•NO + L-CITRULLINE

↓

ONOO- (peroxynitrite anion)

↓ H+

ONOOH

↓

$^{\cdot}OH + NO_2$

↓

$NO_2^- + NO_3^-$

1.2.13.2 Tissue distribution of inducible nitric oxide synthase

Cook *et al.* (1994) have examined the cellular localisation of inducible nitric oxide synthase (iNOS) after experimental endotoxic shock in the rat. They found that after injection with LPS the major site of •NO synthase induction was monocytes and macrophages in multiple organs, principally the liver and spleen. Brachial, bile duct, intestine and bladder epithelium and some hepatocytes also express iNOS. Expression peaked at five hours and had returned to normal by twelve hours post LPS except in the spleen. A semi-quantitative table showing the distribution of immunostaining for iNOS after intraperitoneal LPS is given in table 1.11.

Table 1.11 Location of iNOS in tissues. Nitric oxide is induced by LPS (± faint to +++ intense). Adapted from Cook *et al.*, (1994). ND = not done. Stomach, heart, aorta, vena cavae, small blood vessels and brain were all negative.

Location of iNOS in Tissues	*3 hours*	*5 hours*
Intravascular mononuclear cells (all organs)	+	+++
Liver		
Hepatocytes	±	+
Bile duct epithelium	+	++
Kupffer cells	+	+++
Infiltrating mononuclear cells	-	+
Spleen		
Lymphoid tissue	-	+
Red Pulp	+	+++
Lymph nodes		
Lymphoid tissue	±	ND
Kidney	-	+
Glomeruli	-	+
Tubules	-	-
Pelvic epithelium	+	++
Lung		
Brochial epithelium	±	++
Small intestine & large intestine		
Epithelium	+	++
Bladder		
Epithelium	+	++

1.2.14 Inflammation

An important function of phagocytic cells and phagocytosis is their participation in inflammation, a major component of the body's defense mechanism. Inflammation is a complex process initiated by tissue damage caused by such things as burns, wounds infection by microorganisms, and hypersensitivity reactions. It can be thought of as the metabolic consequence of inflammatory mediator production (Grimble, 1996). The inflammatory response is an important part of both innate and acquired immunity (Abbas *et al.*, 1994). For the most part the inflammatory response is protective and is designed in order to bring the injured tissue back to a normal state. The inflammatory process begins with the activation and increased concentration of pharmacologically powerful substances including a group of proteins known as acute phase proteins, such as C-reactive protein (Grimble, 1990; Abbas *et al.*, 1994). This protein binds to the membrane of certain microorganisms and activates the complement system that results in the lysis of the microorganisms by enhancing phagocytosis by phagocytic cells. The cytokines that play the most important role in the inflammatory response include TNF-α, IL-1, and IL-6. These cytokines are released by activated macrophages and induce adhesion molecule expression on the walls of vascular endothelial cells to which neutrophils, monocytes, and lymphocytes adhere before moving out of the vessel through a process called extravasation, to the inflamed tissues. All of these effects result in the accumulation of fluid (edema) and leukocytic cells in the injured area. These, in turn, amplify the response.

Injury, infection and inflammation are characterized by fever and wasting of peripheral tissues (Grimble, 1996). The wasting results in loss of tissue lipid, protein and micronutrients (Grimble, 1996). Widespread metabolic changes which are part of the wasting process facilitate the delivery of nutrients such as amino acids and fatty acids to the immune system; assist repair of tissues; control cytokine production; protect healthy tissues from the effects of the free radicals and other oxidant molecules, and remove certain nutrients such as iron and zinc, which promote bacterial growth from the bloodstream (Yeh & Schuster, 1999).

If the injury or invasion by microorganisms continues, the inflammatory response will be supplemented and augmented by elements of acquired immunity that include antibodies and cell-mediated immunity. The antibody response will attract and activate even more phagocytic cells to the site of injury as well as antigen-specific lymphocytes. The lymphocytes themselves are capable of destroying some foreign invaders, but more importantly, they release cytokines that activate macrophages and other cells to participate in destroying and removing invaders. Sometimes it is difficult to remove the causes of inflammation. This results in chronic inflammation, which occurs in cases of chronic activation of the immune response such as rheumatoid arthritis (Grimble, 1996).

Common treatments for inflammation include the use of non-steroidal anti-inflammatory drugs (NSAIDS), such as aspirin. NSAIDs inhibit the cyclooxygenase enzymes and lead to decreased production of eicosanoids (DeFreitas *et al.*, 1999) (see figure 1.8). There are two isoforms of cyclooxygenase a constituative form (COX-1) and and an inducible form (COX-2). NSAIDS primarily inhibit COX-1. Eicosanoids such as PGE_2 interact with the immune system in many ways, often promoting or suppressing the induction of cytokines (see figure 1.12). Modulation of eicosanoid synthesis therefore may represent a way to alleviate the cytokine-induced effects of chronic inflammation.

1.2.15 Eicosanoids and the Immune System

1.2.15.1 Prostaglandins

Inflammatory responses are mediated by prostaglandins and leukotrienes produced by monocytes, macrophages and mast cells. Prostaglandins especially those of the 2 series, tend to enhance the inflammatory response and work in synergy with other inflammatory agents such as histamine and bradykinin. Increased levels of PGE_2 are associated with several pathologies, many of which are also associated with the production of pro-inflammatory cytokines (Renz., *et al* 1988). It is possible that decreasing the levels eicosanoids may ameliorate some of the symptoms of these diseases. PGE_2 affects the activity of macrophages (Kinsella, 1990). PGE_2 is synthesized from arachidonic acid (see figure 1.8) and high concentrations of PGE_2 suppress the production of TNF-α, IL-1β and IL-6 (Renz *et al.*, 1988; Knudsen *et al.*,

1986; Spinas *et al.*, 1991). Some of the reported effects of PGE_2 on immunological function are shown in table 1.11 Very low concentrations of PGE_2 (10^{-9} M) are required for normal immune function and T cell differentiation while high concentrations (>10^{-8} M) are immunosuppressive (Renz *et al.*, 1988). Prostaglandins are important regulators of intracellular cAMP concentrations (Goodwin *et al.*, 1981), in particular PGE_2 increases intracellular cAMP levels by binding to a PGE-specific receptor that is known to be G-protein linked (see figure 1.9) (Negishi *et al.*, 1988). G proteins are guanyl nucleotide binding protiens and interconvert between an inactive GDP form and an active GTP form. The activated receptor stimulates the G protein, which carries the excitation signal to adenylate cyclase, resulting in the formation of cAMP. It has been shown that cAMP inhibits the induction of IL-2 but not IL-4 and that PGE_2 inhibits the production of Th-1 cytokines but not Th-2 cytokines by cells from mice (Betz & Fox, 1991) and humans (Gold *et al.*, 1994). Some of the reported effects of PGE_2 are shown in table 1.12.

Table 1.12 Reported Effects of PGE_2 in the Immune System (adapted from Kinsella, 1990)

Immune responses	*Reported effect of PGE_2*
Non-specific immune responses	Regulation of macrophage generation of reactive oxygen species Modification of macrophage receptor function (Ia antigen)
Specific immune responses	↓ Lymphocyte B & T cell proliferation ↓ Antibody IgG production ↑ Lymphocyte migration to inflamed tissue
Roles in signalling	Modulates intracellular cAMP levels Modulates PKC activation Prevents the increase in intracellular calcium important in T cell activation.

1.2.15.2 Leukotrienes and the Immune System

The leukotrienes produced by the LOX enzymes (see figure 1.8) are very potent inflammatory mediators, and are actively involved in the pathology of many inflammatory diseases such as asthma and allergic response (Rola-Pleszczynski *et al.*, 1985; Von Schacky *et al.*, 1993; Calder & Miles, 2000). They are synthesised

predominantly by granulocytes and mediate many of the inflammatory phenomena characteristic of immediate hypersensitivity reactions. AA and EPA are metabolised initially to 5-hydroxy derivatives by the 5-lipoxygenase pathway (see figure 1.8). These are further metabolised to LTA_4 and LTA_5 respectively. Hydrolysis of LTA_4 by a leukotriene A epoxide hydrolase yields LTB_4, a leukotriene with potent inflammatory activities. The action of the same enzyme on LTA_5 yields LTB_5, which has about 10-fold less pro-inflammatory activity than LTB_4 (British Nutrition Foundation, 1992).

1.3 The effects of n-3 fatty acids on the immune system

1.3.1 Historical Background

Epidemiological evidence suggests that the traditional Eskimo diet protects against a number of diseases, including cardiovascular disease (Bang *et al.*, 1971; Bang & Dyerberg, 1972), and diseases involving chronic inflammation such as type I diabetes, psoriasis, and asthma (Kromann & Green, 1980). These studies were based on observations on the pattern of diseases occurring in Eskimos from Greenland as compared to matched controls living in Denmark (see table 1.13 below). The high intake of the long chain n-3 PUFA in the Eskimo diet (around 7 g/day) (Bang & Dyerberg, 1972) was suggested to be the protective component. It was found that Eskimos consuming these diets had remarkable differences in their blood lipid profiles compared to matched controls living in Denmark (Bang & Dyerberg, 1972). It became increasingly clear that consumption of a diet containing long chain n-3 fatty acids would modulate a number of physiological systems in animals. Since the paper by Kromann & Green (1980), a great deal of research has been carried out investigating the effects of different fatty acids on various immune cell types and functions. Much of the work has focused on the n-3 family of fatty acids.

Table 1.13 Distribution of some diseases in Greenland Eskimos (1950-1974).
The expected number of cases is based upon age-specific incidence of each disease in Denmark. Data are taken from Kromann & Green (1980).

Disease	*Number of cases (in 1000)*	*Expected number of cases (in 1000)*
Cancer (all forms)	46	53
Apoplexy	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

The studies on the Greenland Eskimos were pivotal in drawing attention to dietary n-3 fatty acids and their potential protective effects on heart disease and inflammation. Current ideas about dietary fat, although still not entirely clear, owe their revisions in part to these Greenland studies. Trying to discern the interrelationships among different fatty acids has made understanding the effects of dietary fat on the body more complicated. The next sections will focus on some of the studies on the effects of fatty acids on immune cell functions.

1.3.2 Studies of the Effects of n-3 Fatty Acids on the Immune System

It is now accepted that n-3 fatty acids can affect the functioning of immune cells, but the exact mechanisms by which they exert their actions are not yet entirely clear. Dietary fish oils rich in EPA and DHA are capable of altering the membrane fatty acid composition of most animal and human cells (Johnston, 1988; Herold & Kinsella, 1986). These alterations are associated with an increase in the levels of EPA and DHA and a decrease in the AA content of immune cells. Such changes are associated with altered eicosanoid metabolism with consequent effects on immunological function (Drevon, 1992). Several clinical studies have shown a beneficial effect of fish oil supplements in diseases involving cells of the immune system including atopic dermatitis (Bjornobe *et al.*, 1987), rheumatoid arthritis (Kremer *et al.*, 1987), psoriasis

(Bjornobe *et al.*, 1988) and ulcerative colitis (McCall *et al.*, 1989). The next sections will outline some of the studies that have been performed in the area of n-3 rich diets and immune cell function.

1.3.3 The Effect of Different Fatty Acids on Cytokine Production by Macrophages

Modification of the fatty acid profile of macrophages following culture with various fatty acids has been reported (Lee *et al.*, 1985; Calder *et al.*, 1990; Kinsella, 1990), and macrophages incorporate fatty acids obtained through the diet into their cell membranes (Black & Kinsella, 1993). Macrophages possess both COX and LOX activities and many of their functions are related to their ability to produce eicosanoids, or to their response to eicosanoids in their environment. In addition to important proinflammatory characteristics eicosanoids can also suppress numerous leukocyte functions (figure 1.12) especially those that may be mediated by cytokine production. Macrophage-derived eicosanoids play an important role in autoregulation of macrophage functions such as down regulation of cytolytic capacity. For example, the LTB_4 and LTC_4 that are produced by macrophages after stimulation may have an effect on macrophage function such as the ability to lyse target cells (Braun *et al.*, 1993). When animals or humans consume fish oil EPA and DHA replace arachidonic acid in macrophage phospholipids (Black & Kinsella, 1993), thereby decreasing the amount of substrate AA available for eicosanoid synthesis. Changes in type of fatty acids that are present in the diet may affect the relative amounts of both PG and LT produced by providing different substrates for their synthesis. For this reason dietary fatty acids may alter either the type and/or quantity of eicosanoids produced by macrophages. For example, macrophages isolated from the peritoneum of mice consuming a nutritionally complete diet containing 2% safflower oil and 10% menhaden oil for 2 weeks contained elevated levels of EPA and DPA and particularly DHA in all the phospholipid and neutral lipid classes (Kinsella *et al.*, 1990). There was a significant decrease in arachidonic acid and a slight increase in linoleic acid in these cells. Macrophages isolated from mice fed the diet containing menhaden oil synthesised significantly less (< 50%) arachidonic acid derived prostaglandins and thromboxane following stimulation with zymosan (Lokesh *et al.*, 1989), LPS (Yaqoob & Calder, 1995a), or phorbol myristate (Lokesh *et al.*, 1987).

Because of the interactions between eicosanoids and cytokines (see figure 1.12), it follows that if diet can modify eicosanoid production by these cells, it may also modify cytokine production as well. The next section will outline some *in vitro* and *ex vivo* studies that have been carried out to examine this possibility.

1.3.4 *In Vitro* Studies on Macrophages

Baldie *et al.* (1993) have examined the effects of adding individual fatty acids to cultured human monocytes and to the monocytic U937 cell line on IL-1β secretion. AA and EPA enhanced IL-1β production by the U937 cell line and peripheral mononuclear cells, and the authors concluded that these fatty acids may have a pro-inflammatory action but that EPA was found to be less pro-inflammatory than AA. The effects of DHA were not assessed in this study. Sinha *et al.* (1991) have also examined the effects of *in vitro* culture of human mononuclear cells with either AA or EPA. This study found that culture of human mononuclear cells with AA caused a profound increase (+300% compared to control values) in the amount of IL-1β produced upon stimulation of the cells with LPS. However, in contrast to the Baldie *et al.* (1992) study, culture with EPA caused a 70% decrease in the production of IL-1β compared to control (unstimulated) cells. Purasiri *et al.* (1997) investigated the effects of adding EPA, DHA and gamma-linolenic acid (GLA) to cultures of human mononuclear cells on cytokine secretion in response to Con-A and PHA. This study found that the n-3 fatty acids decreased TNF-α and IL-β production by these cells. The effects of AA were, unfortunately, not assessed in this study. Thus, results of *in vitro* studies indicate that fatty acids can modulate cytokine production by monocytes. In summary two *in vitro* studies have shown that AA causes an increase in IL-1β production, while two studies have shown that EPA causes a decrease in IL-1β production and one study has shown that DHA causes a decrease in IL-1β production. Baldie *et al.* (1992) is the only study to show that EPA causes an increase in IL-1β production *in vitro*. However, Tappia *et al.* (1995) have shown that *in vitro* incubation of rat macrophages with DHA or EPA causes an increase in TNF-α and IL-6 production by these cells in response to LPS. IL-1β was not assessed in this study, but as these three cytokines usually follow the similar secretion patterns, an increase in TNF-α and IL-6 production would suggest that IL-1β might be affected by these fatty acids in a similar manner. The effects of

these fatty acids on *in vitro* cytokine production by monocyte/macrophages need to be clarified further.

1.3.5 Dietary Studies on Animals

The studies that have examined the effects of feeding n-3 fatty acids to animals on cytokine production have yielded contradictory findings. Some studies in rodents indicate that dietary supplementation with oils rich in n-3 PUFA reduces the production of TNF-α and IL-1β (Billiar *et al.*, 1988; Renier *et al.*, 1993; Yaqoob & Calder, 1995a) while other studies show no effect (Watanabe *et al.*, 1991) or an increase (Lokesh *et al.*, 1990) in cytokine production. The differences in the results of these studies are probably due to the different experimental protocols used. Some of these include differences in the type of animal (rat, mouse) studied, the stimulus used to activate the macrophages (LPS, IFN-γ), the type of macrophages studied (resident or elicited peritoneal, alveolar, Kuppfer cells), the length of feeding of the n-3 PUFA, the type of n-3 PUFA (18:3 n-3, 20:5 n-3, 22:5 n-3 or a mixture of all three types), the particular cytokine studied (IL-1β, TNF-α etc.), the length of culture (different cytokines are often produced at different stages in the inflammatory response) and the type of assay used to measure the cytokine concentrations (ELISA, bioassay). The differences in the protocols used in these experiments make many of these studies hard to compare. Many studies have shown that n-3 feeding results in increased TNF-α generation after stimulation with LPS, while just as many have shown that n-3 fatty acids decrease TNF-α production (see tables 1.14 and 1.15). The apparent differences in results of the effect of n-3 feeding on cytokine production by macrophages may be in part due to a differences in the activities of the macrophages studied (see section 1.2.5 for an overview of macrophage activation). To examine this, I have divided the studies into those that have used elicited macrophages (table 1.14) and those that have studied resident macrophages (table 1.15). In general, those studies which have examined the effects of feeding n-3 fatty acids to animals on elicited macrophages have shown a decrease in production of inflammatory mediators, especially TNF-α, while those which have examined resident macrophages have shown an increase in production of inflammatory mediators. To date no study has directly compared the two types of macrophage. It is possible that the length of time of feeding of the n-3 fatty

acids may also be an important factor. In studies examining the effects of n-3 rich PUFA diets on elicited macrophages, those studies which have fed the diet for a short period of time (less than 6 weeks) have generally shown no effect on TNF-α and IL-1β production by macrophages, while those which have fed the n-3 diet for lengths of time greater than 6 weeks have generally shown a decrease in the production of these mediators.

Table 1.14 *Ex vivo* studies on the influence of dietary n-3 fatty acids on cytokine production by elicited macrophages

Reference	*Species*	*n-3 level and length of feeding*	*Mediators studied*	*Effect of n-3 PUFA*
Watanabe *et al.*, (1991)	Mouse	4-5 weeks, 100 g/kg perilla oil	TNF-α	No effect
Turek *et al.*, (1991)	Rat	4 weeks, 125 g/kg, linseed oil	TNF-α	No effect
Hardardottir & Kinsella (1992)	Mouse	4-6 weeks, 100 g/kg, sardine oil,	TNF-α	No effect
Renier *et al.*, (1993)	Mouse	6-15 weeks, 100 g/kg, fish oil	TNF-α IL-1β	Decrease Decrease
Tappia & Grimble (1994)	Rat	8 weeks, 23 g/kg diet, Max EPA	TNF-α IL-1β IL-6	No effect Decrease Increase
Somers & Erikson (1994)	Mouse	3 weeks, 100 g/kg, fish oil	TNF-α	Increase
Hubbard *et al.*, (1994)	Mouse	3 weeks, 100 g/kg, fish oil or linseed oil	TNF-α	No effect (tended to decrease)
Yaqoob & Calder (1995b)	Mouse	8 weeks, 200 g/kg, fish oil	IL-6 IL-1β TNF-α	Decrease No effect Decrease

Table 1.15 Ex-vivo studies on the influence of dietary n-3 fatty acids on cytokine production by resident macrophages

Reference	*Species*	*n-3 level and length of feeding*	*Mediators*	*Effect of n-3 PUFA*
Lokesh *et al.*, (1990)	Mouse	4 weeks, 100 g/kg fish oil	TNF-α IL-1	Increase Increase
Watanabe *et al.*, (1991)	Mouse	4-5 weeks, 100 g/kg perilla oil	TNF-α	Increase
Hardardottir & Kinsella (1991)	Mouse	5 weeks, 100g/kg sardine oil,	TNF-α	Increase
Turek *et al.*, (1991)	Rat	4 weeks 125g/kg linseed oil	TNF-α	Increase
Chang *et al.*, (1992)	Mouse	10 weeks, 200g/kg menhaden oil	TNF-α	Increase
Boutard *et al.*, (1994)	Rat	6 weeks, 150g/kg fish oil	TNF-α	Decrease

1.3.6 Human Dietary Supplementation Studies on n-3 Fatty Acids and Cytokine Production

In general, the results of n-3 PUFA feeding on responses of human peripheral blood mononuclear cells are more consistent than the results of animal studies (see table 1.16). Several studies of fish oil feeding have shown a decrease in TNF-α, IL-1β, and IL-6 production by human monocytes *ex vivo* (Meydani *et al.*, 1991; 1993; Endres *et al.*, 1989; Caughey *et al.*, 1996). Caughey *et al.* (1996) studied the effect of diets enriched in n-3 fatty acids from linseed oil or fish oil on TNF-α and IL-1β production. Fish oil feeding was found to cause greater inhibition of IL-1β and TNF-α production than linseed oil. This study also found that there was a significant inverse relationship between TNF-α and IL-1β synthesis and mononuclear cell content of EPA. This fatty acid is derived either from ingested fish oil, or from the metabolism of ingested alpha linolenic acid and most likely accounts for the different potencies of the two oils. Some human studies however show no effect on cytokine production following fish oil feeding. For example Soyland *et al.* (1994) found no effect on IL-6 or TNF-α

production after 16 weeks of feeding n-3 PUFA to patients with psoriasis. Cooper *et al.* (1993) found no effect on TNF-α production by mononuclear cells after feeding 4.5 g fish oil/day for 6-8 weeks, although they did find a decrease in IL-1β and IL-6 production. Molvig *et al.* (1991) also found no effect on IL-1β or TNF-α production following supplementation with similar amounts of fish oil for seven weeks. Blok *et al.* (1997) also found no effect on IL-1β or IL-1Ra production in whole blood cultures after feeding 9 g fish oil/day for one year compared to a placebo. It is clear therefore that some studies have found that n-3 fatty acid feeding decreases TNF-α and IL-1β production, while others have found no effect. As with studies in animals, the unresolved differences in the effect of n-3 fatty acid supplementation of the human diet on cytokine production are most likely due to differences in the length of feeding, the amounts and types of fatty acids fed, and the characteristics of the population under investigation (healthy volunteers or patients with an inflammatory condition, age, gender, habitual intake of fatty acids). In order to standardise these factors and to investigate the effects of increasing n-3 fatty acid intake in a free living population on cytokine production, it is clear that a dose response and time course study is required. To date the only study which has performed a dose response/time course of fish oil supplementation on cytokine production is the study by Blok *et al.* (1997). In this study, populations of Benedictine and Trappist Monks, of varying ages (21-87 years) were fed low (3 g/day) moderate (6 g/day) or high (9 g/day) amount of fish oil for one year. Circulating cytokines were examined in peripheral blood throughout the study and the ability of whole blood cultures to produce cytokines in response to LPS was examined at the two final supplementation time points and up to six months after the cessation of the supplement. No changes in the concentration of IL-1α or β, or TNF-α were found compared to placebo in either the circulating levels of plasma cytokines or in the LPS-stimulated whole blood cultures. It is unfortunate however that the whole blood LPS-stimulated cultures were not assessed at the start at the study or during the first few months of dietary fish oil supplementation period. Due to this omission, it cannot be assessed if the various doses of fish oil caused a change in cytokine production from baseline measurements. In order to assess this point, this study or a similar one needs to be repeated and baseline measurements of cytokine production in response to endotoxin need to be assessed.

Table 1.15 Studies on the effects of n-3 fatty acids on human cytokine production.
'↑' = increase, '↓' = decrease, '=' = no effect

Reference	*Subjects*	*Number in n-3 group*	*n-3 fatty acid dose g/day*	*Duration of diet*	*Control group*	*T cell proliferation*	*CD4/CD8 ratio*	*Macrophage derived cytokines*	*T-cell derived cytokines*
Payan *et al.* (1986)	Patients with persistent asthma	6	4	8 weeks	0.1 g/d EPA	↑	=		
Endres *et al.* (1989)	Healthy men	9	4.6 g EPA+ DHA	6 weeks	Comparison to 4 unsupplemented volunteers			↓ IL-1α ↓ IL-1β ↓ TNF-α	
Kremer *et al.* (1990)	Rheumatoid arthritis patients	49	3.3/6.7	24 weeks	Olive oil	↑			↓ IL-2
Molvig *et al.* (1991)	Healthy men and diabetic men	33	1.7g EPA + DHA	7 weeks	Mixed oil placebo			= IL-1β = TNF-α	
Kelley *et al.* (1991)	Healthy men	10	+/- 20 [1]	8 weeks	Isoenergenic basal diet	↓	=		= IL-2
Virella *et al.* (1991)	Healthy men	6	2.1 EPA/day	6 weeks	Olive oil	=	=		↓ IL-2
Meydani *et al.* (1991)	Healthy older and younger women	12	2.4 g EPA+DHA	12 weeks	None	↓		↓ IL-1β ↓ IL-6	↓ IL-2
Meydani *et al.* (1993)	Healthy men and women over age 40	22	1.2 g EPA+ DHA	24 weeks	Low fat, 0.27 g/d (n-3)FA[3]	↓	↓	↓ IL-1β ↓ IL-6 ↓ TNF-α	↓ IL-2
Cooper *et al.* (1993)	Healthy men and women	15	1.1-1.5 g EPA + DHA	6-8 weeks	Unsupplemented volunteers			↓ IL-1β ↓ IL-6 = TNF-α	

Reference	*Subjects*	*Number*	*n-3 fatty acid type and dose g/day*	*Duration of diet*	*Control group*	*T cell proliferation*	*CD4/ CD8 ratio*	*Macrophage derived cytokines*	*T-cell derived cytokines (or soluble cytokine receptors)*
Soyland *et al.* (1994)	Psoriasis and atopic dermatitis patients	40	5 g EPA + DHA	16 weeks	Corn oil	=			↓ IL-2R [4]
Gallai *et al.* (1995)	Patients with multiple sclerosis	20	4.9 g EPA + DHA	24 weeks	Aged matched controls also supplemented			↓ IL-1β ↓ TNF-α	↓ IL-2 ↓ IL-2R ↓ IFN-γ
Caughey *et al.* (1996)	Healthy men	15	2.7 g EPA + DHA	4 weeks	Sunflower oil			↓ IL-1β ↓ TNF-α	
Schmidt *et al.* (1996)	Healthy men and women	16	0.5 g EPA + DHA	12 weeks	Placebo oil			=IL-1β =TNF-α =IL-6	
Blok *et al.* (1997)	Healthy men	58	1.1 g EPA + DHA	52 weeks	Olive oil and palm oil mix			= IL-1β = TNF-α = IL-6	
Kelley *et al.* (1998)	Healthy men	11	6 g DHA	12 weeks	Isoenergetic basal diet				= IL-2 = IL-2R
Thies *et al.* (2000)	Healthy elderly men & women	8	1.1g EPA + DHA	12 weeks	Placebo oil	=		= TNF-α = IL-1β = IL-6	= IL-2 = IFN-γ
Yaqoob *et al.* (2000)	Healthy men & women	8	3.2g EPA + DHA	12 weeks	Placebo oil	=		= IL-1β = TNF-α	= IL-2 = IFN-γ = IL-10

1. dietary supplementation was given as linseed oil, abundant in linolenic acid (18:3, n-3). The study had a cross over design.
2. ND = not detected
3. The n-3 fatty acids in the control group were plant derived, whereas the n-3 fatty acids in the experimental group were fish derived
4. The expression of interleukin-2 (IL-2) receptor was decreased in the n-3 fatty acids-supplemented group.
5. Dose of the feeding formula was increased during the study period.

1.3.7 Macrophage Function Studies: Cytotoxicity

Cytotoxicity (cytolytic capacity) can be defined as the ability of immune cells to kill foreign cells invading the host organism. In the case of macrophages this is usually carried out through phagocytosis, production of ROS (part of the respiratory burst), and through cytokine and eicosanoid production. The results of studies in this area are presented in table 1.16. Macrophages acquire the capacity to destroy tumour cells upon exposure to IFN-γ and LPS (Somers *et al.*, 1989). However, the mechanisms as to how this happens are unresolved. Various functional capacities, including tumoricidal activity may be down regulated by either exogenous or endogenous PGE_2 (Somers *et al.*, 1989; Kunkel *et al.*, 1986; Roper & Phipps, 1994), suggesting that FO should increase cytotoxicity by macrophages. Studies by Somers *et al.* (1989) and Hubbard *et al.*, (1994) have investigated the effects of dietary fatty acids on macrophage ability to kill P815 cells, a cell line killed by nitric oxide. Hubbard *et al.* (1994) found that after feeding mice a diet of fish oil (100g /kg for 3 weeks) macrophage cytolysis of P815 cells decreased. Somers *et al.* (1989) fed mice for four weeks on diets containing one of three fats, fish oil, safflower oil or coconut oil. Mice fed the fish oil diet had a reduced capacity to kill P815 cells upon activation with IFN-γ and LPS. Although nitric oxide production was not investigated in this study, the results obtained implied that fish oil feeding decreases nitric oxide production. This contradicts what has been found in other studies that show that fish oil feeding increases nitric oxide production (Yaqoob & Calder, 1995b; Chaet *et al.*, 1993 see 1.2.13 for a review on nitric oxide). The effect of n-3 feeding on the cytotoxic capacity of macrophages to kill L929 cells, a murine fibroblast cell line killed by TNF-α, has also been investigated (Black & Kinsella 1993, Renier *et al.*, 1993). Cytotoxicity is decreased towards these cells after fish oil feeding (see table 1.17). This implies that TNF-α production or secretion is reduced by these cells following consumption of an n-3 rich diet. The effect of n-3 feeding on TNF-α production by macrophages is not entirely clear (see section 1.3.5). Macrophages involved in the killing of pathogens, and infected, damaged, apoptotic or cancerous cells would most likely be those in a higher state of activation and most studies which have examined elicited (inflammatory) macrophages have shown that TNF-α production is reduced following n-3 feeding (see section 1.3.5). This would correlate well with reduced cytotoxic capacity towards cells that are killed by TNF-α.

Table 1.17 The effects of n-3 PUFA on macrophage cytotoxicity

Reference	*Species and macrophage type*	*Diet*	*Time fed*	*Cytotoxicity towards target cells*
Somers *et al.* (1989)	Mouse elicited peritoneal Mϕ	100 g/kg fish oil	4 weeks	Decreased killing of P815 cells
Black & Kinsella (1993)	Mouse elicited peritoneal Mϕ	15 g n-3 PUFA/kg	2 weeks	Decreased killing of L929 cells
Renier *et al.* (1993)	Mouse elicited peritoneal Mϕ	100 g/kg fish oil	6-15 weeks	Decreased killing of L929 cells
Hubbard *et al.* (1994)	Mouse elicited peritoneal Mϕ	100 g/kg fish oil	3 weeks	Decreased killing of P815 cells

1.3.8 Fatty Acids and Cytokine Production by Lymphocytes

Lymphocytes play an important role in the development and progression of a number of autoimmune disorders, which are characterized by the presence of activated T cells and cytokines at the site of tissue injury and in the circulation. As n-3 PUFAs have been shown to be associated with a decrease in inflammatory disease (Kromann & Green, 1980), it may be that they are having an effect through modulating cytokine production (Yaqoob & Calder, 1995a). It is possible that this may occur through n-3 modulation of eicosanoid production. Gold *et al.* (1994) found that IL-2 and IFN-γ production by Th-1 cells was inhibited by PGE_2, while production of IL-4 and IL-10 by Th-2 cells was not effected by PGE_2. This may be one way in by which modulation of fatty acids might affect cytokine production by these cells - modulation of PGE_2 levels. However, other studies have shown that cytokine production in some immune cells can also occur through a prostaglandin-independent mechanism (Santoli & Zurier, 1989). The next sections will outline some of the studies that have previously been carried out examining the effects of diet on lymphocyte functions and cytokine production.

1.3.9 *In Vitro* Studies on Lymphocytes

A large number of studies have investigated the addition of individual fatty acids to lymphocytes cultures *in vitro* and their effects on subsequent mitogen-stimulated lymphocyte proliferation (Mertin *et al.*,, 1974; Offner & Clausen, 1974; Mertin & Hughes, 1975; Mihas *et al.*, 1975; 1977; Buttke, 1984; Virella *et al.*, 1989; Calder *et*

al., 1990a; 1991, 1992; Calder & Newsholme, 1992 a, b; Soyland *et al.*, 1993; Rotondo *et al.*, 1994; Purasiri *et al.*, 1997) and cytokine production (Calder & Newsholme 1992a,b; Rotondo *et al.*, 1994; Purasiri *et al.*, 1997). The inhibition of lymphocyte proliferation by unsaturated fatty acids is dependent on the concentration of fatty acid used, the method of addition of fatty acid (either as free fatty acids or complexed to albumin), and the duration of exposure of the cells to fatty acid (Calder, 1995). Most studies agree that the extent of inhibition is also partly dependent on degree of unsaturation of the fatty acid, with chain length also being important. The approximate order of potencies appear to be oleic < linoleic = α-linolenic < γ-linolenic = dihomo-γ-linoleic acid= docosahexaenoic < arachidonic = eicosapentaenoic acid (Calder, 1995).

1.3.10 Dietary Fatty Acids and Lymphocyte Proliferation

There are a number of studies in animals and humans examining the effect of dietary fat on lymphocyte proliferation (see table 1.18). These have been reviewed in detail by Calder (1995; 1998) and a few studies will briefly be described here. Many of the studies have shown that fish oil feeding causes a decrease in proliferation of rodent lymphocytes and human blood mononuclear cells. Lymphocyte proliferation is dependent on the cytokine IL-2 (Smith, 1988; Abbas *et al.*, 1994). Prostaglandins and leukotrienes have also been shown to inhibit some lymphocyte responses such as cytokine-stimulated proliferation (Hwang, 1989; Gold *et al.*, 1994; Whelan *et al.*, 1995; Ring *et al.*, 1997). Interference with the biosynthesis of prostaglandins after immune activation, especially by related fatty acids, would result in modification of lymphocyte proliferation. Calder *et al.* (1992; 1995) however have pointed out that most of the experiments in which the effect of eicosanoids on lymphocyte proliferation have been examined have used pharmacological levels of prostaglandins that would be unlikely to be found in the cellular environment under normal conditions. In *in vitro* experiments, when inhibitors of enzymes involved in the synthesis of eicosanoids (such as indomethacin are included in the media along with individual unsaturated fatty acids, the inhibitory effects of fatty acids are still observed. Calder *et al.* (1992) Soyland *et al.* (1993) and Rotondo *et al.* (1994) have concluded that the suppressive effects of PUFA on stimulated lymphocyte proliferation are not mediated through the production of eicosanoids, because the addition of a COX inhibitor to the culture medium did not alter the effect of the PUFA on cell proliferation. Addition of

inhibitors of PLA_2 and LOX such as quinacrine (inhibits PLA_2), caffeic acid and nordihydroguaiaretic acid (inhibit LOX) also did not alter the suppressive effect of unsaturated fatty acids on lymphocyte proliferation (Calder *et al.*, 1992; Soyland *et al.*, 1993; Kumar *et al.*, 1992). More work needs to be done to elucidate how fatty acids are exerting their effects on lymphocyte proliferation. It may be that fatty acid modulation of certain lipid signalling cascades, or cascades other than ones traditionally thought to involve lipids, might be responsible for the eicosanoid-independent effects.

Table 1.18 Animal studies investigating the effects of n-3 fatty acids on lymphocyte proliferation

Reference	*Cell source*	*n-3 fat level and length of feeding*	*Stimulus*	*Effect of n-3 PUFA*
Marshall & Johnston (1985)	Rat spleen	100 g/kg 18:3n-3; 3 weeks	Con-A, PHA	Decrease
Alexander & Smythe (1988)	Mouse spleen	200 g/kg fish oil; 3 weeks	Con-A, PHA	Decrease
Kelley *et al.* (1988)	Rabbit spleen	76 g/kg fish oil; 20 weeks	Con-A, PHA, LPS	Decrease
Fernandes *et al.* (1994)	Mouse spleen	100 g/kg fish oil; 6 months	Con-A, LPS, superantigen	Increase
Yaqoob & Calder (1995b)	Mouse spleen	200 g/kg fish oil; 8 weeks	Con-A	Decrease
Jeffery *et al.* (1996)	Rat spleen	200 g/kg linseed oil; 10 weeks	Con-A	Decrease
Jolly *et al.* (1997)	Mouse spleen	30 g/kg safflower oil + 20 g/kg EPA or DHA; 10 days	Con-A	Decrease
Peterson *et al.* (1998)	Rat spleen	178 g/kg diet containing 4.4 g or 6.6 g EPA + DHA; 6 weeks	Con A	Decrease

1.3.11 Effects of n-3 Fatty Acids on Cytokine Production by Lymphocytes: Animal Feeding Studies

A few studies have shown that feeding rodents and other animals on n-6 and n-3 fatty-acid rich diets will affect cytokine production (see table 1.19), and the expression of certain cytokine receptors. In a study of non-human primates (Wu *et al.*, 1996) the effects of marine and plant derived n-3 PUFA were examined. IL-2 production was significantly increased following feeding of a diet containing 3.3% EPA and DHA, and PGE_2 production was suppressed. The conclusion of these authors was that after adjustment for tocopherol concentration, marine-derived PUFA, but not plant-derived n-3 PUFA increased the T cell-mediated mitogenic response and IL-2 production. The effect of dietary fat on the lymphocyte-derived cytokines IL-2, IFN-γ, IL-4 and IL-10 was investigated in a study by Yaqoob & Calder (1995a). They fed weanling mice for eight weeks on a low fat diet or one containing 20% by weight hydrogenated coconut oil, olive oil, safflower oil or fish oil. Fish oil feeding had no effect on IL-2, IL-4, IFN-γ or IL-10 production by these lymphocytes. As mentioned in the previous section, the effect of diets high in in-3 PUFA on lymphocyte proliferation consistently show a decreased proliferation, implying a decrease in IL-2 production. Proliferation of lymphocytes is triggered by the interaction of IL-2 with its specific receptor following T-lymphocyte activation. Diets high in n-3 fatty acids have also been shown to decrease the expression of the IL-2 receptor in both humans and animals (Soyland *et al.*, 1994; Jolly *et al.*, 1998). The mechanisms of action of how FO may be affecting IL-2 production, secretion and receptor expression require further investigation as it appears that dietary n-3 fatty acids may alter IL-2 secretion, IL-2 kinetics, IL-2 receptor expression and IL-2 messenger RNA (Jolly *et al.*, 1997, 1998). It is therefore possible that more than one mechanism may be modulating the effects of n-3 fatty acids on IL-2.

Table 1.19 The effect of n-3 feeding on cytokine generation by animal cells.

Reference	*Cell source*	*n-3 fat level and length of feeding*	*stimulus*	*Effect of n-3 PUFA*
Fernades *et al.*, 1994	Mouse spleen	100 g/kg FO, 6.5 months	Con-A	IL-2 decreased
Yaqoob & Calder, 1995b	Mouse spleen; culture in foetal calf serum	200 g/kg fish oil, 8 weeks	Con-A	IL-2 increase (ns) IL-4 increase (ns) IL-10 no effect IFN-γ no effect
Yaqoob & Calder, 1995a	Mouse spleen; culture in autologous plasma	200 g/kg fish oil, 8 weeks	Con-A	IL-2 no effect IL-4 no effect IL-10 no effect IFN-γ no effect
Jolly *et al.*, 1997	Mouse spleen culture in foetal calf serum	30 g/kg safflower oil + 20 g/kg EPA or DHA; 10 days	Con-A	IL-2 decreased in EPA and DHA groups

1.3.12 Effects of n-3 PUFA on T Cell Cytokines: Human Dietary Supplementation Studies

IL-2 production by mononuclear cells has been shown to be decreased following addition of fish oil to the human diet (Meydani *et al.*, 1991; Endres *et al.*, 1993; Gallai *et al.*, 1993; see table 1.16). Meydani *et al.* (1991) reported a decrease in the production of IL-2 by human peripheral blood mononuclear cells (PBMC) following fish oil supplementation in a group of older women compared with a group of young women. While the results of the study are often quoted, there are several ways in which this study can be criticised. This study did not include a placebo group. Therefore it cannot be know for certain if the results obtained were indeed the result of the supplementation. While there were significant differences in IL-2 production by lymphocytes from young and older women during fish oil supplementation, there were actually no differences compared with the initial baseline measurements for each group. Thus fish oil supplementation did not affect the concentrations of IL-2 produced by lymphocytes from either young or older women. Another criticism in this study is a bioassay was used to measure concentrations of IL-2 in the culture medium, which also contained 10% (v/v) autologous plasma. The type of serum or plasma in culture

medium has a significant effect on the IL-2 bioassay (Yaqoob & Calder, 1995b) but this was not accounted for in the above study. It is possible that the apparent decrease in IL-2 production was in fact due to an effect of plasma from fish oil supplemented subjects on the proliferation of the target cell line. The study by Endres *et al.* (1993) used an ELISA to measure the IL-2 concentration in the medium of cultured PBMCs during fish oil supplementation of the diet. There was a significantly lower IL-2 concentration in the medium of these cells only at 10 weeks post supplementation compared with baseline; no differences were observed during the supplementation period itself. The protocol in this case involved nine subjects all of whom consumed the supplement for 6 weeks and again there was no placebo group. It is possible that subtle differences in the conditions of the 10 weeks post supplementation experiment alone could have given rise to lower IL-2 concentrations measured at this time point. Furthermore it is difficult to reconcile this result with observations that the washout period for the general effects of fish oil supplementation is between 8 and 12 weeks. The effects of fish oil supplementation of the human diet on IL-2 production are therefore are not as clear cut as they might appear, as two of the major studies reporting an effect can be criticised in their design and execution. Given the importance of this cytokine to T cell clonal expansion and the immune response, the long term effects of dietary supplementation with n-3 fatty acids need to be investigated. Also no human studies to date have investigated the effects of n-3 fatty acids on the production of Th-2 cells by the cells of the immune system.

1.3.13 Studies Investigating the Effects of Dietary Fat on Macrophage •NO production: *Ex Vivo* Studies.

•NO is involved in many inflammatory reactions (see section 1.2.13). There have been a number of studies investigating the effects of dietary fish oil on nitric oxide production by macrophages. However, the results of these studies are conflicting (table 1.20). Two studies show decreased production of •NO, two studies show no effect and three show increased •NO production (table 1.20). Both studies that showed a decrease in •NO production used resident macrophages. The results of the studies on elicited or alveolar macrophages are inconclusive as one study shows no effect of n-3 feeding and three show an increased production of •NO.

Table 1.20 Ex vivo animal studies on the effects of n-3 fatty acids on •NO production

Reference	*Species*	*n-3 fat level and length of feeding*	*Macrophage type*	*Effect of n-3 PUFA*
Joe & Lokesh (1994)	Rat	80 g/kg fish oil, 6 weeks	Resident peritoneal	Decrease
Boutard *et al.* (1994)	Rat	150 g/kg fish oil, 6 weeks	Resident peritoneal	Decrease
Hubbard *et al.* (1994)	Mouse	100 g/kg fish oil, 3 weeks	Thioglycollate elicited peritoneal	No effect
Carbonell *et al.* (1997)	Rats	50 g/kg fish oil, 5 weeks	Blood monocytes	No effect
Renier *et al.* (1993)	Mouse	50 g/kg fish oil or 100 g/kg fish oil, 6 to 15 weeks	Thioglycollate-elicited peritoneal	Increase
Yaqoob & Calder (1995b)	Mouse	200 g/kg fish oil, 8 weeks	Thioglycollate elicited peritoneal	Increase
Chaet *et al.* (1994)	Rat	90 g/kg fish oil, 12 days	Alveolar	Increase

1.3.13.1 In Vitro Studies on Macrophage •NO Production

Results from *in vitro* studies of the effects of individual fatty acids added to cultures of macrophages and examining subsequent •NO production and iNOS transcription are more consistent than the feeding studies. In general n-3 fatty acids suppress •NO production *in vitro*. This is shown in a study by Ohata *et al.*, (1997) which examined the effects of DHA, EPA and ALNA on an LPS-stimulated macrophage cell line (RAW264 cells). This group found that each of the n-3 fatty acids caused suppression of •NO production in a dose- dependent manner. In contrast no inhibition was observed with linoleic, oleic or stearic acids. This group also suggest that the suppression is due to a suppression of induction of the iNOS gene. Lu *et al.*, (1998) also found that DHA inhibited •NO production by murine macrophages. DHA inhibited the induction of the iNOS gene in murine macrophages stimulated *in vitro* with IFN–γ plus LPS.

The effects of n-3 fatty acids on iNOS expression and •NO secretion might be explained by an *in vitro* study on a murine macrophage cell line by Milano *et al.*, (1995). This study has determined that PGE_2 at concentrations between 1 and 10 ng/ml is able to stimulate the expression of iNOS and the release of •NO, while higher concentrations (> 50 ng/ml) are inhibitory. This study showed that the inhibitory effect of PGE_2 on •NO synthesis depends on the strength of the activation signal given to the macrophage. As fish oil feeding can influence the amount of PGE_2 produced by macrophages upon stimulation, it may be that differences in the concentration of PGE_2 produced by cells of different dietary origins might be responsible for the conflicting results seen with •NO production and fish oil feeding. It may also be possible that PGE_3 produced by the cells might effect NO production but this possibility has never been examined. As n-3 fatty acids appear to modulate NO production, manipulating the ratio of n-3/n-6 fatty acids in the diet may be of use in the treatment of some diseases where the production of •NO may be beneficial or detrimental.

1.4 Dietary Fatty Acids and Inflammatory Mediator Production By Murine and Human Immune Cells

Earlier sections show that there have been a number of studies of the influence of dietary n-3 fatty acids on functional responses by various immune cell types and that many of these studies have focused on cytokine production. Despite the number of studies, the data do not provide a consistent picture. Some of these differences are most likely due to different methodologies that do not make comparisons between studies possible. Other differences are due to comparisons being made to differing placebos and also in the amounts and types of n-3 fatty acids under investigation. There is a need to systematically examine the influence of dietary fatty acids on cytokine production using comparable methodologies in order to determine what the precise effects of n-3 fatty acids are and to identify the factors which gives rise to the inconsistencies in the literature. Therefore, the purpose of the current study, is to investigate the effects of

fatty acids on the production of inflammatory mediators by murine and human immune cells. The hypothesis is that n-3 dietary fatty acids will decrease the production of pro-inflammatory mediators and that this will elicit a change in cell function.

The reasons for performing this study are that previous studies have provided contradictory results, most likely because they have differed in a number of key variables. The objectives of this study are to normalise these variables in order:

1. to examine the effect of dietary fatty acids on inflammatory mediator production by elicited murine peritoneal macrophages;
2. to examine the effect of dietary fatty acids on inflammatory mediator production by resident murine peritoneal macrophages;
3. to examine the effect of dietary fatty acids on macrophage cytotoxicity towards target cell lines;
4. to examine the effect of the type of serum or plasma used in culture on the production of inflammatory mediators by macrophages;
5. to examine the effect of dietary fatty acids on inflammatory mediator production and proliferation by spleen lymphocytes;
6. to investigate the effects of environmental stress in combination with diet on inflammatory mediator production by spleen lymphocytes;
7. to investigate the effect of supplementation of the diet of healthy human subjects with different doses of fish oil on peripheral blood mononuclear cell inflammatory mediator production and proliferation;

This will be the first time that the production of inflammatory mediators by both resident and elicited peritoneal macrophages from mice fed the same diets has been directly compared. By studying a range of mediators a better picture should emerge as to how dietary fatty acids, stimulus, and cell type interact to affect the types of mediators produced by each cell. The potencies of various fatty acids to modulate cytokine production, and the dose of fatty acid needed to do so, should be elucidated. The experiments reported in this thesis represent the most comprehensive study to date of the effect of dietary fatty acids on *ex vivo* production of inflammatory mediators by some of the key cells of the immune system.

Chapter 2: The effects of dietary fatty acids on inflammatory mediator production by murine macrophages in different activation states.

2.1 Introduction

As discussed in sections 1.2.4 and 1.2.5, macrophages secrete a number of mediators upon activation by inflammatory stimuli such as bacterial lipopolysaccharide (LPS). Tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) are the classical pro-inflammatory cytokines released by activated monocytes and macrophages (Auger, 1989). These cytokines serve to activate other cells involved in the immune response such as neutrophils, T and B lymphocytes, antigen presenting cells and endothelial cells. In addition, these cytokines mediate the systemic effects of inflammation such as fever and hepatic acute phase protein synthesis. The production of appropriate amounts of these cytokines is beneficial in the response to infection (Abbas *et al.*, 1994). However, inappropriate or overproduction of these inflammatory mediators can be dangerous and TNF-α, IL-1β and IL-6 are implicated in causing some of the pathological responses which occur in endotoxic shock, adult respiratory distress syndrome and other acute and chronic inflammatory conditions (Grimble, 1996). Eicosanoids such as PGE_2 are synthesised from AA as discussed in section 1.1.9. High concentrations of PGE_2 suppress the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Renz *et al.*, 1988; Knudsen *et al.*, 1986; Spinas *et al.*, 1991). LTB_4 is also synthesised from AA and in contrast to the effects of PGE_2, high concentrations of LTB_4 enhance the production of the above cytokines (Gagnon *et al.*, 1989; Schade *et al.*, 1989; Dubois *et al.*, 1989; Rola-Pleszczynski & Lemaire, 1985). Diets high in FO, rich in the n-3 fatty acids EPA and DHA, have been shown to lead to the replacement of a proportion of the AA in immune cell membrane phospholipids with EPA. This leads to a decrease in the amount of PGE_2 and LTB_4 produced by these cells (see section 1.1.9), and therefore it is possible that dietary fatty acids may influence the production of cytokines by immune cells through modulating LTB_4 and PGE_2 levels. The effect of dietary fatty acids on the production of inflammatory mediators by murine macrophages has been examined over many years. However, there are many conflicting reports in the literature (see section 1.3.5). Studies in this area are difficult to compare because of different experimental protocols used.

As discussed in section 1.3.5, previous studies have differed in the amounts and/or types of dietary fat fed to mice; the type of mouse studied (C57BL/6, Balb C etc.); the activation state of the macrophage (resident, elicited); the agent used to elicit the macrophages (thioglycollate, Complete Freunds' Adjuvant (CFA)); the anatomical site from which the macrophages were obtained (peritoneum, liver, lung); the stimulus given to activate the macrophages in culture (LPS, IFN-γ), the mediators measured (cytokines, eicosanoids, free radicals); the length of time the cells were cultured, the type of plasma or serum used in the culture medium (either autologous plasma or foetal calf serum), and the method of measurement of the mediators (ELISA, RIA) (Calder, 1995). The activation state of the macrophages studied may have a profound impact on the observed results of a feeding study. In general, studies that have examined the effects of diets high in n-3 fatty acids elicited (activated) macrophages have found decreased pro-inflammatory cytokine production (Yaqoob & Calder, 1995; Renier *et al.*, 1993). In contrast, studies which have examined the effects of diets high in n-3 fatty acids on resident macrophages have found increased pro inflammatory cytokine production (see section 1.3.5). The effects of diets rich in n-3 fatty acids on cytokine production by macrophages in the different state of activation have never been directly compared. This was done in the current study for the first time. The study reported in this chapter used a single mouse species (C57BL/6) fed a range of types of fat at a single total fat concentration (that which is close to the typical UK diet in terms of percent calories from fat), two types of macrophage (resident and elicited) isolated from one anatomical location (peritoneum) were studied and the production of a range of mediators (PGE_2, IL-1β, IL-6, TNF-α, IL-10, IL-12, •NO, LTB_4, LTC_4) was studied. By investigating the production of a range of mediators under the same conditions, it was hoped to clarify the exact effects of the different families of dietary fatty acids on inflammatory mediator production, and also to further elucidate the relationship between the eicosanoids and cytokines produced by these cells.

2.2 Objective

To examine the effects of diets rich in certain fatty acids on mediator production by stimulated and unstimulated macrophages. It was hypothesised that the apparent contradictory effects observed for resident and thioglycollate-elicited macrophages

from fish oil-fed rodents relate to different capacities of the different types of cells to produce pro-inflammatory eicosanoids such as PGE_2 and LTB_4. To examine this mice were fed a variety of high fat diets including one rich in FO and the production of TNF-α, IL-1β, IL-6, NO, IL-10, IL-12, LTB_4, and LTC_4, and PGE_2 by resident and elicited peritoneal macrophages was measured. This is the first time this comparison has been made.

2:3 Methods

2.3.1 Animals and diets

Male C57BL/6 mice weighing between 20 and 25 grams were purchased from Charles River, Margate, UK. They were housed in individual cages in the climate controlled animal house at the School of Biological Sciences, University of Southampton; a 12-hour light/dark cycle was used. They were allowed access ad libitum to water and to one of five experimental diets (prepared by ICN Biomedicals, High Wycombe, Bucks, UK) for a seven week period. These were a low fat (LF) diet which contained approximately 2.5% by weight of corn oil and four high fat diets which contained 20% by weight of hydrogenated coconut oil (CO), olive oil (OO), safflower oil (SO) or menhaden (fish) oil (FO) (plus 1% corn oil to prevent essential fatty acid deficiency). All diets contained 20% protein, 20% starch, 29.6% sucrose, and 0.12% vitamin E (250 international units/gram). The fatty acid compositions of the diets are shown in table 2.1. The LF diet most closely resembles standard mouse chow. Mice used for preliminary experiments were fed on standard laboratory chow.

Table 2.1 Fatty acid composition of the diets used (g/100g fatty acids; nd indicates not detected)

Diet	*Total fatty acids (g/kg diet)*	*10:0*	*12:0*	*14:0*	*16:0*	*16:1 n-7*	*18:0*	*18:1 n-9*	*18:2 n-6*	*18:3 n-3*	*20:5 n-3*	*22:6 n-3*
LF	25	nd	3.4	3.5	13.7	nd	3.3	21.9	54.3	nd	nd	nd
CO	210	6.8	56.5	17.6	7.8	nd	5.1	2.0	2.3	nd	nd	nd
OO	210	nd	nd	nd	14.9	2.2	3.7	60.0	19.1	nd	nd	nd
SO	210	1.6	4.3	1.1	8.7	nd	3.9	19.4	61.0	nd	nd	nd
FO	210	nd	nd	10.3	22.0	14.5	4.2	11.8	9.0	3.5	10.6	10.1

2.3.2 Chemicals

Sodium nitrite, RPMI, napthylethylenendiamine, sulfanilamide, lipopolysaccharide (LPS), glutamine, penicillin, streptomycin, foetal calf serum (FCS), histopaque, and phosphoric acid were all obtained from Sigma Chemical Co. (Poole, UK). Brewer's thioglycollate broth was obtained from Difco, East Moseley, UK. The PGE_2 ELISA kits were obtained from Neogen (Lexington, USA). LTB_4 and LTC_4 ELISA kits were obtained from Cayman Chemical (Ann Arbor, USA). The cytokine ELISA kits were from Biosource International (Camarillo, USA).

2.3.3 Preparation of Brewer's thioglycollate broth

Thioglycollate broth was prepared according to the manufacturer's instructions. Briefly, 4.05 g was dissolved in 100 ml distilled H_20 and then heated over a bunsen burner for 15 minutes until the solution turned golden. The solution was allowed to cool and was then autoclaved and stored in the dark until use.

2.3.4 Preparation of culture medium

Cell culture medium consisted of 500 ml RPMI, 2 mM glutamine, 50 ml sterile, heat-inactivated FCS and 0.5 ml of a 1:1 50μg/ml mixture of streptomycin and penicillin.

2.3.5 Thioglycollate-elicited macrophage preparation

Mice were injected intraperitoneally with 1 ml of Brewer's thioglycollate broth prepared as described above. The thioglycollate causes macrophages to migrate to the peritoneal cavity and differentiate into a semi-active state. The mice were killed four days later by CO_2 asphyxiation and the peritoneal cell population was collected by washing out the peritoneal cavity with 4 ml of sterile phosphate-buffered saline. The cells were collected by centrifugation, poured through sterile lens tissue paper to remove any contaminating debris, pelleted again by centrifugation (5 minutes at 1500 rpm) and re-suspended in 1 ml culture medium. The cells were then counted and diluted to a density of 1 x 10^6 cells/ml with culture medium. Analysis of the preparation by flow cytometry indicated that 95% of the cells collected in this manner were macrophages (results not shown).

2.3.6 Resident macrophage preparation

Peritoneal cells were collected from mice in a manner similar to the collection of elicited macrophages described above, except that no thioglycollate injection was given prior to sacrifice. Cells were plated on petrie dishes and placed in an incubator at 37°C for four hours in order for macrophages to adhere to the petrie dish surface. Non-adhering cells were then removed by gentle washing and adhering macrophages were removed from the bottom of the petrie dish with the use of a sterile cell lifter. These cells were re-suspended in culture medium, counted and finally diluted to a concentration of 1 x 10^6 cells/ml.

2.3.7 Macrophage culture

Macrophages (resident or elicited) were suspended at a density of 1 x 10^6 cells/ml in culture medium. 1 ml of cells was added to each of two wells of a 24 well plate along with 800 µl of RPMI and 200 µl 100 µg/ml LPS (to give a final LPS concentration of 10 µg/ml) or RPMI. Preliminary experiments using a range of LPS concentrations (0, 5, 10, 20, 50, 100 µg/ml) indicated that 10 µg/ml LPS resulted in maximal production of TNF-α, IL-1β, IL-6 and PGE_2 by thioglycollate elicited macrophages from chow-fed mice (data not shown). The cells were then incubated at 37°C in an atmosphere of 19:1 air:CO_2. After 24 hours the plate was centrifuged for 5 minutes at 1500 rpm. The supernatants were collected and aliquoted into sterile eppendorf tubes. These aliquots were frozen at –20°C for later measurement of mediator concentrations.

2.3.8 Cytokine and eicosanoid assays

The concentrations of IL-1β, IL-6, IL-10, IL-12, TNF-α, PGE_2, LTB_4, LTC_4 and •NO were measured in macrophage culture media generated according to section 2.3.7. The PGE_2, LTB_4 and LTC_4 ELISAs operated on the basis of competition between the enzyme conjugate and the eicosanoid in the sample for a limited number of binding sites on the antibody-coated plate. The cytokine ELISAs kits use a biotin-avadin enhanced immunoassay. For all assays the manufacturer's instructions were followed. Assay standard curves are given in appendix 1.

2.3.9 Nitrite assay

In aqueous solution •NO is converted to NO_2 (nitrite) and NO_3 (nitrate). This means that either or both of these can be used as a measure of •NO production. Samples (standards of sodium nitrite dissolved in RPMI at concentrations up to 60 μM or macrophage culture supernatants) were analysed by adding 100 μl of standard or sample to an equal volume of Griess reagent (0.5 g napthylethylenediamine and 0.5 g sulfanilamide in 100 ml of 2.5 (v/v) phosphoric acid) in a 96 well flat-bottomed tissue culture plate. After 10 minutes the absorbance was read on a plate reader at a wavelength of 550 nm. Nitrite concentrations were calculated by reference to a standard curve of sodium nitrite prepared in parallel.

2.3.10 Fatty acid analysis by gas chromatography

Frozen cells were resuspended in 0.5 ml PBS, transferred to glass tubes, and 3 ml of chloroform/methanol (2:1 v/v) added. The tubes were sealed under nitrogen, vortexed and left for 15 minutes. They were then gently centrifuged. This separated the sample into three layers: an aqueous layer, a layer of cellular debris and a bottom layer of chloroform, which contained the lipid soluble fractions of the sample. The lipid layer was transferred by glass pipette into clean glass tubes. The procedure was repeated on the original sample tube. The extracts (approximately 4 ml) were then washed twice with 8.8 g/l potassium chloride. Briefly 2 ml of the 8.8 g/l KCl mixture was added to the sample tubes. The tubes were then sealed under nitrogen, vortexed and centrifuged gently to separate the layers. The top layer was then removed by aspiration. This process was repeated for two KCl washings. After the second washing, the extracts were dried down under nitrogen, and then 100 μl of methanol and 1 ml of 0.5 M KOH was added. The sample was once again sealed under nitrogen and then placed in a water bath at 80°C for 3 hours in order for a saponification reaction to take place. After completion and cooling, the reaction mixture was neutralised by the addition of 50 μl 5M H_2SO_4.

The choloroform:methanol extraction was then repeated as were the KCl washings. The samples were then transferred to clean glass vials, and dried under nitrogen. The samples were then methylated by adding 10 μl of methanol and then 1 ml

diazomethane in ether. The samples were dried under nitrogen, resuspended in 10 µl hexane, and 2 µl was injected via a Hamiliton syringe into a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, Avondale, PA) fitted with a 30 m x 32 mm BPX70 capillary column, film thickness 0.25 µm. Helium at 2.0 ml/min was used as the carrier gas, and the split/splitless injector was used with a split:splitless ratio of 10:1. The separation was recorded with HP GC Chemstation software. Fatty acid methyl esters were identified by comparison with standards run previously.

2.3.11 Statistical analysis

Statistical analysis was performed using SPSS. Means and standard errors are reported. Differences between the groups were determined by one-way ANOVA followed by the least significant difference post-hoc test; $P<0.05$ was taken to indicate significant differences. Spearman correlation coefficients were used to determine associations between cytokine and eicosanoid concentrations.

2.4.Results

2.4.1 The effects of dietary lipids on food intake and weight gain

Figures 2.1 and 2.2 show the growth and food intake of mice fed the different diets over the seven week period. There were no significant differences in body weights between the dietary the groups at baseline. However, by the end of the seven week period, the mice fed on the OO and the SO diets were significantly heavier than the mice on the other diets (figure 2.1). Throughout the study period the mice fed on the LF diet ate significantly more food than the mice fed on the other diets (figure 2.2). This was probably due to the fact that this diet was lower in calories than the high fat diets and therefore the mice would have had to eat a greater amount in order to meet their caloric requirements. The FO and CO fed mice, despite having similar food intakes to the other high-fat fed mice, did not have a similar pattern of weight gain. Mean weight gain and food intake per week are shown in table 2.2.

Figure 2.1 Weight of mice fed the different diets.
Data are means for 6 animals per diet

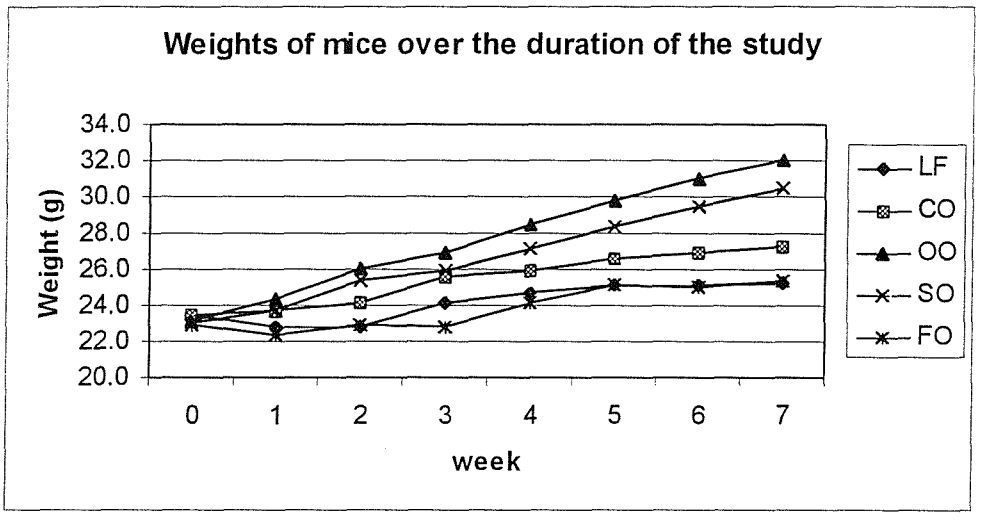


Figure 2.2 Food intake of mice on the different diets
Data are means for 6 animals per diet.

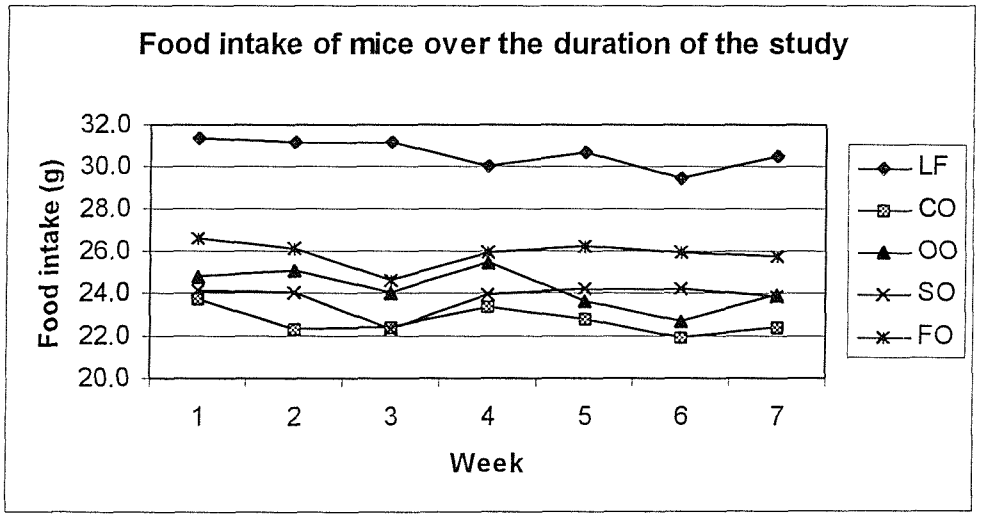


Table 2.2 Weight gain and food intake of mice fed on the different diets (n=6 mice per dietary group). Values in a column not sharing the same superscript letter are significantly different.

Diet	*Weight gain (g/ week)*	*Se*	*Food intake (g/ week)*	*se*
LF	0.33^{a}	0.05	30.6^{a}	0.05
CO	0.54^{ab}	0.03	22.8^{b}	0.06
OO	1.26^{b}	0.02	24.1^{b}	0.11
SO	1.07^{b}	0.02	23.6^{b}	0.13
FO	0.35^{a}	0.02	25.6^{b}	0.07

2.4.2 Total fatty acid composition of resident and elicited macrophages

The fatty acid composition of the diet markedly affected the fatty acid composition of the elicited macrophages (see table 2.3). Elicited macrophages from mice fed the LF diet contained a significantly higher proportion of 20:4n-6 than those from the mice fed the OO, SO or FO diets. Macrophages from mice fed the CO diet contained a higher proportion of 14:0 than mice fed each of the other diets except for FO. Macrophages from mice fed the OO diet contained a significantly higher proportion of 18:1n-9 than those from mice fed each of the other diets. Macrophages from mice fed the SO diet had a significantly higher proportions of 18:2n-6 and significantly lower incorporation of 16:1n-7 than those from mice fed each of the other diets. Macrophages from mice fed the FO diet had significantly lower proportions of 18:1n-9, 18:2n-6, 20:3 n-6 and 20:4n-6 than macrophages from mice fed each of the other diets. These macrophages contained higher proportions of 20:5n-3, 22:5n-3, and 22:6n-3. The elicited macrophages from mice fed the LF, CO, OO, and SO diets contained an unknown fatty acid in their cell membranes. However, this fatty acid was not detected in the macrophages from mice fed the FO diet. This may indicate that the unknown fatty acid is one of the long chain n-6 fatty acids.

The fatty acid composition of resident macrophages was not as strongly affected by diet (table 2.4). The only fatty acid which was different between the groups was 18:2n-6 which was highest in the macrophages from mice fed the LF and CO diets, and

lowest in the macrophages from mice fed the FO diet. The n-3/n-6 ratio was significantly higher in resident macrophages from mice fed the FO diet.

Table 2.3 Fatty acid composition of elicited macrophages (g/100g fatty acid). All values are means and Standard errors (SE) for 4-6 animals fed each diet. Values across a row not sharing the same alphabetical superscript are significantly different.

Fatty Acid	*LF*	*Se*	*CO*	*Se*	*OO*	*Se*	*SO*	*Se*	*FO*	*Se*
14:0	0.4^{a}	0.4	3.2^{bc}	0.4	0.2^{a}	0.2	0.6^{a}	0.4	1.9^{ab}	1.0
16:0	20.5^{bc}	1.1	20.9^{ab}	1.3	16.0^{a}	1.0	19.3^{ab}	0.9	19.4^{bc}	0.3
16:1n-7	1.7^{bc}	0.6	3.1^{bc}	0.9	1.0^{ab}	0.3	0^{a}	0	2.6^{bc}	0.2
18:0	15.2^{ab}	0.6	17.7^{bc}	2.2	12.5^{a}	0.9	16.8^{bc}	0.6	16.7^{bc}	0.3
18:1n-9	14.8^{c}	0.6	15.1^{c}	0.7	28.7^{d}	1.8	10.7^{b}	0.2	7.9^{a}	0.4
cis-18:1	3.9^{b}	0.8	4.5^{a}	1.2	3.4^{b}	0.1	2.0^{b}	0.1	3.9^{b}	0.1
18:2n-6	12.0^{c}	0.3	9.8^{b}	0.4	9.2^{b}	0.4	22.6^{d}	0.4	6.9^{a}	0.3
20:3n-6	0.8^{ab}	0.4	1.1^{bc}	0.5	1.5^{bc}	0.1	2.0^{dc}	0.2	0^{a}	0
20:4n-6	20.1^{d}	0.9	18.1^{cd}	0.9	15.1^{b}	1.1	16.0^{bc}	1.0	8.7^{a}	0.4
20:5n-3	0^{b}	0	0^{b}	0	0^{b}	0	0^{b}	0	23.1^{a}	11.3
22:5n-3	0.3^{b}	0.3	0.2^{b}	0.2	0^{b}	0	0^{b}	0	10.7^{a}	0.3
22:6n-3	0.8^{b}	0.6	0.6^{b}	0.6	1.8^{b}	0.1	0^{b}	0	20.4^{a}	11.2
Unknown	7.5^{c}	0.3	3.5^{b}	0.9	6.1^{c}	0.7	7.4^{c}	0.3	0^{a}	0
n-3/n-6	0.06^{b}	0.04	0.04^{b}	0.04	0.1^{b}	0	0^{b}	0	3.7^{a}	0.1

Table 2.4 Total fatty acid composition of resident macrophages (g/100g fatty acid). Data are based on 3 samples consisting of mixtures of cells obtained from 4 mice within each dietary group. Values are means and Se. Values across a row not sharing the same alphabetical superscript are significantly different.

Fatty acid	*LF*	*Se*	*CO*	*Se*	*OO*	*Se*	*SO*	*Se*	*FO*	*Se*
14:0	4.5	0.4	4.5	0.4	4.1	1.5	3.8	0.6	3.8	0.3
16:0	30.9	5.6	24.2	3.2	28.0	1.9	28.2	0.7	29.8	1.6
16:1n-7	2.7	1.6	2.3	0.03	3.2	0.1	2.1	0.3	3.3	0.4
18:0	24.9	7.1	20.7	1.6	16.7	2.5	24.2	0.4	18.7	1.6
18:1n-9	10.6	1.5	15.0	0.1	26.3	7.4	13.8	4.4	23.3	3.7
18:2n-6	7.8^{a}	1.1	8.2^{a}	4.6	5.0^{ab}	1.0	5.6^{ab}	0.7	2.0^{b}	0.2
18:3n-3	0	0	0	0	0	0	0	0	0.8	0.8
20:4n-6	13.8	6.9	19.9	3.8	11.8	2.7	16.8	3.7	13.0	1.4
20:5n-3	3.1	0.5	2.7	1.0	2.3	0.5	3.0	0.3	3.3	0.7
22:6n-3	1.6	0.6	2.7	1.0	2.4	0.9	2.3	1.0	2.0	0.3
n-3/n-6	0.2^{b}	0.1	0.2^{b}	0	0.3^{b}	0	0.2^{b}	0	0.4^{a}	0.1

2.4.3 Mediator production by peritoneal macrophages from mice fed different diets

2.4.3.1 PGE_2 Production

Figures 2.3 and 2.4 show the concentrations of PGE_2 produced by peritoneal macrophages. PGE_2 production was substantially greater (at least 7-fold and up to 30-fold) by LPS-stimulated elicited macrophages than for unstimulated elicited macrophages or LPS-stimulated resident macrophages. Unstimulated elicited macrophages produced similar amounts of PGE_2 regardless of their dietary origin. This was similar to the amount of PGE_2 produced by LPS-stimulated resident macrophages. LPS-stimulated elicited macrophages from the FO-fed mice had significantly lower PGE_2 production than those from each of the other groups. Among the other groups PGE_2 production by the OO and SO groups were greater than by the CO and LF groups. Resident macrophages from mice fed the FO diet produced the lowest amount of PGE_2. This was significantly lower than the amount of PGE_2 produced by resident macrophages from mice fed the CO, SO and LF diets.

2.4.3.2 LTB_4 Production

Figures 2.5 and 2.6 show LTB_4 production by macrophages fed the different diets. All cell types produced similar amounts of LTB_4 whether or not they had been stimulated by LPS. This may be due to the fact that the 5-LOX enzyme is not induced by LPS stimulus (Nettleton, 1995). There were no statistically significant differences among the dietary groups for either unstimulated or LPS-stimulated elicited macrophages. Unstimulated elicited macrophages fed the FO diet however tended to produce the lowest amount of LTB_4. Resident macrophages from mice fed the FO diet produced the lowest amount of LTB_4 upon LPS stimulation; this was significantly different from the LF group.

2.4.3.3 LTC_4 Production

Figures 2.7 and 2.8 show LTC_4 production by the murine macrophages. Unstimulated elicited macrophages from mice fed the SO diet tended to produce the greatest amount of LTC_4 and cells from the LF-fed mice the least, although there were no statistically

significant differences among the groups. LPS-stimulated elicited macrophages from the SO fed mice significantly higher concentrations of LTC_4 than the CO and LF groups. There were no statistically significant differences in LTC_4 production by LPS-stimulated resident macrophages from mice fed the different diets. LPS-stimulated resident macrophages produced about 10-fold more LTC_4 than elicited macrophages.

Figure 2.3 PGE_2 production by unstimulated and LPS-stimulated elicited macrophages. Data are means ± for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the stimulated macrophage group are significantly different. Bars not sharing the same number across the unstimulated macrophage group are significantly different.

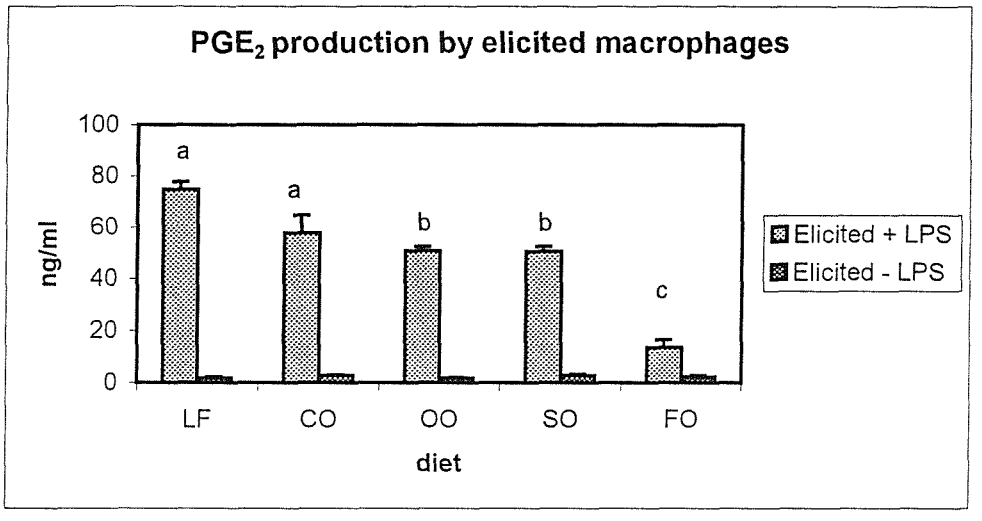


Figure 2.4 PGE_2 production by LPS-stimulated resident and elicited macrophages. Data are means ± for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the elicited macrophage group are significantly different. Bars not sharing the same number across the resident macrophage group are significantly different.

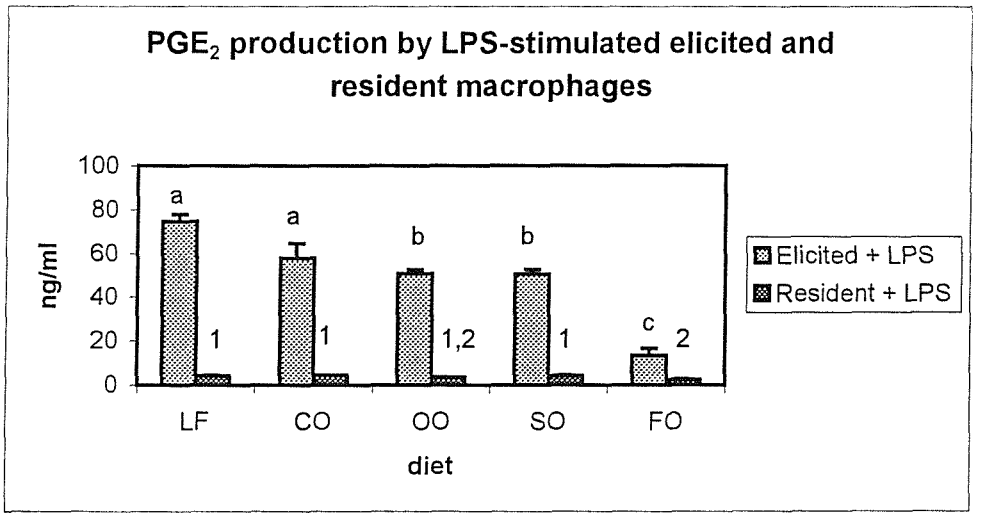


Figure 2.5 LTB_4 production by unstimulated and LPS stimulated elicited macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well.

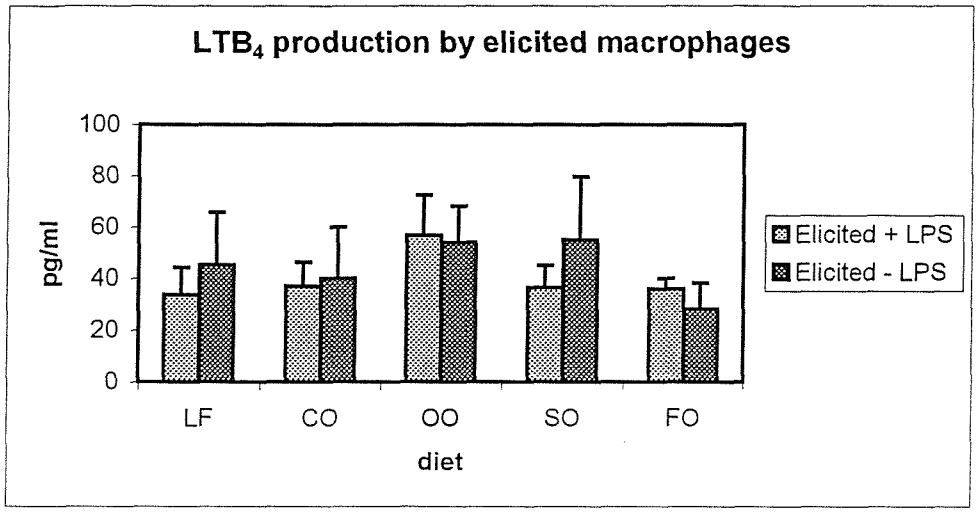


Figure 2.6 LTB_4 production by LPS-stimulated elicited and resident macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Columns not sharing the same number are significantly different.

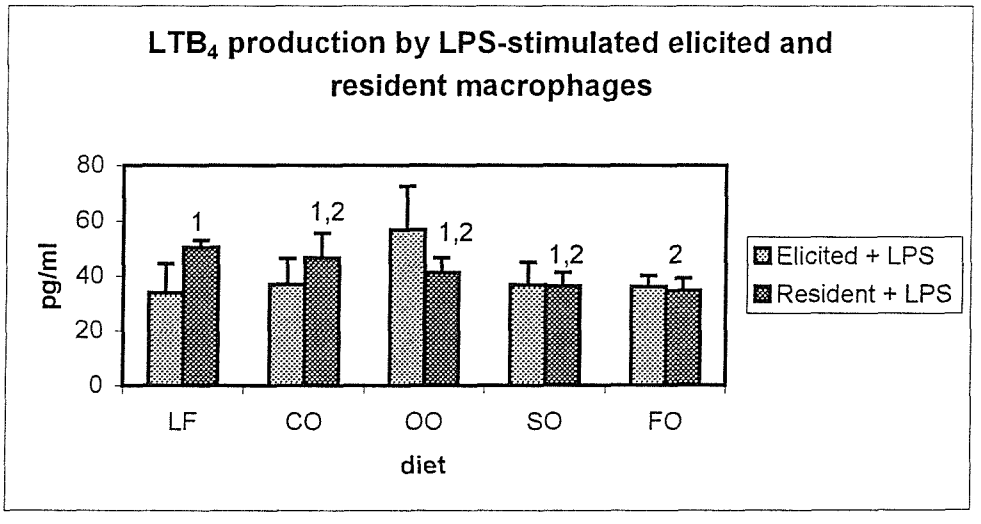


Figure 2.7 LTC_4 production by unstimulated and LPS-stimulated elicited macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the stimulated macrophage group are significantly different.

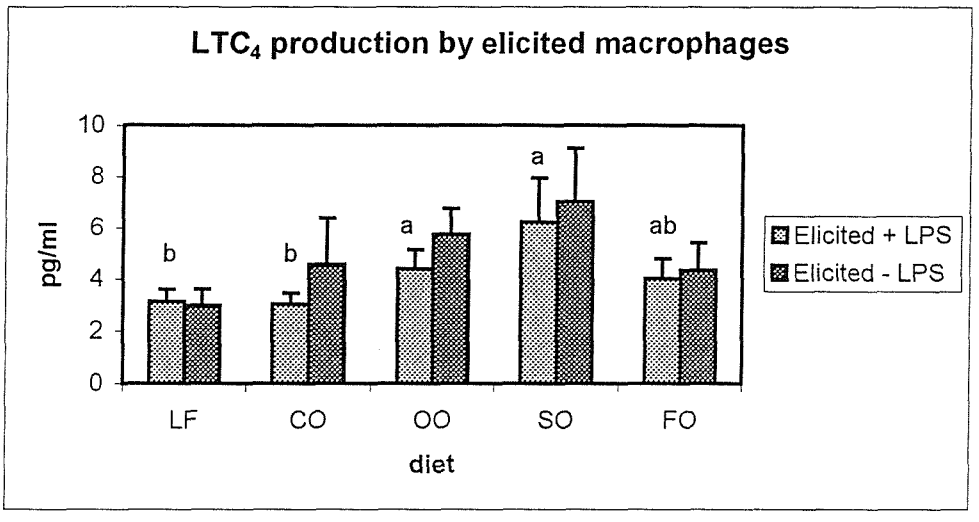
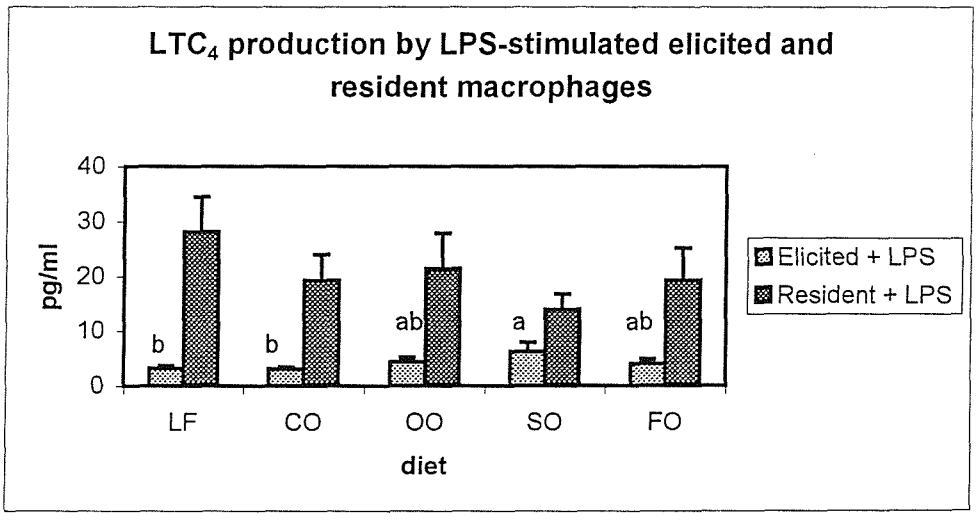


Figure 2.8 LTC_4 production by LPS-stimulated elicited and resident macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the elicited macrophage group are significantly different.



2.4.4 Cytokine production

2.4.4.1 TNF-α Production

Figure 2.9 and 2.10 show TNF-α production by macrophages. LPS significantly increased the concentration of TNF-α in the media of elicited macrophages from mice fed the LF or SO diets but not from those fed CO, OO or FO. LPS-stimulated elicited macrophages from mice fed the FO diet produced the lowest concentration of TNF-α. This was significantly lower than the concentrations produced by LPS-stimulated macrophages from animals fed the LF, CO or OO diets. Elicited macrophages from mice fed the SO diet and cultured with LPS also had decreased production of TNF-α compared to those from LF or CO fed animals. The concentrations of TNF-α in the supernatants of unstimulated elicited macrophages was lowest for those from mice fed the SO and FO diets. LPS-stimulated elicited macrophages produced a greater concentration of TNF-α than did LPS-stimulated resident macrophages, except for those from the FO fed mice. Resident macrophages from mice fed the FO diet produced a higher concentration of TNF-α when stimulated with LPS, and those from animals fed the CO diet the least. The pattern of TNF-α production from the macrophages from mice fed the FO diet was the opposite to that for macrophages from mice fed the other diets.

2.4.4.2 IL-1β Production

Figure 2.11 and 2.12 illustrate IL-1β production by macrophages. LPS-stimulated elicited macrophages from mice fed the FO diet produced significantly less IL-1β than those from mice fed each of the other diets. Unstimulated elicited macrophages from mice fed the LF, CO and OO diets produced significantly more IL-1β than did those from mice fed the SO and FO diets. LPS stimulation of elicited macrophages caused a significant increase in IL-1β production by macrophages from mice fed the LF, CO and SO diets, but not the OO or FO diets. There were no significant differences between the concentration of IL-1β produced by resident macrophages from the different dietary groups.

2.4.4.3 IL-6 Production

Figure 2.13 and 2.14 show IL-6 production by macrophages. Unstimulated elicited macrophages from mice fed the OO diet produced the greatest amount of IL-6. This was significantly greater than the amount of IL-6 produced by macrophages from mice fed the CO diet. IL-6 production by LPS-stimulated macrophages was significantly greater than from macrophages that were not exposed to LPS. LPS-stimulated macrophages from mice fed the FO diet tended to produce less IL-6 than those from mice fed the CO or OO diets. However, there were no significant differences in IL-6 production between the groups. Resident macrophages from FO and LF fed mice produced significantly less IL-6 than those from mice fed the SO diet. Resident macrophages produced about half the amount of IL-6 produced by elicited macrophages upon stimulation with LPS.

Figure 2.9 TNF-α production by unstimulated and LPS-stimulated elicited macrophages. Data are means ± se for 6 animals per diet, and $2x10^6$ cells per well. Bars not sharing the same letter across the stimulated macrophage group are significantly different. Bars not sharing the same number across the unstimulated macrophage group are significantly different.

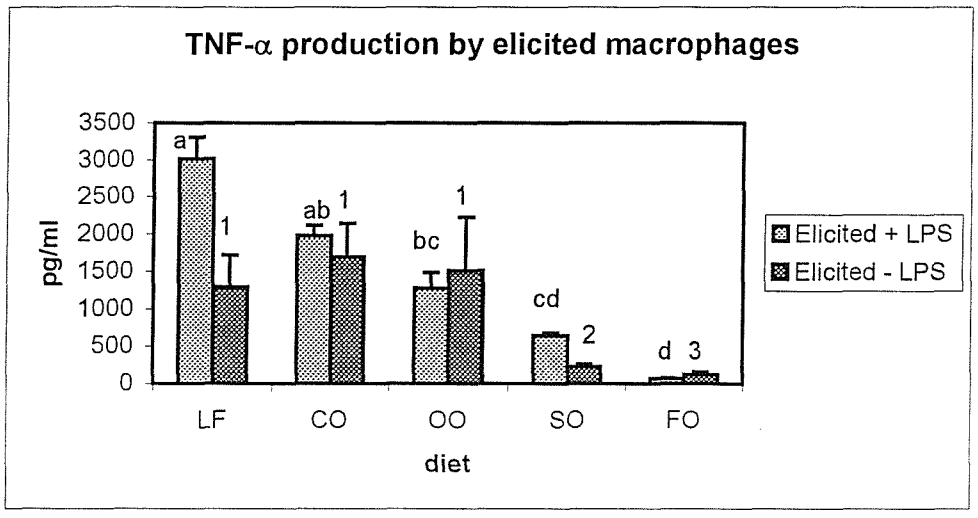


Figure 2.10 TNF-α production by LPS-stimulated elicited and resident macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the elicited macrophage group are significantly different. Bars not sharing the same number across the resident macrophage group are significantly different.

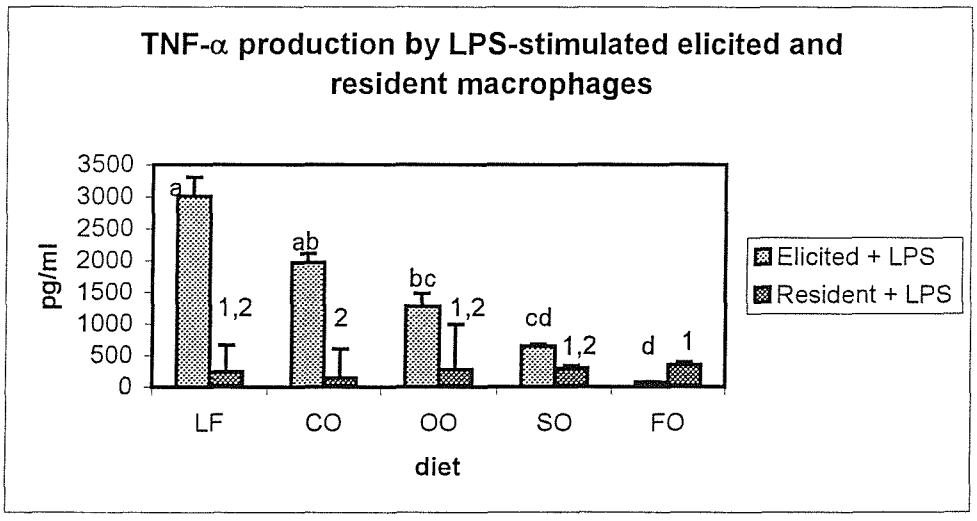


Figure 2.11 IL-1β production by unstimulated elicited, and LPS-stimulated elicited macrophages. Data are means (se) for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the stimulated macrophage group are significantly different. Bars not sharing the same number across the unstimulated macrophage group are significantly different.

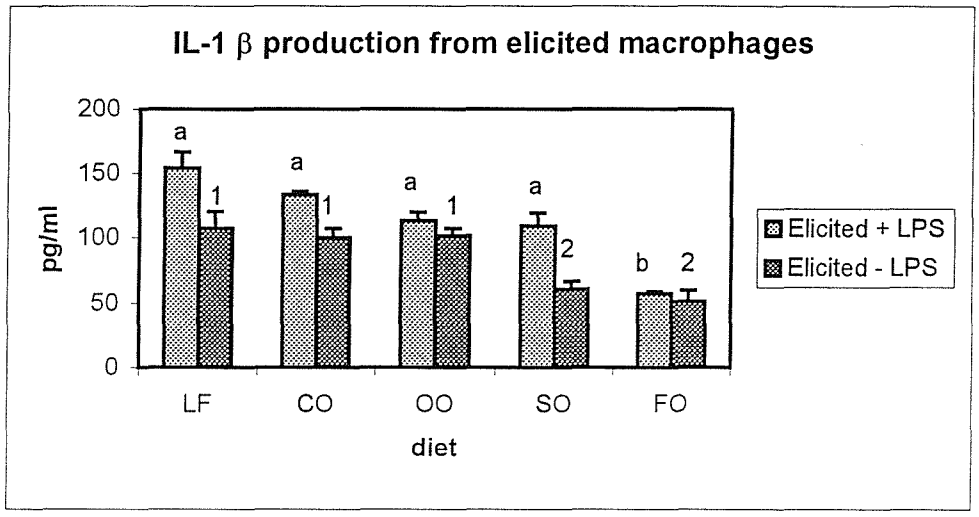


Figure 2.12 IL-1β production by LPS-stimulated elicited and resident macrophages. Data are means (se) for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the elicited macrophage group are significantly different.

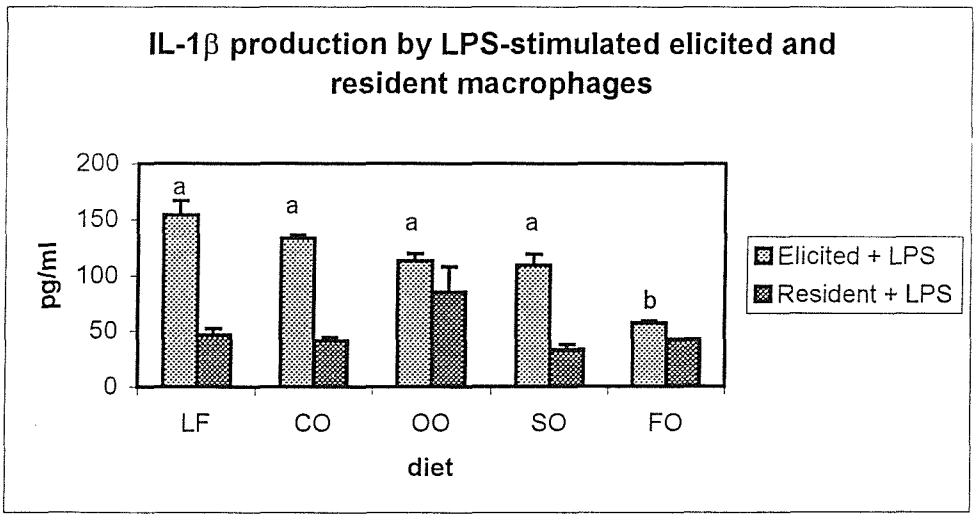


Figure 2.13 IL-6 production by unstimulated elicited macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the unstimulated macrophage group are significantly different. (For IL-6 production by LPS-stimulated elicited macrophages see figure 2.14)

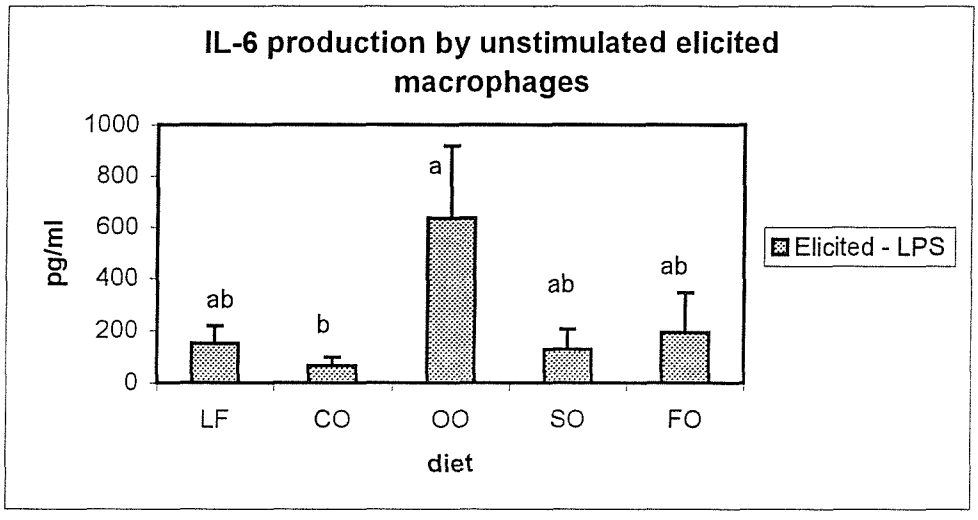
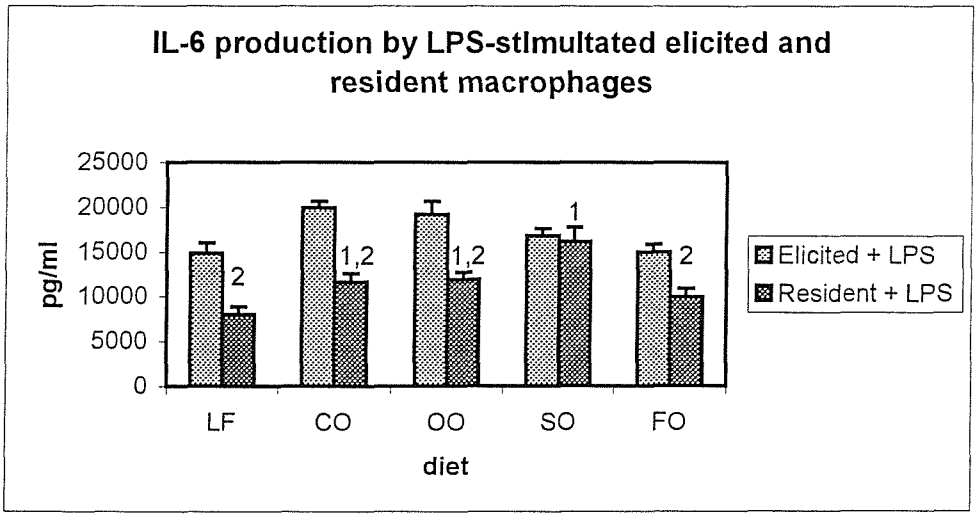


Figure 2.14 IL-6 production by LPS-stimulated elicited and resident macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same number across the resident macrophage group are significantly different.



2.4.4.4 IL-10 Production

Figures 2.15 and 2.16 show IL-10 production by macrophages. LPS-stimulated elicited macrophages from mice fed the CO diet produced significantly less IL-10 than those from mice fed the LF diet. Unstimulated elicited macrophages from mice fed the LF and FO diets produced significantly more IL-10 than macrophages from mice fed the OO diet. LPS stimulation did not greatly alter the amount of IL-10 produced by elicited macrophages from mice fed the CO and FO diets. LPS doubled the amount of IL-10 produced in the OO group and increased the amount produced by the LF and SO groups by almost 50%. Resident macrophages from mice fed the SO and FO diet produced significantly more IL-10 than those from mice fed the other diets.

2.4.4.5 IL-12 Production

Resident macrophages did not produce detectable levels of IL-12. Figure 2.17 shows IL-12 production by elicited macrophages. IL-12 production by LPS-stimulated elicited macrophages was 2-fold higher than that from unstimulated elicited macrophages. Macrophages from mice fed the LF diet tended to produce the greatest amount of IL-12, although there were no statistically significant differences between the groups.

2.4.4.6 •NO Production

Figures 2.18 and 2.19 show •NO production by macrophages. LPS-stimulated elicited macrophages from mice fed the CO diet produced significantly less •NO than those from mice fed the other diets. Unstimulated elicited macrophages from LF-, OO- and FO-fed mice produced significantly more •NO than unstimulated macrophages from mice fed the SO or CO diets. Resident macrophages from mice fed FO produced the lowest amount of nitric oxide and those from mice fed the LF diet produced the greatest amount. These two groups were significantly different ($P<0.05$). LPS-stimulated elicited macrophages produced four times as much •NO as compared to LPS-stimulated resident macrophages.

Figure 2.15 IL-10 production by unstimulated elicited, and LPS-stimulated elicited macrophages. Data are means (se) for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the stimulated macrophage group are significantly different. Bars not sharing the same number across the unstimulated macrophage group are significantly different.

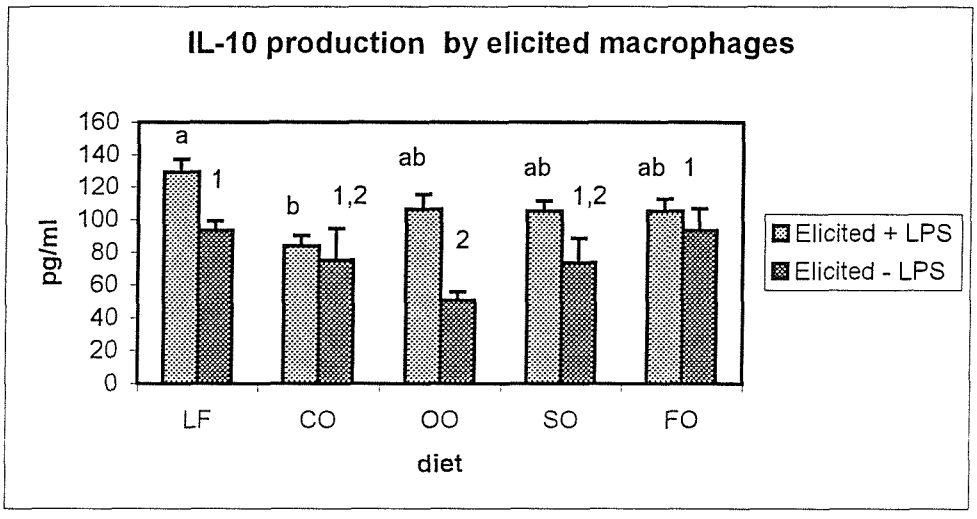


Figure 2.16 IL-10 production by LPS-stimulated elicited and resident macrophages. Data are means (se) for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the elicited macrophage group are significantly different. Bars not sharing the same number across the resident macrophage group are significantly different.

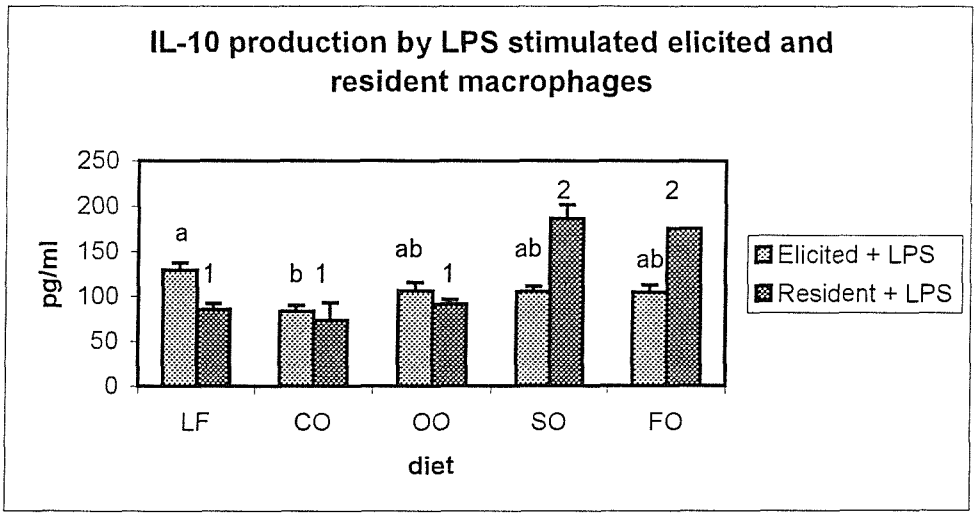


Figure 2.17 IL-12 production by elicited macrophages.
Data are mean ± se for 6 animals per diet and $2x10^6$ cells per well.

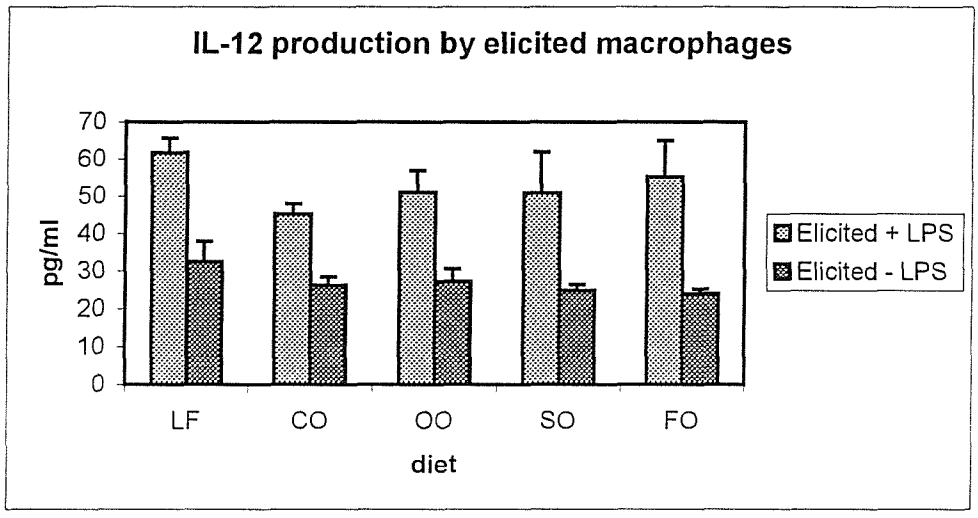


Figure 2.18 •NO production by unstimulated and LPS-stimulated elicited macrophages. Data are means ±se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the stimulated macrophage group are significantly different. Bars not sharing the same number across the unstimulated macrophage group are significantly different.

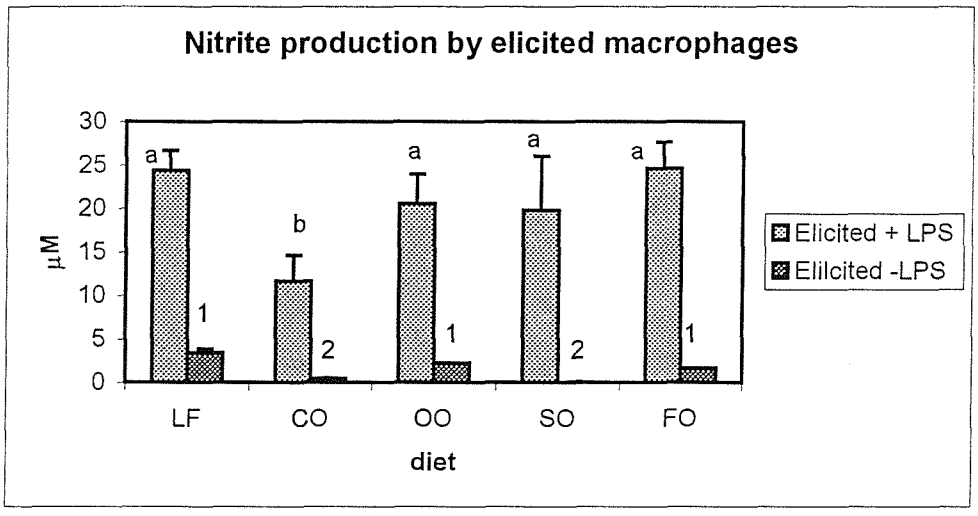
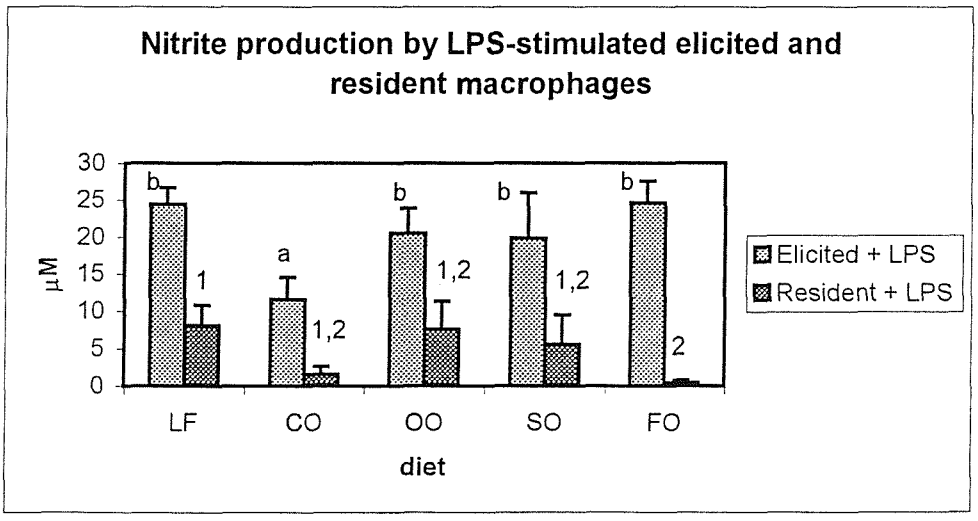


Figure 2.19 •NO production by LPS-stimulated elicited and resident macrophages. Data are means ± se for 6 animals per diet and $2x10^6$ cells per well. Bars not sharing the same letter across the elicited macrophage group are significantly different. Bars not sharing the same number across the resident macrophage group are significantly different.



2.4.4.7 Summary of results of mediator production by macrophages

Tables 2.5 and 2.6 show a summary of the effects of high fat feeding on the production of mediators by resident and elicited macrophages. Both resident and elicited macrophages FO feeding caused a significant decrease in PGE_2 production compared to SO feeding. •NO production and IL-6 production were decreased compared to SO feeding in the resident macrophages but not in the elicited macrophages. Production of IL-1β and TNF-α were decreased following FO feeding compared to SO feeding in the elicited macrophages, but not in the resident ones.

Table 2.5. The effects of the high fat diets on mediator production by resident macrophages (statistically significant changes compared to the SO diet. ↔ = no significant difference; ↓ significant decrease)

Mediator	*CO*	*OO*	*FO*
PGE_2	↔	↔	↓
•NO	↔	↔	↓
TNF-α	↔	↔	↔
IL-1β	↔	↔	↔
IL-6	↔	↔	↓
IL-10	↓	↓	↔
LTB_4	↔	↔	↔
LTC_4	↔	↔	↔

Table 2.6. The effects of the high fat diets on elicited macrophages (statistically significant changes compared to the SO diet. ↔ = no significant difference; ↓ significant decrease; ↑ significant increase)

Mediator	*CO*	*OO*	*FO*
PGE_2	↑	↔	↓
•NO	↓	↔	↔
TNF-α	↑	↔	↓
IL-1β	↔	↔	↓
IL-6	↔	↔	↔
IL-10	↔	↔	↔
IL-12	↔	↔	↔
LTB_4	↔	↔	↔
LTC_4	↑	↔	↔

2.4.5 Relationships between eicosanoid and cytokine production by macrophages

There was a significant correlation between the proportion of AA in elicited macrophages and the amount of PGE_2 produced after LPS stimulation of these cells (see figure 2.18). This relationship did not exist for the LPS-stimulated resident macrophages (see figure 2.19). The diets fed in this study resulted in a change in production of many inflammatory mediators studied and as such they enable the relationship between the production of these mediators to be investigated. For elicited macrophages the concentration of TNF-α and IL-1β produced was positively correlated with the concentration of PGE_2 produced; TNF-α production however correlated negatively with PGE_2 production by the resident macrophages. There was no correlation with PGE_2 production and IL-1β production in the resident macrophages however. This indicates that resident and elicited cells may respond differently to PGE_2 (table 2.7). There were no significant associations between the leukotrienes in the medium and the production of TNF-α or IL-1β.

Table 2.7 Correlations between the production of eicosanoid and cytokines by stimulated macrophages.

	Elicited Mϕ		*Resident Mϕ*	
	r	p	r	p
IL-1β vs. PGE_2	0.62	< 0.001	-0.09	0.69
IL-1β vs. LTB_4	0.08	0.71	-0.13	0.55
IL-1β vs. LTC_4	-0.14	0.51	-0.16	0.45
IL-1β vs. PGE_2/LTB_4	0.42	< 0.04	0.06	0.78
IL-1β vs. PGE_2/LTC_4	0.47	< 0.02	0.13	0.57
TNF-α vs. PGE_2	0.70	< 0.001	-0.52	< 0.009
TNF-α vs. LTB_4	0.03	0.87	-0.23	0.26
TNF-α vs. LTC_4	-0.33	0.10	-0.11	0.61
TNF-α vs. PGE_2/LTB_4	0.51	< 0.009	-0.18	0.41
TNF-α vs. PGE_2/LTC_4	0.78	< 0.001	-0.19	0.37

Figure 2.20 PGE_2 production by elicited macrophages correlates with cell AA content.

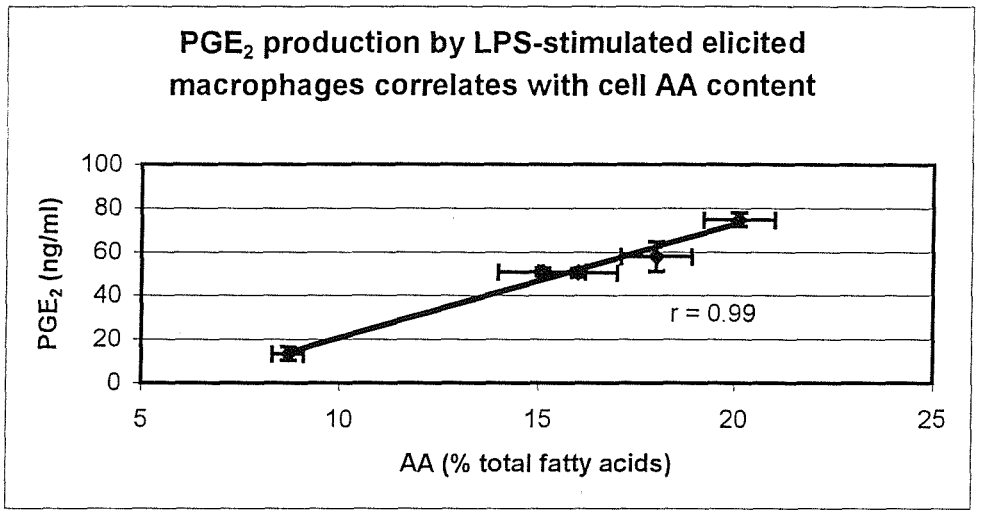
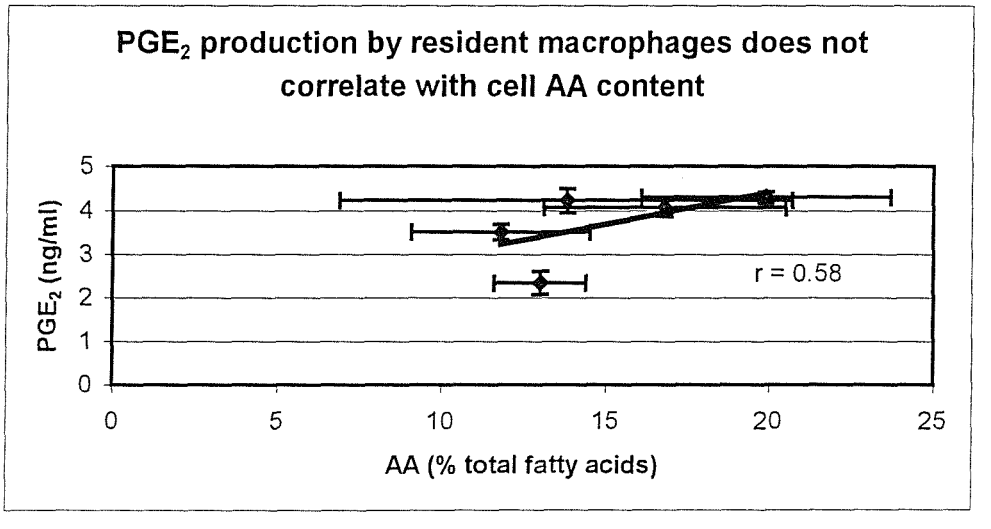


Figure 2.21 PGE_2 production by resident macrophages does not correlate to cell AA content.



2.5 Discussion

Macrophages are active cells that are capable of producing a wide range of inflammatory mediators. Macrophages have different levels of activity depending on their state of activation and differentiation, which is determined by their exposure to various stimuli such as LPS or cytokines released from other cells (Adams & Hamilton, 1992). Macrophages can be subdivided into three different subclasses, resident, elicited (inflammatory) and activated each with differing abilities to produce inflammatory mediators and differing rates of membrane turnover. Activated macrophages secrete a variety of products including nitric oxide, eicosanoids and cytokines (Abbas, 1994), and these play a key role in macrophage functions such as the killing of tumour cells, the regulation of T and B cell functions and the regulation of the inflammatory response (Adams & Hamilton, 1992). Over the past decade there have been a great number of studies investigating the effects of dietary fatty acids (especially FO) provided to either animals or humans on the production of inflammatory mediators by macrophages or monocytes (see section 1.3.5). There are a large number of inconsistencies in the literature as to whether these fatty acids provided in the diet cause an increase or decrease in the production of inflammatory mediators. For example, some of studies have reported an increase in the production of TNF-α by macrophages as a result of feeding rodents a diet high in n-3 fatty acids (Lokesh *et al.*, 1990; Watanabe *et al.*, 1991; Hardardottir & Kinsella, 1991; Turek *et al.*, 1991; Chang *et al.*, 1992; Somers & Erickson, 1994), while others have reported a decrease (Renier *et al*, 1993; Boutard *et al*, 1994; Yaqoob & Calder, 1995b) or no effect (Watanabe *et al.*, 1991; Turek *et al*, 1991; Hardardottir & Kinsella, 1992; Hubbard *et al.*, 1994) (see section 1.3.5). Studies are often hard to compare however because they differ greatly in the experimental protocols used. What seems to emerge however when the literature is examined is that some of the contradictions in the effects of dietary fatty acids on inflammatory mediator production (at least with regard to their effect on TNF-α production) may be explained by the state of activation of the macrophage studied. The purpose of this study therefore, was to standardize a number of factors in order to investigate how diets of varying fatty acid composition could influence the production of inflammatory mediators (such as TNF-α) from macrophages in differing states of activation. To do this resident macrophages, elicited

macrophages not exposed to LPS and elicited macrophages exposed to LPS were examined.

2.5.2 Mice weights and food intakes

Mice fed the OO and SO diets gained the most weight and those fed the LF and FO diets gained the least. The CO-fed animals gained an intermediate amount of weight, about half of the amount gained by the OO- and SO- fed mice and twice the amount gained by the FO- fed mice. The fact that the high fat groups did not gain the same amount of weight despite eating similar amounts of food, and therefore having similar caloric intakes, is unusual. It was observed that the mice fed the FO-fed diet were much more active during the day time than were the mice fed the OO, SO, and CO diets. No attempt was made in this study to assess the nocturnal activity levels of the mice. If however the FO fed mice maintained their high activity level during the night, it would help to explain why these mice did not put on the same amount of weight as those in the other high fat groups. Mice in the LF group ate more food than those in the other groups; yet did not gain as much weight. This diet was lower in energy (and total fat) than the high fat diets, so it follows the mice would have to eat a greater amount of food in order to meet their caloric requirements. The differences in weight gain between the dietary groups may also suggest that the fatty acids are being metabolised differently, i.e. some are being sequestered for storage more efficiently than others, while some are perhaps being preferentially metabolised for energy. The involvement of PPAR-α and PPAR-γ (peroxisome proliferator activated receptor-α and γ) in the regulation of energy balance in the mice fed the different diets has been investigated by another member of our group (Donnellan, 1999). Significantly increased PPAR-α mRNA expression has been found in the livers, and significantly increased PPAR-γ mRNA has been found in the adipose tissue of mice fed the FO diets compared to the other diets (Donnellan, 1999). Changes in the expression of these transcription factors in the livers and adipose of the mice may be in part responsible for the dramatically different patterns of weight gain seen in mice fed those high fat diets of similar caloric content.

2.5.3 Macrophage lipid profiles

The thioglycollate stimulus as well as drawing other cells from the circulation to the site of inflammation, causes the relatively quiescent resident macrophages to differentiate and become partially activated (Adams & Hamilton, 1992). This results in a number of metabolic demands and changes on the cells that may cause them to take up lipids from their surroundings and incorporate them into membrane phospholipids (Chapkin & Miller, 1990). Activated macrophages secrete a variety of products including •NO, eicosanoids and cytokines (Abbas, 1994). It may be that dietary manipulation had a greater effect on elicited macrophages than on resident macrophages due to their increased metabolic activity. A comparison of the fatty acid composition of resident and elicited macrophages from mice fed the LF diet provides an indirect indication of the effect of activation of the cells in vivo. Resident macrophages had a higher content of saturated fatty acids and a lower content of unsaturated fatty acids than did elicited macrophages (see 2.4.2). This suggests that activation *in vivo,* as would occur in response to thioglycollate injection results in an increase in unsaturated fatty acid content of macrophages. Black & Kinsella (1993) also found that elicited macrophages contain a greater percentage of unsaturated fatty acids in their cell membranes than the resident macrophages do. In the current study, fatty acid composition analysis clearly showed that diet has an effect on total lipid profile of macrophages, which has previously been reported (Kinsella *et al.,* 1990; Brouard & Pascaud, 1990; Black & Kinsella, 1993). Elicited macrophages from mice fed the CO, OO, and SO diets showed a single key enrichment in the fatty acid found in high levels in the diet. However, FO feeding induced a number of changes in fatty acid composition, the major ones being a decrease in the content of all n-6 fatty acids, including AA, and increase in the proportion of n-3 fatty acids. The difference in macrophage arachidonic acid levels in elicited macrophages accorded with the amount of PGE_2 produced upon LPS-stimulation: the proportion of AA in the elicited macrophages correlated with the PGE_2 produced upon LPS stimulation ($P=0.001$ and $r^2=0.98$). IL-1β production by these cells also correlated strongly with the content of AA present in cells ($P=0.001$, $r^2=0.98$). There were negative correlations between the n-3/n-6 ratio of the macrophage lipids and PGE_2 production ($P=0.04$, $r^2=0.90$) and IL-1β production ($P=0.06$, $r^2=0.87$) by the LPS-stimulated elicited macrophages. These

correlations did not exist for the unstimulated elicited macrophages or the resident macrophages. This may account for some of the differences in the literature between the effects of diet on the production of inflammatory mediators by different types of murine macrophages (see section 1.3.3). TNF-α production by the LPS-stimulated elicited macrophages from the mice fed the different diets did not correlate with the n-3/n-6 ratio of the macrophage lipids. It did however correlate strongly and positively with the production of PGE_2 and the ratio of PGE_2/LTC_4 produced by the macrophages (P= 0.001). This suggests that TNF-α production is strongly regulated by the production of the different n-6 derived eicosanoids produced upon stimulation with LPS. The correlation was negative for resident macrophages, suggesting that these relationships are specific to elicited macrophages, and that different relationships exist between eicosanoids and cytokines for cells in a lower state of activation.

The dramatic effects of n-3 fatty acid incorporation seen for elicited macrophages were not as apparent for resident macrophages where there was only a significant dietary effect on the amount of 18:2 n-6 present in the macrophages from the FO-fed mice compared to the LF- and CO-fed mice. This may explain some of the differences seen in behavior between resident and elicited cells. While differences in fatty acid composition between resident and elicited macrophages have been observed previously (Brouard & Pascaud, 1990; Black & Kinsella, 1993) they have never been shown for this large a range of diets. Black & Kinsella (1993) found that the effect of n-3 fatty acid feeding on macrophage phospholipid fatty acid composition was more dramatic in resident macrophages than elicited macrophages. They found that elicited macrophages maintained a high proportion of n-6 fatty acids in their phospholipids, and suggested that elicited macrophages may have altered ability to acylate, elongate and desaturate and deacylate long chain n-3 fatty acids. In the current study total cellular fatty acid composition was assessed rather than phospholipid composition, and so the distribution of fatty acids within the cells was not clearly elucidated. Nevertheless, the data from this study contrast with Black & Kinsella (1993). Indeed, their idea of the impaired ability of elicited macrophages to effectively incorporate and turnover n-3 fatty acids conflict with the *in vitro* observations that such macrophages can readily incorporate long chain fatty acids into their membranes (Chapkin & Miller, 1990; Calder *et al*, 1990), and other studies which show that dietary fish oil dramatically alters the fatty

acid composition of macrophages (Brouard & Pascaud, 1990; Surette *et al.*, 1995). Macrophages can stay in tissues for many months, and a large proportion of the resident macrophages collected may have been present in the peritoneal cavity for a long time and may not have been so strongly effected by diet.

2.5.4 Mediator Production by Macrophages

The effects of dietary fatty acids upon macrophage functions apart from the production of eicosanoids, are unclear, with many contradictions in the literature (see section 1.3). This study directly compares the effects of feeding mice diets rich in saturated, monounsaturated, n-6 polyunsaturated and n-3 polyunsaturated fatty acids upon the production of PGE_2, IL-1β, TNF-α, IL-6, IL-10, IL-12, LTB_4, LTC_4 and •NO by both resident and elicited macrophages. In general, LPS stimulation of elicited macrophages increased the production of PGE_2, TNF-α, IL-1β, IL-6, IL-10, IL-12 and •NO, but not of LTB_4 or LTC_4, but the response to LPS was affected by the type of fat in the diet. Generally, elicited macrophages produced more PGE_2, TNF-α, IL-1β, IL-6, IL-12 •NO but less LTC_4 after LPS stimulation than did resident macrophages; the production of IL-10 and LTB_4 was similar between the two types of macrophages after LPS-stimulation. The effect of the amount of fat in the diet on macrophage mediator production can be discerned by comparing the LF diet to the SO diet since both these diets are rich in linoleic acid. High fat feeding did not appear to affect the production of IL-6, IL-10, IL-12 and •NO by either type of macrophage, but high fat feeding decreased the production of TNF-α and IL-1β by LPS-stimulated elicited macrophages and increased the production of IL-6 and IL-10 by the LPS-stimulated cells. In terms of the effect of the specific high fat diets on the production of inflammatory mediators, the effects can be determined by comparing the CO, OO and FO diets with SO. The FO diet had the greatest influence on mediator production. FO feeding caused a decrease in the production of PGE_2, •NO and IL-6 by resident macrophages but an increase in the production of TNF-α and IL-10 by these cells. The concentration of the mediators produced by the resident macrophages from mice fed the OO and CO diets were not greatly different from those produced by resident macrophages from mice fed the SO diet. The only difference was that •NO production was decreased in macrophages from the CO group compared to the macrophages from the SO group. The effects of FO

feeding on the LPS stimulated elicited macrophages were to decreased PGE_2, TNF-α, and IL-1β production (with a trend to decrease IL-6 production). FO feeding increased •NO production by these cells compared to the SO diet. CO feeding caused a decrease in •NO and IL-10 production compared to the SO diet. CO feeding and OO feeding did not decrease TNF-α production as much as SO feeding. Therefore these diets partially reverse or prevent the effect of high fat feeding.

The capacity to produce inflammatory mediators is influenced by the state of activation of the macrophages. The FO diet caused a significant decrease in production of PGE_2, IL-1β, TNF-α and IL-6 by the thioglycollate-elicited macrophages cultured with LPS. No effect of FO feeding was seen on the production of IL-10 or IL-12 by these macrophages. This is the first study to investigate the effect of dietary fat on IL-10 and IL-12 production by peritoneal macrophages. IL-10 is a potent anti-inflammatory cytokine that suppresses TNF-α and IL-1β production and drives the Th-2 type response (Howard *et al.*, 1993). IL-12 is important in stimulating lymphocytes, especially natural killer cells and cytotoxic lymphocytes (Sutterwala & Mosser, 1999) and helps to drive the Th-1 type immune response (Ma *et al.*, 2000). Circulating IL-12 levels have recently been investigated by Fritsche *et al.* (1999) in mice fed diets rich in lard, soybean oil, or a fish oil:corn oil mix. They found that circulating IL-12 levels were significantly lower following challenge with *Listeria monocytogenes* in the mice fed the fish oil:corn oil mix diet than in mice fed the other two diets. No effect of FO feeding on IL-12 production by elicited macrophages was seen in the current study. This may indicate that the effects seen by Fritsche *et al.*, (1999) on circulating IL-12 levels following FO feeding may be due to FO modulation of a cell type other than monocyte-macrophages, perhaps B-cells which also produce IL-12, or may be due to differences in response to the type of challenge: live bacteria given to the mouse *in vivo* compared with LPS to purified macrophage cells *ex vivo*.

The observations of a FO-induced decrease in production of PGE_2, IL-1β, TNF-α and IL-6 by elicited macrophages agree with other studies which have found that FO feeding causes a decrease in production of these pro-inflammatory mediators (Billar *et al.*, 1988; Renier *et al.*, 1993; Yaqoob & Calder, 1995). There might be several

mechanisms by which the dietary fat is acting in order to elicit these effects. The changes in cytokine production may be due to a change in eicosanoid production, through direct effects of n-3 fatty acids on cytokine production, or through modulation of enzyme activity/function or lipid mediators in signaling pathways. The precise biochemical pathways involved in the modulation of cytokine production by dietary fatty acids remains to be clarified. However, the production of some pro-inflammatory cytokines clearly correlate to changes in the amounts and types of eicosanoids produced by stimulated macrophages.

2.5.4 Resident Macrophages Compared to Elicited Macrophages

For both cell types FO feeding caused a decrease in PGE_2 production upon LPS stimulation, although for the resident macrophages this decrease was not significant. There are a number of differences between the responses of the two cell types from mice fed the different diets when stimulated with LPS. FO feeding caused an increase in •NO production by the elicited macrophages, and a decrease in production by the resident cells. The reason for the different effect of diet on •NO production is unclear, but it may relate to an interaction between the iNOS and cyclooxygenase (COX) pathways. An important link between the iNOS and COX enzymes is that •NO activates the COX enzyme resulting in an augmented production of prostaglandins (Salvemini, 1997). In the current study •NO and PGE_2 production were highest by LPS-stimulated elicited and resident macrophages from animals fed the LF diet, slightly less in macrophages from the CO-fed animals, and similar in macrophages from OO and SO fed animals. Elicited macrophages from the FO-fed animals produced the lowest amount of PGE_2, but a high amount of •NO, while resident macrophages from FO-fed animals produced the lowest amount of •NO. LPS has been shown to induce iNOS in cultured rat peritoneal macrophages (Sonoki *et al.*, 1997). The •NO produced by iNOS could in turn stimulate COX to produce prostaglandins such as PGE_2. Differences in the types of prostaglandins produced as a result of the different availability of precursor substrates through dietary manipulation may in turn modulate subsequent •NO production. Ohata *et al.* (1997) have shown that •NO production by an LPS-stimulated macrophage cell line is suppressed by n-3 fatty acids *in vitro*. Khair El Din *et al.* (1996) showed that transcription of the iNOS gene in a macrophage cell

line is inhibited *in vitro* by DHA, a component of FO incorporated into the cell membranes of mice fed a FO diet (see section 2.4). These studies suggest that FO should decrease •NO production. Resident macrophages from the FO fed mice produced the least amount of •NO upon stimulation with LPS, which agrees with what Boutard *et al.* (1994) found with rat resident peritoneal macrophages. This may indicate that the n-3 PUFA suppress the induction of iNOS or its activity. This suppression does not appear to occur in the elicited macrophages, and NO production was increased in elicited macrophages from mice fed the FO diet. Chaet *et al.* (1994) studied •NO production by rat alveolar macrophages and found that macrophages from FO fed rats stimulated with interferon-γ and LPS produced more •NO that than macrophages from rats fed SO. In the Chaet *et al.* (1994) study, when indomethicin was added to the culture medium the difference in •NO production between the dietary groups was ablated, indicating that dietary fatty acids influence the production of •NO in this cell type via production of eicosanoids. Iwabuchi *et al.* (1997) have also shown that indomethacin inhibits the production of •NO by the macrophage cell line J774 and that PGE_2 increased nitrite production by LPS J774 macrophages in a dose-dependent manner. This indicates that one or more cyclooxygenase products, including PGE_2, enhance •NO production by macrophages. PGI_2 has also been shown to dose-dependently inhibit •NO production by LPS-stimulated J774 cells (Marotta *et al.*, 1992). The effect of the n-3 prostaglandin PGE_3 on •NO production has not been investigated. The 5-lipoxygenase product LTB_4, which was also lowered by FO feeding, has been shown not to affect •NO production by the macrophage cell line RAW 264.7 (Hulkower *et al.*, 1996). LTB_4 and LTC_4 are strong inducers of •NO release in human neutrophils however (Larfars *et al.*, 1999). The effect of LTB_5 and LTC_5 on •NO production by macrophages has not been investigated.

One very interesting result was that resident macrophages do not produce IL-12 upon stimulation with LPS, while elicited macrophages produce around 50 pg/ml. While there were no statistically significant differences between the groups, macrophages from mice fed the LF diet tended to produce the greatest amount and macrophages from mice fed the CO diet tended to produce the lowest amount of IL-12. The effect of dietary fat in the regulation of IL-12 production in murine macrophages has not been

well studied although Maya *et al.*, (1997) have examined IL-12 production in whole blood cultures from patients with HIV infection and found that it did not relate to PGE_2 production or IL-10 production. *In vivo* studies have shown that IL-12 production is amenable to dietary manipulation (Fritche *et al.*, 1999). Therefore, the effect of diet on the production of IL-12 by macrophages needs to be examined in more detail.

One of the most interesting results obtained in this study is the difference in production patterns of TNF-α by the macrophages from mice fed the FO diet compared to those fed the other diets. Secretion of TNF-α was remarkably lower by LPS-stimulated elicited macrophages, intermediate by the unstimulated elicited macrophages and highest by the LPS-stimulated resident cells (see figures 2.6a and b). As diets high in FO lower PGE_2 production, it may be possible that the effects of FO feeding on TNF-α might be occurring through modulating the quantities of PGE_2 produced. PGE_2 causes a concentration dependent inhibition of TNF-α production by inflammatory macrophages (Renz *et al.*, 1988; Scales *et al.*, 1989). The concentrations of PGE_2 observed in the medium of macrophages in this study (i.e. 14-80 ng/ml or 45-250 nmol/l) are most likely sufficient to significantly alter TNF-α production. This was not found to be the case however: FO feeding significantly reduced both PGE_2 and TNF-α production. This implies that dietary FO must lower TNF-α production by a mechanism other than alterations in PGE_2 production. Renier *et al.*, (1993) showed that dietary FO significantly lowered TNF-α mRNA in LPS-stimulated murine macrophages, while Chandrasekar & Fernandes, (1994) found that a FO rich diet resulted in significantly lowered TNF-α mRNA in the kidneys of autoimmune disease-prone mice. These observations indicate that a component of FO (or a derivative of a component of fish oil) is able to inhibit the expression of TNF-α gene. This might come about through the inhibition of the nuclear transcription factor NFκB. NFκB is activated by the phosphorylation and subsequent dissociation of its inhibitory subunit (IκB) (see section 1.2.12). This phosphorylation occurs through PKC-mediated reactions. PKC is activated by diacylglycerol released by PLC-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate. The n-3 fatty acids EPA and DHA, components of FO, have been shown by to directly inhibit PKC from rat lymphocytes (May *et al.*, 1993) and macrophages (Tappia *et al.*, 1995) and FO feeding has also been shown to

inhibit PLC activity (Sanderson & Calder, 1998) and to decrease DAG concentrations (Jolly *et al.*, 1997) after stimulation in lymphocytes. These effects of FO would serve to decrease NFκB activation and so reduce TNF-α gene transcription. Recently EPA has been shown to inhibit the degradation of the inhibitory subunit of NFkB, IκB (Ross *et al.*, 1999), thereby providing a mechanism by which activation of NFκB might be prevented in cells enriched with this fatty acid. Thus mechanisms exist, independent of altered eicosanoid metabolism, to explain the decreased production of TNF-α by macrophages after FO feeding.

The dramatic difference in the pattern of production of TNF-α by the cells from the mice fed the FO diet suggest that these cells respond to LPS in different ways depending upon their state of activation (elicited vs. resident). Macrophage activation occurs in response to a number of factors (see section 1.2.5), and recently PPAR-γ has been shown to be a negative regulator of macrophage activation (Ricote *et al.*, 1998), and has been shown to suppress inflammatory cytokine production by human monocytes (Jiang *et al.*, 1998). Naturally occurring compounds such as the fatty acid DHA, and eicosanoids PGJ_2 and LTB_4 a have been shown to act as ligands for PPAR-γ (Clarke *et al.*, 1999). The down-regulation of TNF-α production in the elicited macrophages from mice fed the FO diet might occur as a result of activation of PPAR-γ by n-3 fatty acids present in the cellular environment and released from the membrane by phospholipases in response to LPS. Elicited macrophages contained substantially more n-3 fatty acids than did the resident macrophages (see section 2.4.7), perhaps due to the fact that resident macrophages are relatively quiescent cells which reside in tissues until encountering a stimulus and differentiating into elicited (inflammatory) macrophages. Membrane turnover would be low in these cells until encountering a stimulus. When resident cells encountered the thioglycollate broth, they would have differentiated into inflammatory macrophages. In doing so they would have undergone various morphological and membrane changes and additional monocyte/macrophages from the bloodstream would have moved into the peritoneum. For the FO-fed mice this would have occurred in an environment high in n-3 fatty acids. The other groups would not have had exposure to these fatty acids in any appreciable quantities. The cells from mice fed the FO diet have incorporated these fatty acids, and this might have resulted

in the inhibition of proper activation and/or the down regulation of TNF-α production which resulted in the lower production of this mediator by these cells. Indeed Renier *et al.* (1993) found decreased TNF-α mRNA in elicited macrophages from FO fed mice. LPS stimulation of elicited macrophages from FO fed mice actually tends to decrease TNF-α production, in direct contrast to the effects of LPS stimulation of the elicited macrophages from each of the other groups. Resident cells encountering their first stimulus to differentiate (the LPS challenge added *ex vivo* to the culture medium), along with FCS which would contain fatty acids which might not normally be present in the cellular environment *in vivo*, and which certainly would not represent the fatty acid profile of the diet the mouse would have been eating, would be theoretically less likely to activate PPAR-γ or to induce PPAR-γ expression. The levels of PPAR-γ have not been examined in the macrophages in this experiment, but PPAR-γ has been shown to be induced by FO feeding in adipocytes (Jump *et al.*, 1997). Indeed the adipose tissue from the mice used in this study had elevated levels of PPAR-γ mRNA (Donnellan, 1999). Elevated expression of PPAR-γ coupled with higher levels of a potent ligand for PPAR-γ might explain the overall reduced inflammatory mediator response in the elicited macrophages from the FO group.

2.5.6 Implications

This study is the first to directly compare the effects of dietary lipids including FO on cytokine production by resident and elicited (inflammatory) macrophages. It shows that the effect of dietary lipids depends upon the activation state of the macrophage used. The most dramatic effects are caused by FO feeding: this diet increased production of TNF-α by resident cells and decreased TNF-α and IL-1β production, and tended to decrease IL-6 production, by elicited macrophages. The contradictions in the literature with regard to the influence of dietary lipids on pro-inflammatory cytokine production have sometimes been considered to be due to differences in the species, strain or gender of animal used, the amount of fat fed, the duration of feeding, the anatomical site of origin of the macrophages studied, the type and concentration of the stimulus used to elicit the cytokine production, the type of serum used in the *ex vivo* culture and its concentration, the number of cells used in culture, and the type of assay used to measure cytokine concentrations. In the current study each of these factors was

normalized; the only difference was in the activation-state of the macrophage at the start of the culture period. This study suggests that it is the difference in the activation state of the macrophage used which accounts for the different outcomes reported in the literature. Certainly most of the studies which report that dietary FO enhances TNF or IL-1 production used resident macrophages (see section 1.3.5), while those which report that dietary FO decreases TNF-α, IL-1 and IL-6 production used elicited (inflammatory) macrophages, Kupffer cells or monocytes.

One application of these findings, particularly those concerning the inflammatory macrophages relates to the possible modulation of mediator production in chronic inflammatory diseases. Such diseases are characterized by inappropriate production of pro-inflammatory cytokines (Grimble, 1996) often from activated macrophages. It would be of use, in some situations, therefore to intervene either nutritionally or pharmacologically in the process of macrophage activation and mediator production. FO has been shown to decrease the pathology and increase survival in animal models of lupus (Chandrasekar & Fernandes, 1994; Prickett *et al.*, 1983; Robinson *et al.*, 1986), arthritis (Leslie *et al.*, 1991; Cathcart & Gonnerman, 1991), inflammatory bowel disease (Vilaseca *et al.*, 1990; Guarner *et al.*, 1992; Murch & Walker-Smith, 1998) and acquired immune deficiency syndrome (Xi *et al.*, 1998). In some cases this has been associated with diminished production of pro-inflammatory cytokines either *in vivo* (Chandrasekar & Fernandes, 1994) or *ex vivo* (Xi *et al.*, 1998). FO has been used to treat patients with rheumatoid arthritis (see Belch & Muir, 1998 for a review), inflammatory bowel disease (Lorenz *et al.*, 1989; Belluzzi *et al.*, 1996; Stenson *et al.*, 1992; Hawthorne *et al.*, 1992), IgA nephropathy (Donadio *et al.*, 1994; Donadio *et al.*, 1997), lupus (Das, 1994) and pancreatic cancer (Wigmore *et al.*, 1996). Most of these studies report clinical improvements but few have reported circulating cytokine concentrations or *ex vivo* cytokine production by mononuclear cells. Two studies report that FO decreases IL-1 concentrations in the circulation of patients with rheumatoid arthritis (Esperson *et al.*, 1992; Krémer *et al.*, 1990), while FO decreased *ex vivo* TNF-α and IL-6 production by mononuclear cells from pancreatic cancer patients (Wigmore *et al.*, 1996). It is possible that the marked clinical improvement reported in some of the above studies might be due to FO-induced decrease in the production of pro-inflammatory mediators which are associated with the pathology of these diseases.

The second application of these findings is to endotoxemia or sepsis induced by LPS released from Gram negative bacteria, which is characterized by elevated concentrations of TNF-α and IL-1β in the circulation (Grimble, 1996). FO feeding decreases the lactic acidosis (Pomposelli *et al.*, 1990), fever (Pomposelli *et al.*, 1990), and anorexia (Hellerstein *et al.*, 1989) which follows LPS challenge in experimental animals and markedly enhances survival to a normally lethal dose of LPS (Mascioli *et al.*, 1988). Injection of cytokines such as TNF-α and IL-1β into animals mimics the effects of LPS- induced shock (Tracey *et al.*, 1986; Mathison *et al.*, 1988; Okusawa *et al.*, 1988) and neutralization of these cytokines protects animals from bacterial or LPS induced shock (Beutler *et al.*, 1985; Alexander *et al.*, 1991; Tracey *et al.*, 1987). Thus, it is clear that TNF-α and IL-1β play a key role in the progression of endotoxemia, suggesting that inhibition of their production might prevent the pathological changes which are characteristic of endotoxemia. The current study suggests that FO might exert its protective effects towards endotoxemia by decreasing the production of the pro-inflammatory cytokines that are central to the *in vivo* response to LPS.

2.6 Conclusions

Dietary fatty acids affect inflammatory mediator production by macrophages, but the effects of the dietary fatty acids depends upon the state of activation of the macrophages studied. In general, FO feeding causes a decrease in the production of pro-inflammatory mediators produced by LPS-stimulated elicited macrophages (PGE_2, IL-1β, TNF-α), and an increase in some of the pro-inflammatory mediators produced by LPS-stimulated resident macrophages (IL-1β, TNF-α). Production of some of these mediators correlates with the production of eicosanoids, but the exact relationship depends on the activation state of the macrophage used. The data presented in this chapter help to clarify some of the contradictions in the literature with regard to the effects of fatty acids on inflammatory mediator production.

Chapter 3: The effects of dietary fatty acids on macrophage cytotoxicity towards tumour cells.

3.1. Introduction

Macrophages produce a range of inflammatory mediators when activated (see section 1.2.4) some of which are cytotoxic to foreign or cancerous cells (Abbas *et al.*, 1994). Cytotoxic mediators released by macrophages include cytokines, such as TNF-α, and reactive nitrogen species such as •NO. Both of these mediators have been shown to be toxic to certain tumour cell lines *in vitro* (Fidler & Ichinose, 1989; Feinman *et al.*, 1987; Keller & Keist, 1989; Keller *et al.*, 1990; Rees & Parry, 1992). The production of both TNF-α and •NO by macrophages is upregulated by immunological stimuli such as LPS (Auger & Ross, 1992), and can be affected by the state of activation of the macrophage under investigation (see chapter 2). Modulation of the production of TNF-α and •NO might therefore influence the ability of macrophages to kill cancerous cells. Fatty acid mediators such as PGE_2 and ceramide can regulate the production of TNF-α and •NO (Abbas *et al.*, 1994), and both PGE_2 and ceramide have been shown to be influenced by the type of fat present in the diet (Jolly *et al.*, 1997; see chapter 2). Therefore, the type of fat present in the diet might lead to increased/decreased capacity of macrophages to be cytotoxic to cancer cells. In recent years, experimental and epidemiological studies have provided evidence linking dietary fat with increased risk for some cancers. For instance some epidemiological studies have reported a positive correlation between breast cancer and dietary fat intake (Prentice, 1994). Erickson & Hubbard (1994) have suggested that specific dietary fatty acids influencing macrophage tumouricidal activity might be responsible for such associations. While a number of studies have previously investigated the effect of dietary fatty acids on the production of TNF-α and •NO by macrophages (see section 1.3), very few have coupled this with an assessment of cellular function, such as mediator-induced cytotoxicity. At the present time only two studies have investigated the effect of dietary fatty acids on macrophage-mediated killing of L929 cells (Renier *et al.*, 1993; Black & Kinsella, 1993) or P815 cells (Somers *et al.*, 1989; Hubbard *et al.*, 1994), which are killed by TNF-α and •NO, respectively. One of the studies which investigated the killing of L929 cells did not measure TNF-α production (Black *et al.*, 1993), while one

of the studies which investigated P815 cell killing did not measure •NO production (Somers *et al.*, 1989). Macrophage-mediated cytotoxicity towards both L929 and P815 cells has not previously been compared within a single study. Thus, the purpose of the current study was to combine the measurement of production of two cytotoxic mediators by macrophages with a functional assessment of their production, the inhibition of proliferation of target tumour cells.

3.2 Objective

To investigate the effect of dietary fatty acids on cytotoxic mediator production by macrophages and to combine this with a functional assessment of their production, the inhibition of proliferation of target tumour cell lines.

3.3 Methods

3.3.1 Animals and Diets

Both the animals and the diets used in this experiment were identical to those described in section 2.3.1. Mice (n=6 per diet) were fed the different diets for six weeks.

3.3.2 Chemicals

Glutamine, FCS, streptomycin, penicillin, *Eschericia coli* LPS, RPMI culture medium, napthylethylenediamine, sulfanilamide, sodium nitrite, sodium nitroprusside and *N*-mono-methyl-L-arginine (NMMA) were purchased from Sigma Chemical Co, Poole, UK. Brewers thioglycollate broth was purchased from Difco, East Molesey, UK. A rabbit anti-murine TNF-α antibody was purchased from Serotec, Oxford, UK.

3.3.3 Culture Medium

Culture medium used to culture both the cell lines and the macrophages was identical to that used in the previous chapter (see section 2.3.4).

3.3.4 Tumour Cell Lines

P815 mastocytoma lines and L929 murine fibroblasts were gifts from the Sir William Dunn School of Pathology, University of Oxford. The P815 cells were seeded at a concentration of 1 x 10^5 cells/ml and the L929 cells were seeded at a concentration of 2

x 10^5 cells/ml. The cells were always handled in sterile conditions and were sub-cultured every three days.

3.3.5 Preparation of Thioglycollate

This was as described in section 2.3.3.

3.3.6 Thioglycollate-Elicited Macrophage Preparation

This was as described in Section 2.3.5.

3.3.7 Measurement of Cytotoxic Mediators

Macrophages were cultured for 24 hours in an identical manner to the conditions described in section 2.3.7. After 24 hours of culture, the culture plates were centrifuged, supernatants collected and PGE_2 and TNF-α concentrations were measured using commercially available ELISA kits as described in section 2.3.11. •NO was measured in an identical manner to section 2.3.9.

3.3.8 Preliminary experiments.

Preliminary experiments were carried out to determine if indeed the proliferation of the L929 cells and the P815 cells were inhibited in the presence of TNF-α and •NO respectively. The cells were incubated along with increasing concentrations of either TNF-α (1 μg/ml – 0.01 μg/ml) or the •NO donor sodium nitroprusside (0.5 mM-2.5 mM) for 24 hours along with (0.2 μCi/well) tritiated thymidine. Cells were then harvested onto filter paper and counted in a β scintallation counter.

3.3.9 Measurement of Tumour Cell Killing

Macrophage cytotoxicity towards the L929 and P815 cell lines was determined by measuring the incorporation of tritiated thymidine into the target cell lines in the presence and absence of added macrophages. Macrophages did not incorporate tritiated thymidine in the presence or absence of LPS (results not shown). Macrophages (5 x 10^4/well) and target cells (1 x 10^4/well), both in complete medium, were added to the wells of a 96-well, flat-bottomed micro-titre plate and tritiated thymidine (0.2 μCi /well) and LPS (final concentration 10 μg/ml) were added; total culture volume was

200 µl. Thus, the macrophage to target cell ratio used in the experiments investigating the effect of diet was 5:1; some preliminary experiments also used lower macrophage to target cell ratios were varied by decreasing the number of macrophages. After 24 hours the contents of the wells were transferred to glass fiber filters and washed and dried using a Skatron Cell Harvester (Skatron, Lier, Norway). The filters were transferred to counting vials and 100 µl scintillant added to each; radioactive incorporation was measured using a liquid scintillation counter. The percentage cell survival was calculated as:

$$\% \text{ cell survival} = \frac{^{3}H \text{ thymidine incorporation into target cells incubated with macrophages}}{^{3}H \text{ thymidine incorporation into target cells alone}} \times 100$$

3.3.10 Statistical analysis

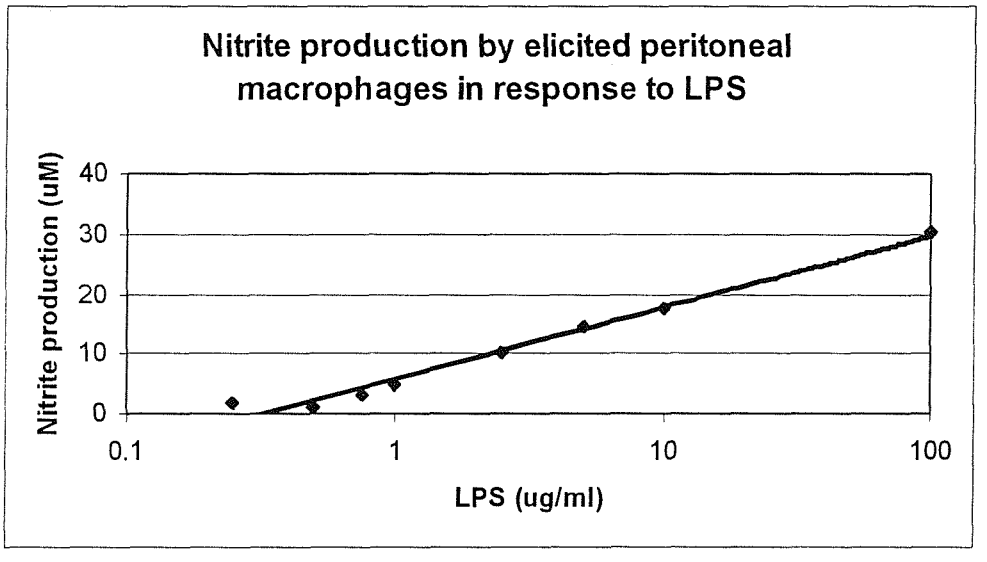
Means and standard errors are reported. Differences between groups were determined by one-way ANOVA followed by a post-hoc least significant difference test. Statistical analysis was performed using SPSS version 6.0 (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was taken to indicate statistical significance.

3.4 Results

3.4.1 LPS-stimulated •NO Production by Macrophages

Only low concentrations of •NO were produced at LPS concentrations below 0.5 μg/ml; as the LPS concentration increased above this •NO concentration increased. (figure 3.1) •NO production was completely abolished by inclusion of the iNOS inhibitor NMMA (1mM) in the culture medium (results not shown).

Figure 3.1 Nitrite production by elicited peritoneal macrophages in response to LPS



3.4.2 Inhibition of proliferation of P815 cells by macrophages

Macrophages and P815 cells were incubated together at ratios of 1.25, 2.5 and 5; these ratios were achieved by varying the number of macrophages present and keeping the number of P815 cells constant at 1 x 10^4 per well. Thymidine incorporation was decreased as the macrophage:P815 ratio increased but only if the macrophages were stimulated with LPS (figure 3.2). This indicates that the proliferation of the P815 cells is inhibited by an agent released by macrophages upon LPS stimulation. Inclusion of 1 mM NMMA in the media maintained thymidine incorporation at the level seen in the absence of LPS simulation (see figure 3.3) indicating that P815 cell proliferation is inhibited by •NO. In conformation of this, proliferation of P815 cell was inhibited by incubation with the •NO donor sodium nitroprusside in the absence of macrophages (see figure3.4).

Figure 3.2 P815 cells proliferation is inhibited by macrophages

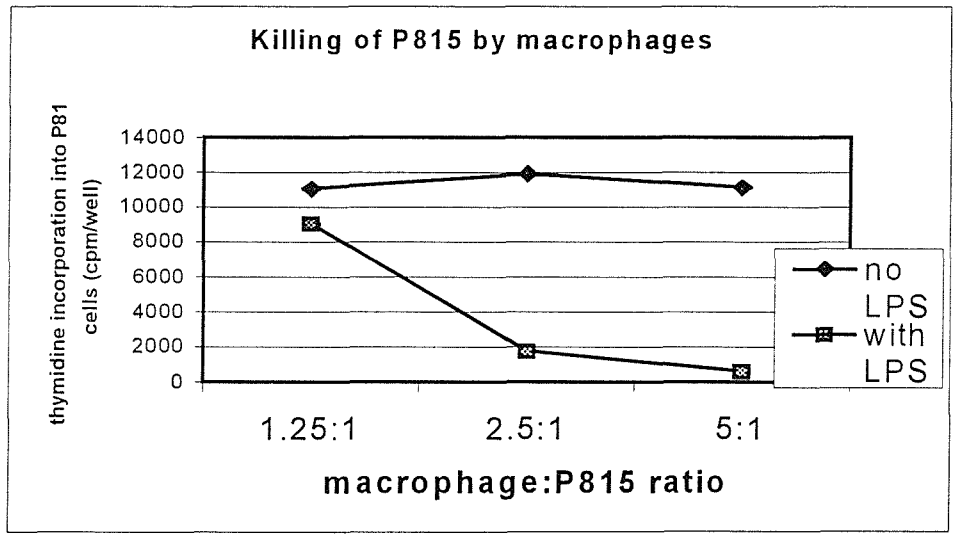


Figure 3.3 Proliferation of P815 is inhibited by nitric oxide

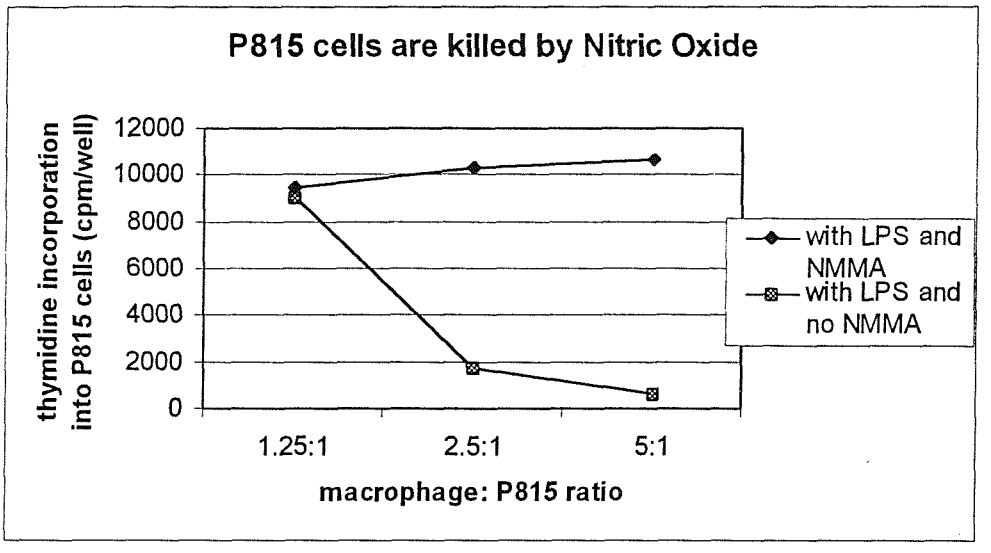
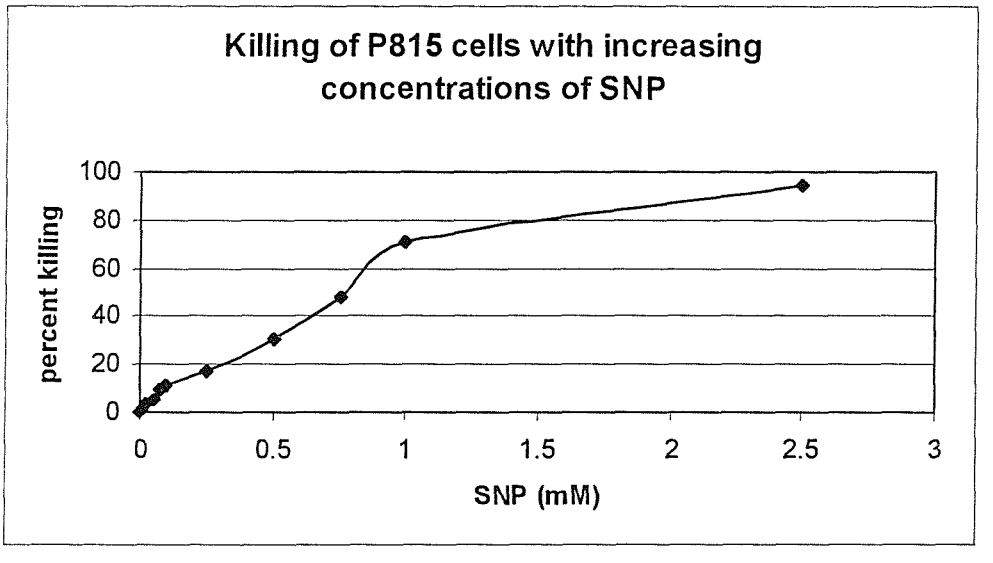


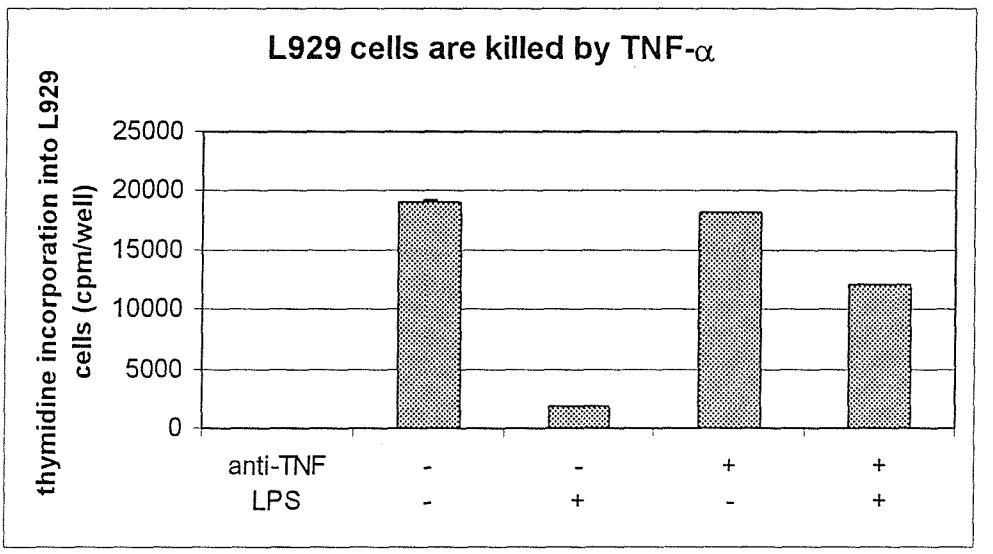
Figure 3.4 Proliferation of P815 cells is inhibited by increasing concentrations of SNP.



3.4.3 Proliferation of L929 Tumour Cells is inhibited by Macrophages

Proliferations of L929 cells by macrophages was decreased by LPS stimulation and was largely abolished by inclusion of a TNF-α blocking antibody (1 μg/ml) in the medium (see figure 3.5). This indicates that proliferation of L929 cells are inhibited by TNF-α released from LPS-stimulated macrophages.

Figure 3.5 Proliferation of L929 Tumour cells is decreased by Macrophages



3.4.4 Production of cytotoxic mediators by macrophages from mice fed different diets.

The concentrations of TNF-α, PGE_2 and •NO produced by LPS-stimulated macrophages taken from mice fed different diets are presented in Table 3.1. Macrophages from mice fed the FO diet produced significantly less PGE_2 and TNF-α than macrophages from mice fed each of the other diets. Macrophages from mice fed the SO diet produced significantly less •NO than macrophages from mice fed the other diets.

Table 3.1 Production of cytotoxic mediators by macrophages.
Data are mean and se for 6 mice per diet. Values in a column not sharing a common superscript letter are significantly different

Diet	*PGE_2 (ng/ml)*	*Se*	*TNF-α (ng/ml)*	*Se*	*Nitrite (μmol/L)*	*Se*
LF	82^{a}	10	3.1^{a}	0.6	42.2^{a}	3.7
CO	58^{ab}	14	2.0^{ab}	0.3	25.9^{bc}	1.5
OO	51^{b}	4	1.3^{b}	0.4	20.7^{bd}	1.6
SO	50^{b}	4	0.7^{b}	0.1	17.9^{d}	1.9
FO	14^{c}	6	0.12^{c}	0.02	30.2^{c}	2.5

3.4.5 Cytotoxicity of Macrophages from mice fed different diets.

Macrophages from mice fed the five experimental diets were cultured with or without 10 μg/ml LPS and either P815 cells or L929 cells. Mean percent survival of the cell lines after incubation with macrophages is shown in table 3.2. Cells from mice fed the LF diet had the greatest cytotoxic activity towards both the target cell lines, and cells from mice fed the SO diet had the least cytotoxic activity towards both the target cell lines. The survival of P815 cells correlated negatively with the amount of •NO produced by the macrophages ($p < 0.025$; $r = 0.81$, see figure 3.6). Thus, the diet-induced effects on the ability of macrophages to produce •NO are responsible for the effects of diet on the ability of macrophages to inhibit the proliferation of P815 cells. Survival of the L929 cells however was correlated to TNF-α production only if the data from the FO-fed animals were omitted ($p < 0.025$; $r=0.94$) (figure 3.7). Cells from these animals showed a greater cytotoxic capacity than what would be expected from the amount of TNF-α produced

Table 3.2 Percent survival of tumour cell lines incubated with macrophages form mice fed different diets.

Data are mean ± SEM for 6 mice fed on each diet. Values in a column not sharing a common superscript letter are significantly different

Diet	*% survival of P815 cells*	*% survival of L929 cells*
LF	14.2 ± 1.7^{a}	21.4 ± 2.1^{a}
CO	55.6 ± 8.2^{b}	36.8 ± 3.2^{ab}
OO	55.9 ± 9.9^{b}	54.9 ± 8.1^{b}
SO	78.6 ± 6.4^{c}	55.3 ± 11.1^{b}
FO	18.9 ± 5.1^{a}	36.4 ± 9.5^{ab}

Figure 3.6 Nitrite production by macrophages vs. P815 survival.

Data are for 6 mice per diet. Means and standard errors are reported.

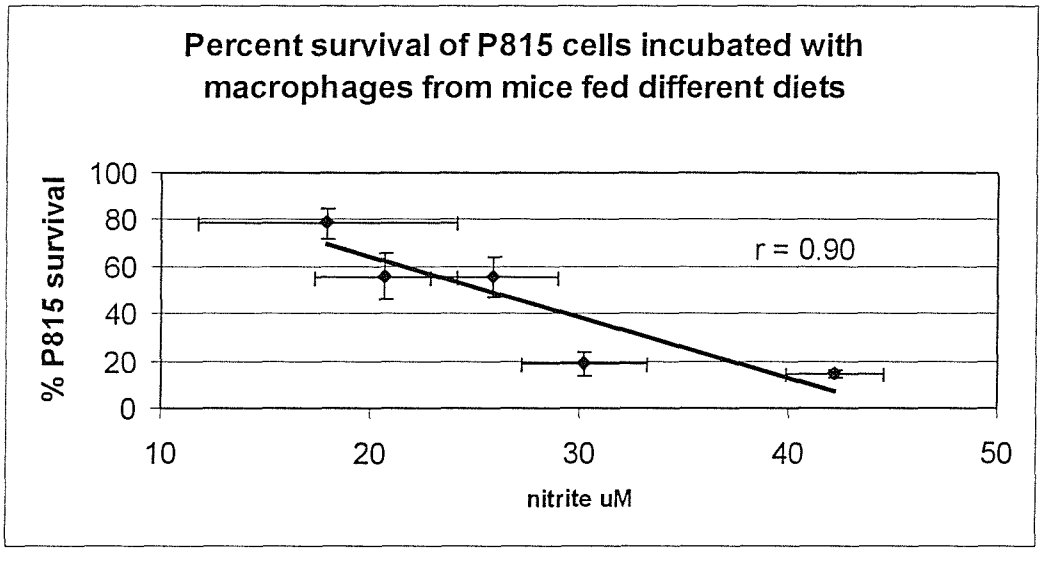
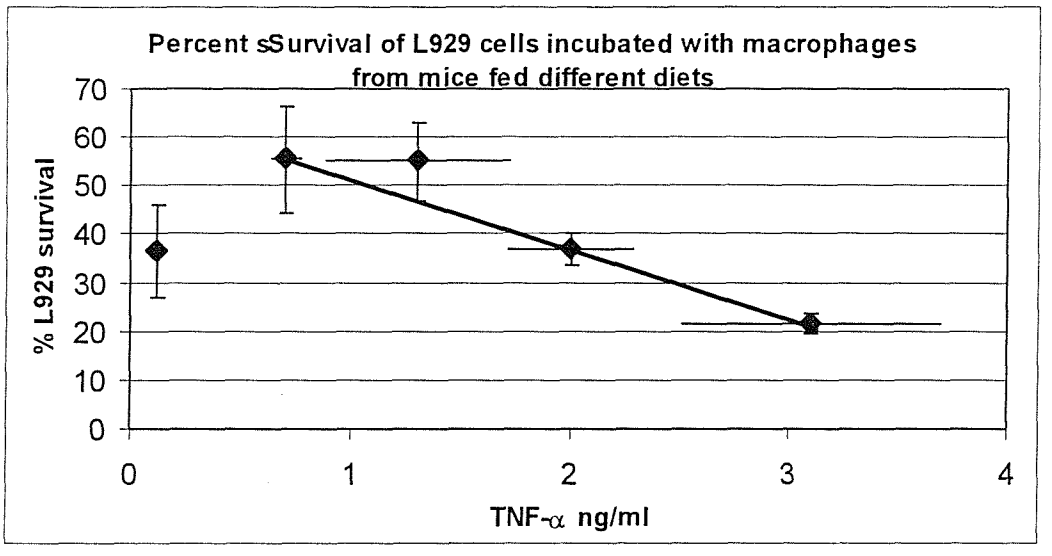


Figure 3.7 TNF-α production by macrophages vs. L929 survival.

Data are for 6 mice per diet. Means and standard errors are reported. Data for the FO fed mice has been excluded from the regression line.



3.5 Discussion

This study investigated the effects of the amount and type of fat in the mouse diet upon the production of mediators PGE_2, TNF-α and •NO by LPS-stimulated thioglycollate-elicited macrophages and upon one functional effect of TNF-α and •NO, the inhibition of proliferation of target tumour cell lines. A number of studies have previously investigated the effect of dietary fats on TNF-α and •NO production by rodent macrophages (see sections 1.3.5, 1.3.6, 1.3.7 and 1.3.13 for references), but few have investigated macrophage-mediated cytotoxicity. This study shows that both the amount and type of fat in the diet can affect the production of various mediators by macrophages cultured *ex vivo* and can alter the ability of macrophages to inhibit the growth of tumour cells.

In this study, the effect of the amount of fat in the diet can be determined by comparing the LF and SO diets: these diets have very similar fatty acid compositions as they are both rich in linoleic acid (table 2.3.1), but they contain 2.5 % and 21 % fat by weight respectively. The effect of the type of fat in the diet can be determined by comparing

respectively. The effect of the type of fat in the diet can be determined by comparing the CO, OO, SO and FO diets, since the principal fatty acids in these diets represent the major fatty acid classes (saturated, monounsaturated, n-6 PUFA and n-3 PUFA respectively: see section 2.3 for the fatty acid composition of the diets). These diets contribute 40% of energy of the diet as fat, similar to that found in most Western diets. Increasing the amount of fat in the mouse diet decreased the production of TNF-α and •NO and decreased macrophage-mediated cytotoxicity. Across mice fed each of the diets •NO production significantly correlated with the ability of macrophages to kill the •NO-sensitive P815 target cell line. Among macrophages from the high fat-fed mice, those from mice fed FO had the greatest cytotoxicity towards the P815 cells and those from mice fed SO had the least.

There are a number of mechanisms as to how dietary fatty acids might affect •NO production by macrophages. One mechanism is through fatty acid modulation of the production of the eicosanoid PGE_2. PGE_2 is produced from its precursor fatty acid AA by cyclooxygenase. Mice fed the FO diet have been shown to replace a proportion of AA present in macrophage membranes with the n-3 fatty acids EPA and DHA (see chapter 2). This correlates with a reduced ability of elicited macrophages from mice fed the n-3 fatty acid diets to produce PGE_2 upon LPS stimulation (see chapter 2). Although many studies have shown PGE_2 affects •NO production, the precise effects reported are contradictory. For example, Iwabuchi *et al.* (1997) showed that indomethacin, a cyclooxygenase inhibitor, decreased •NO production by a LPS-stimulated macrophage cell line, and that PGE_2 increased nitrite production by this cell line in a dose dependant manner. Albina *et al.* (1991) also found that inhibiting cyclooxygenase with indomethacin caused LPS-stimulated murine resident peritoneal macrophages to produce less •NO. However, Chaet *et al.* (1994) found that inhibition of cyclooxygenase with indomethacin increased •NO production by LPS-stimulated alveolar macrophages. PGE_2 and PGI_2, which is also produced by cyclooxygenase, have been shown to dose- dependently inhibit nitric oxide production by LPS-stimulated J774 macrophages (Marotta *et al.*, 1992). Furthermore, indomethacin increased, and PGE_2 decreased, the killing of P815 cells by macrophages (Taffet *et al.*, 1981; 1982). These studies indicate that cyclooxygenase products such as PGE_2 inhibit

•NO production. If this is correct then a fish oil rich diet, which has been shown to lower PGE_2 production by macrophages (see chapter 2), should increase •NO production by these cells. Among the high fat diets in this study, macrophages from mice fed the FO diet produced the greatest amount of nitric oxide and the lowest amount of PGE_2. Therefore, the enhanced production of •NO by macrophages from mice fed these diets and the enhanced ability of these macrophages to inhibit the proliferation of P815 cells might be due to modulation of PGE_2 levels. There was, however, no correlation across the diets between PGE_2 and •NO production. This would indicate that perhaps it is not only diet-induced changes in PGE_2 production which are having an effect on LPS-stimulated •NO production by these cells. The answer to this dichotomy might lie in the absolute amounts of PGE_2 produced. Milano *et al.* (1997) have found that PGE_2 can regulate iNOS activity in LPS-stimulated J774 macrophages: it was found that PGE_2 concentrations between 1 and 10 ng/ml were able to stimulate the expression of iNOS and the release of •NO, but higher concentrations (> 50 ng/ml) were inhibitory. In the current study, the amount of PGE_2 produced by LPS-stimulated macrophages from mice fed the FO diet was approximately 10 ng/ml, a concentration which would stimulate iNOS leading to greater •NO production and decreased P815 cell survival. The concentrations of PGE_2 produced by macrophages from mice fed each of the other diets the other diets were greater than 50 ng/ml and thus would lead to inhibition of iNOS, decreased •NO production and increased P815 cell survival. While this may explain the observation made for the LPS-stimulated macrophages from mice fed the high fat diets, it does not explain the high levels of P815 cell killing and increased •NO production by macrophages from mice fed the LF diet. Macrophages from mice fed this diet had the greatest production of PGE_2 (82 ng/ml) and by the above reasoning, should produce the least •NO in response to LPS and should therefore result in the greatest increase in P815 survival. This was not the case as P815 cells incubated with LPS-stimulated macrophages from mice fed the LF diet had the lowest percent survival. It cannot be ruled out that that diet-induced changes in the production of other eicosanoids may be responsible for the observed effects on macrophage-mediated cytotoxicity. PGI_2 has been shown to be 10-fold more potent than PGE_2 at inhibiting •NO production by a LPS-stimulated macrophage cell line Marotta *et al.* (1992). In this current study this metabolite was not measured.

decreased the production of PGI_2 (measured as its stable breakdown product 6-keto-$PGF_{1\alpha}$) by murine macrophages stimulated with LPS about the same extent as it decreased PGE_2 production. It is also possible that the effect on •NO production by stimulated macrophages seen with fish oil feeding might be mediated by eicosanoids generated produced from n-3 fatty acids. To my knowledge the effect of eicosanoids produced from the n-3 family of fatty acids on •NO production or iNOS activity has not been examined. It is possible that •NO production by macrophages might be modulated by other mechanisms. Macrophages secrete arginase. Arginase is the enzyme which breaks down the amino acid arginine into ornithine and urea. Incubation of tumour cells with arginase results in tumour cell death and it has been proposed that macrophages kill tumour cells by depleting them of a source of arginine (Currie, 1978). However it might also be possible that macrophages kill tumour cells by secreting arginase, resulting in the 'channeling' of the intracellular arginine substrate towards the production of •NO. It might therefore be the increased •NO production which is responsible for increased macrophage cytotoxicity towards tumour cells rather than the secretion of arginase per se. It is also possible that other mediators secreted by macrophages might be responsible for increased macrophage cytotoxicity towards tumour cells. Macrophages secrete other mediators such as hydrogen peroxide and superoxide as part of their respiratory burst which can affect cell function and may lead to cell death (Adams & Hamilton, 1992). These mediators have not been investigated in the current study.

The effects of dietary fatty acids on the ability of macrophages to inhibit the proliferation of L929 cells was also investigated in the current study. L929 cell proliferation is inhibited by TNF-α and the ability of cells or the components of cell supernatants to kill the L929 cell line is often used as a bioassay to infer the production of TNF-α by cells. Macrophages from mice fed the low fat diet produced the greatest amount of TNF-α, while those from mice fed the fish oil diet produced the least. TNF-α production significantly correlated with the ability of macrophages to kill the TNF-α sensitive L929 cell line only if the data for the fish oil-fed animals were excluded.

fish oil-fed animals produce a large amount of TNF-α early after stimulation, and that perhaps the levels decrease by the 24 hour time point, when the supernatants in the cultures studied here were collected. Hardardottir & Kinsella, (1992) found that cell-associated TNF-α production increased earlier in resident peritoneal macrophages from mice fed a 1.5% n-3 fatty acid diet compared to macrophages from mice fed an equivalent amount of n-6 PUFA. Erickson & Hubbard (1994) also investigated the effect of dietary fatty acids on TNF-α production by resident peritoneal macrophages. They fed mice fish oil as a source of n-3 fatty acids or safflower oil as a control. They found that production of TNF-α by macrophages from both groups of mice peaked at 6-8 hours after LPS stimulation, and that macrophages from mice fed fish oil produced significantly more TNF-α than the macrophages from the safflower oil fed mice at 24 hours. Erickson & Hubbard (1994) found that the relatively high levels of TNF-α produced by macrophages from fish oil-fed mice at 24 hours did not appear to be due to continuous production of bioactive TNF-α. It is important to note here that both of these studies used resident peritoneal macrophages rather than the elicited ones used in the present study, and fed a lower level of dietary fat. Furthermore, in chapter 2 it was shown that the effect of dietary fat on TNF-α production by resident and elicited macrophages is different. A time course of TNF-α production by elicited macrophages would be needed in order to assess if fish oil feeding caused a change in the kinetics of TNF-α production by the cells studied here. Alternatively the decreased level of L929 survival by macrophages from mice fed fish oil, despite low levels of TNF-α production, may indicate that the cells are producing another compound which is affecting the cells proliferative ability, or perhaps producing a compound which potentiates the action of TNF-α. The identity of this possible mediator was not investigated in the present study. However, if macrophages from mice fed FO do make a mediator other than TNF-α which is toxic to L929 cells, then there are serious implications for the measurement of TNF-α by the commonly used bioassay based upon cytotoxicity of cell culture supernatants towards target L929 cells. The current study measured TNF-α concentrations using a specific ELISA. Most studies which report that FO feeding decreases TNF-α production have used ELISAs to measure the amount of

cytokine (Renier *et al.*, 1993; Yaqoob & Calder, 1995b; Grimm *et al.*, 1994), while those that report that FO feeding increases TNF-α production have used the L929 killing bioassay (Chaet *et al.*, 1994; Lokesh *et al.*, 1990; Chang *et al.*, 1992; Somers & Erickson, 1994). One other point of interest is that Tonetti *et al.*, (1997) have shown that L929 cells themselves can produce previously unknown soluble mediators which can have regulatory effects on TNF-α production by a macrophage cell line. It may be that macrophages from mice fed the fish oil diet could be more sensitive to these soluble factors produced by the target cells, and this might explain any possible changes in TNF-α production by the elicited macrophages under study.

One earlier study found that elicited macrophages obtained from mice fed a diet containing FO (10% by weight) for 15 weeks had decreased tumouricidal activity towards L929 cells compared to macrophages from mice fed a saturated fat diet (consisting of 10% palm oil and cholesterol) or a control diet (containing 5% palm oil and 5% fish oil) (Renier *et al.*, 1993). It is interesting to note that this group fed their mice for a longer time period than the current study, and that the control diet contained n-3 fatty acids, while the control diet in the current study did not.

The relationship between the amount of fat in the human diet and monocyte/macrophage-mediated cytotoxicity towards tumour cells has not been investigated, although putting healthy volunteers on a low fat diet (less than 30% or 22% of total energy as fat) significantly increased natural killer cell activity (Barone *et al.*, 1989; Hebert *et al.*, 1990) suggesting that high fat consumption may suppress natural killer cell activity in man. A diet high in the n-3 fatty acid DHA has also recently been shown to decrease natural killer cell activity in healthy male volunteers (Kelley *et al.*, 1998). The modulation of immune cell-mediated cytotoxicity by the amount and type of fat in the diet has implications for host defense, particularly against tumour cells. There are significant positive correlations between total fat content of the human diet and the incidence of breast, colon and prostate cancers (Rose, 1997; Reddy, 1992; Willett *et al.*, 1990). Ecologic, case-control, and cohort studies all support a positive relation between the consumption of certain types of fat (especially animal fat) and some forms of cancer (see Willett, 1997 for a review). For example Japan, a

country whose traditional diet is high in n-3 fatty acids, has on a population basis adopted a more Western type diet over the past 20 years, and this correlates with increasing rates of breast, colon and prostate cancers (Lands *et al.*, 1990). The observations that total fat, saturated fat and n-6 PUFA increase cancer incidence are supported by animal studies which have shown that the incidence and growth of colon and mammary tumours is higher in animals fed high fat compared with low fat diets and fed high fat diets rich in saturated fat or n-6 PUFA (Reddy, 1992). Specific fatty acids in the diet may be important: Animal experiments suggest that linoleic acid promotes colorectal carcinogenisis (Zaridze 1983; Sakaguchi *et al.*, 1984) and that a low fat diet rich in EPA has an inhibitory effect on colon cancer (Minoura *et al.*, 1988). Calder *et al.*, (1998) have shown that the enhancing effects of feeding diets rich in fat, saturated fat and n-6 PUFA on the growth of implanted human mammary and colon tumours are retained in athymic mice. Thus, if the mechanism by which fatty acids increase tumour growth is via a decrease in immunity towards the tumour, this immunological effect cannot involve T-cell-driven cell-mediated immunity, and may involve diminished macrophage activity towards tumours. Feeding fish oil did not promote colon tumour growth in athymic mice (Calder *et al.*, 1998). This could be explained in part by the fish oil-mediated enhancement in macrophage activity towards tumour cells demonstrated in the current study.

3.6 Conclusions

In conclusion, in agreement with the previous chapter, this study has illustrated that both the amount and type of dietary fat can affect the ability of macrophages to produce inflammatory mediators, and that this has implications for the anti-tumour effects of macrophages. An n-6 PUFA-rich diet decreased macrophage-mediated cytotoxicity to the greatest extent, while a FO- rich diet prevented the effects normally associated with high fat feeding.

Chapter 4: The effect of the type of serum/plasma used in cell culture on production of inflammatory mediators by macrophages.

4.1 Introduction

Macrophages are one of the major phagocytic cells of the immune system and play a key role in the modulation of inflammatory responses (see section 1.2.4). Dietary fatty acids can affect mediator production by macrophages, but many of the reports in the literature are conflicting (see section 1.3.5). Some of this conflict can be explained by differences in the activation state of the macrophages studied (see chapter two). There are however other methodological differences between studies such as length of feeding, species and strain of animal studied, level of fat fed, and stimulus used to elicit the macrophage response (see section 1.3.5). Another difference between some of the studies on the production of inflammatory mediators is the source of serum or plasma used in the *ex-vivo* cultures. Studies investigating the effects of dietary fatty acids on mediator production by macrophages have used either serum-free conditions (Hardardottir *et al.*, 1992), which denies the cells any fatty acids which would normally be present in their environment; foetal calf serum, (Yaqoob & Calder, 1995b), which contains high concentrations of EPA and DHA (see table 4.1), fatty acids which perhaps would not normally be present in high concentrations in the macrophage cellular environment; or autologous plasma (Brouard & Pascaud, 1990), which essentially provides the fatty acids in the physiological form and type which would normally be present in the cellular environment. Fatty acids are normally present in bloodstream as TAGs, cholesterol esters and phospholipids contained in lipoproteins and non-esterified fatty acids (NEFAs) bound to albumin (see section 1.1.5). Serum and plasma contain a range of lipids and fatty acids, which reflect the diet of the animal. *In vitro* studies that have examined the effects of fatty acids provided in culture medium on inflammatory mediator production by peripheral blood leukocytes or by peritoneal macrophages have shown that the type of fatty acid available in the culture medium can affect the ability of these cells to produce inflammatory mediators (Baldie *et al.*, 1993; Purasiri *et al.*, 1997; Tappia *et al.*, 1994). Autologous plasma also contains growth factors and hormones that would normally be presented to the cell *in vivo*. Serum-free conditions clearly lack many such factors while FCS will contain other

types and concentrations of bovine factors. Stimulated cells have a high level of membrane turnover, and would be likely to take up fatty acids from the culture medium not only for energy, but to replace some of the fatty acids lost from the rapidly turning over plasma membranes. Calder *et al.* (1994) showed this for Con-A stimulated lymphocytes cultured with various individual fatty acids. Stimulation with the mitogen Con-A caused significant changes in the fatty acid composition. When AA, EPA and DHA were included in the culture medium, they tended to be incorporated into membrane lipids in substantial quantities, especially in the case of DHA.. The types of fatty acid and other factors present in the culture medium may therefore be masking at least in part, the effects of the fatty acids provided in the diet. However, the influence of the type of serum or plasma present in the cell culture medium upon functional outcomes is not clear in the context of dietary manipulation. It is likely to be highly important and will be investigated in this chapter.

4.2 Objective

The objective of this study was to investigate the effects of the type of serum or plasma in the culture medium on the production of PGE_2, TNF-α and IL-1β by murine macrophages taken from mice fed different diets.

4.3 Methods

4.3.1 Animals and Diets

Male C57BL6 mice (n= 6 per diet) were obtained from Charles River, Margate, UK. Mice were fed diets identical to those described in section 2.3.1. Blood was collected by cardiac puncture into sterilized eppendorf tubes immediately after sacrifice.

4.3.2. Plasma Preparation

Autologous plasma was prepared by centrifuging the blood at 13,000 rpm for 6 minutes. The plasma was transferred into sterile eppendorf tubes.

4.3.3 Fatty Acid Compositions of the Diets and of FCS

Determination of the fatty acid composition of the diets has been previously described (see chapter 2). The fatty acid composition of the FCS was determined as described in section 2.3.10

4.3.4 Culture for Mediator Production

Thyioglycollate elicited macrophages (see section 2.3.5) were cultured in the presence of 10 μg/ml LPS and either 10% (v/v) foetal calf serum or 2.5 % autologous plasma (AP) in a manner identical to that described in section 2.3.5. Cell supernatants were collected after 24 hours and frozen at –20°C until analysis of the concentrations of PGE_2, TNF-α and IL-1β by commercial ELISA.

4.3.5 Determination of Triacylglycerol and Non-esterified Fatty Acid

Triacylglycerol concentrations were determined using a TAG assay kit (Sigma Chemical Co, Poole, Dorset) and NEFA concentrations were determined using a NEFA assay kit (Boehringer, Lewes, East Sussex).

4.3.6 Statistical analysis

Statistical analysis of dietary differences within a given serum/plasma condition was performed using a one way analysis of variance followed by the least significant difference test. Statistical differences between the two culture treatments within a given dietary group were analysed using an independent samples Student's t-test. A P value of < 0.05 was used to indicate statistical significance. The statistical computer package SPSS was used for all analyses.

4.4 Results

4.4.1 Fatty Acid Composition of the Diets and of FCS.

The fatty acid compositions of the diets are reported in section 2.3.1. Previous work has shown that the fatty acid composition in the serum/plasma of rodents is strongly influenced by the fatty acid composition of the diet (Yaqoob *et al.*, 1995). The fatty acid composition of the FCS used is shown in table 4.1.

Table 4.1 Fatty acid composition of the FCS as determined by gas chromatography. Fatty acids are given as g/100g of total fatty acids present in the sample.

	14:0	*16:0*	*18:0*	*18:1 n-9*	*18:2 n-6*	*20:4 n-6*	*20:5 n-3*	*22:6 n-3*
FCS	1.6	24.1	22.4	22.4	5.4	5.6	5.3	6.9

4.4.2 TAG and NEFA Levels in Plasma

In order to know the level of fat added to the culture medium by the addition of autologous plasma (AP), the concentrations of TAG and NEFA were measured. FO feeding decreased both TAG and NEFA levels compared to each of the other high fat diets (table 4.2). The concentrations of TAG and NEFA in one batch of FCS used in our laboratory were 0.83 mg/ml and 0.16 mmol/l, respectively (P.C. Calder, personal communication).

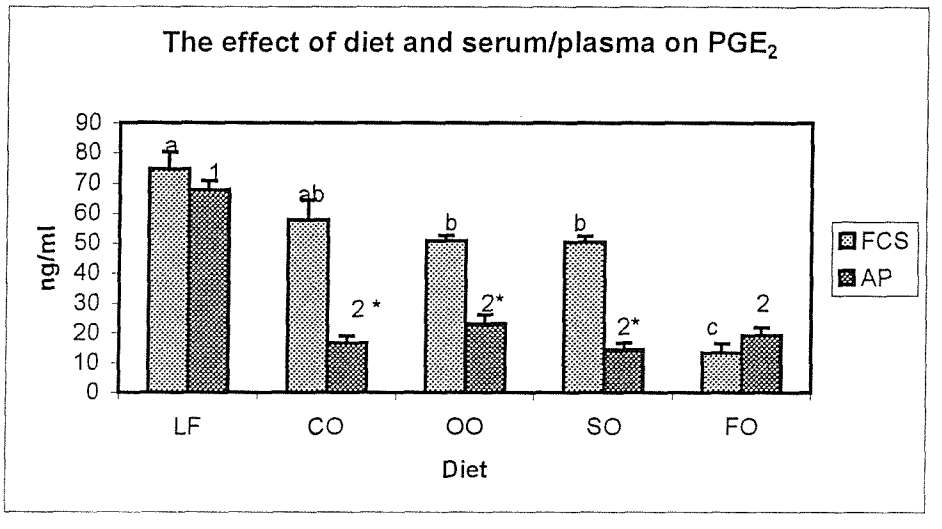
Table 4.2 TAG and NEFA concentrations in the autologous plasma from mice fed the different diets. Numbers not sharing a common letter within a column are significantly different ($P<0.05$).

Diet	*TAG (mg/ml)*	*Se*	*NEFA (mmol/l)*	*Se*
LF	0.6^{b}	0.1	0.3^{a}	0.1
CO	0.7^{b}	0.1	0.8^{c}	0.1
OO	0.7^{b}	0.1	0.6^{b}	0.03
SO	0.6^{b}	0.1	0.6^{b}	0.04
FO	0.4^{a}	0.03	0.3^{a}	0.02

4.4.3 PGE_2 Production by Macrophages

When cultured in FCS, cells from mice fed the LF diet produced significantly more PGE_2 than cells from the OO, SO and FO groups, while cells from mice fed the CO, OO and SO diets produced significantly more PGE_2 than those from mice fed FO (see figure 4.1). When cultured in AP, cells from mice fed the LF diet produced significantly more PGE_2 than those from each of the other groups. Culture with AP rather than FCS caused a significant decrease in the amount of PGE_2 produced by cells from mice fed the CO, OO and SO diets, but did not affect PGE_2 production by cells from mice fed the FO and LF diets. All cells were stimulated with 10 μg/ml LPS.

Figure 4.1 PGE_2 production by macrophages. Mice were fed one of five experimental diets and cultured with either FCS or AP. Data are mean ± SEM for 6 animals/diet. Within the FCS treatment group, columns not sharing the same alphabetic letter are significantly different. Within the AP treatment group, columns not sharing the same number are significantly different. Within each dietary group, * indicates significant differences between FCS and AP. All cells were stimulated with 10 μg/ml LPS



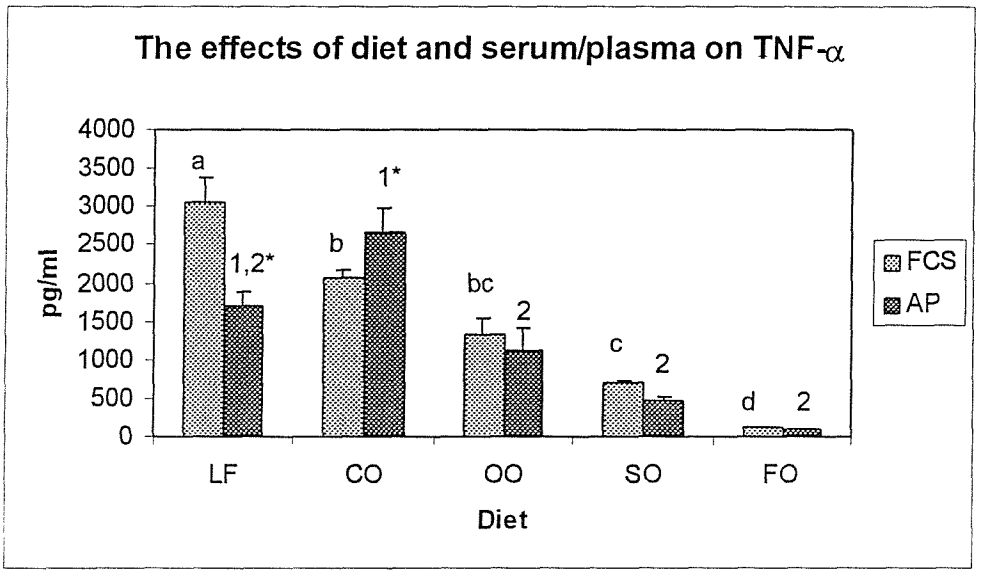
4.4.4 TNF-α Production by Macrophages

When cultured in FCS, cells from mice fed the LF diet produced significantly more TNF-α than cells from each of the other groups, while cells from mice fed the CO diet produced significantly more TNF-α than cells from mice fed the SO and FO diets (see figure 4.2). Cells from mice fed FO diet produced significantly less TNF-α than cells from mice fed each of the other diets. When cultured in AP, cells from mice fed the CO diet produced significantly more TNF-α than cells from mice fed the OO, SO and FO diets, while cells from mice fed the FO diet produced significantly less TNF-α than cells from mice fed the LF and SO diets. Culture with AP rather than FCS caused a decrease in the amount of TNF-α produced by cells from mice fed the LF and SO diets and caused an increase in the amount of TNF-α produced by cells from mice fed the

CO diet. TNF-α production by cells from mice fed the OO or FO diets was not affected by the type of serum/plasma.

Figure 4.2: TNF-α production by macrophages

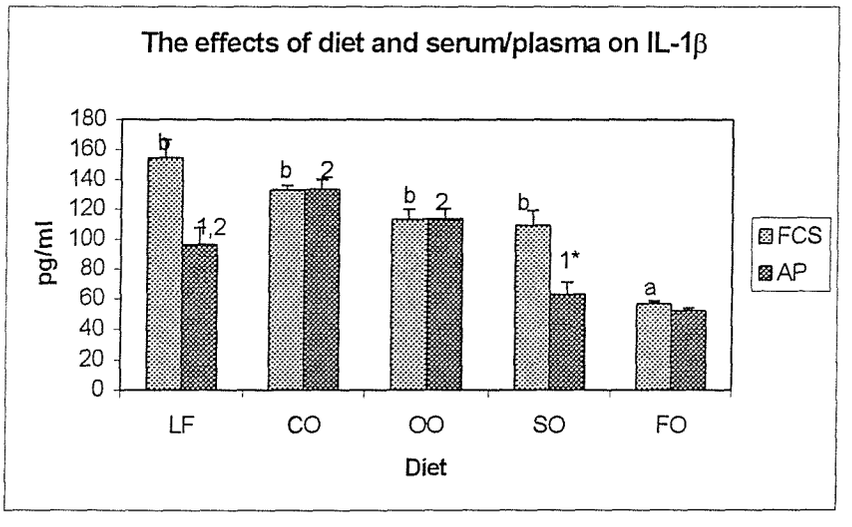
Mice were fed one of five experimental diets and cultured with either FCS or AP. Data are mean ± SEM for 6 animals per diet. Within the FCS treatment group, columns not sharing the same alphabetic letter are significantly different. Within the AP treatment group, columns not sharing the same number are significantly different. Within each dietary group, * indicates significant differences between FCS and AP. All cells were stimulated with 10 μg/ml LPS



4.4.5 IL-1β Production by Macrophages

When cultured in FCS, cells from mice fed the FO diet produced significantly less IL-1β than cells from mice fed each of the other diets (see figure 4.3). When cultured in AP, cells from mice fed either the SO or FO diets produced significantly less IL-1β than cells from mice fed the CO and OO diets. Culture with AP rather than FCS caused a significant decrease in the amount of IL-1β produced by cells from mice fed the LF and SO diets, but did not affect IL-1β production by cells from mice fed the CO, OO or FO diets. All cells were stimulated with 10 μg/ml LPS.

Figure 4.3 IL-1β: IL-1β production by macrophages. Mice were fed one of five experimental diets and cultured with either FCS or AP. Data are means ± SEM for 6 animals/diet. Within the FCS treatment group, columns not sharing the same alphabetic letter are significantly different. Within the AP treatment group, columns not sharing the same number are significantly different. Within each dietary group, * indicates significant differences between FCS and AP. All cells were stimulated with 10 μg/ml LPS



4.5 Discussion

This study examined the effects of culture in the presence of either FCS or AP on inflammatory mediator production by elicited peritoneal macrophages from mice fed one of four high fat diets or a low fat diet. This is the first time the effect of the type of serum/plasma present in the cell culture media on the production of macrophage-derived mediators has been investigated. It was found that the type of serum/plasma present could alter the outcome of dietary investigations of the effects of some types of dietary oils. It appears that cells from animals fed oils rich in n-6 fatty acids are particularly sensitive to the *ex vivo* culture conditions. In contrast, FO feeding caused a decrease in the amounts of PGE_2, IL-1β and TNF-α produced, and these decreases were not significantly affected by *ex vivo* culture conditions.

Mediator production by the FO group was the most resistant to change under the different conditions. Cells from FO-fed mice had the highest levels of n-3 fatty acids present in their membranes and addition of the FCS, with its high levels of n-3 fatty acids did not appreciably change the production of mediators. Mediator production by macrophages from the mice fed the OO and CO diets were also resistant to changes induced by *ex vivo* culture conditions. This may be due to the fact that FCS is also high in saturated fatty acids and oleic acid, fatty acids which would also be present in high concentrations in the AP from mice fed these diets. Therefore, the types of fatty acids presented to the cells cultured with FCS would be similar to those presented to cells cultured with AP and one would not expect to see much difference in mediator production between the cells cultured under the different *ex vivo* conditions. In contrast, the cells from mice fed the LF and SO diets had low levels of n-3 fatty acids present in their membranes and showed an enhancement of mediator production in most cases when the high n-3 fatty acid FCS was added to the culture medium.

In vitro studies have shown that the addition of fatty acids to culture medium affects the production of some pro-inflammatory mediators (IL-1, IL-6) by macrophages (Baldie *et al.*, 1993; Tappia *et al.*, 1995). The conditions in which the macrophages are

cultured might therefore either abrogate or enhance any effects of dietary manipulation on the production of inflammatory mediators by this cell type. The production of inflammatory mediators (cytokines) by Con-A stimulated murine lymphocytes (Yaqoob & Calder, 1995b) and the proliferation of rat lymphocytes (Yaqoob *et al.*, 1994) were shown to be affected by the presence of either FCS or AP in culture. The current study showed that culturing cells in FCS rather than AP had an overall stimulatory effect on production of some mediators by macrophages in some of the dietary groups. Some of the statistical differences in mediator production of some mediators seen in the presence of AP were abrogated or enhanced compared to culturing the cells with FCS. For example, macrophages from mice fed the LF diet produced significantly more IL-1β and TNF-α when the culture medium contained FCS rather than AP. However the type of serum/plasma present in the culture media did not affect the ability of macrophages from mice fed the LF diet to produce PGE_2. In general (but not always) macrophages from mice fed the various diets were more responsive and produced a higher concentration of mediators upon LPS stimulation when the culture medium contained FCS rather than AP. It is possible that FCS contains a factor that promotes macrophage activity and that macrophages from mice fed FO not sensitive to this, or have already become tolerant to it having encountered that factor *in vivo*. The identity of this factor(s) is unknown but it is possible that enzymes or hormones (perhaps stress hormones) could be responsible for the modulation seen in mediator production.

The increase in cytokine (IL-1β and TNF-α) production with culture in the presence of FCS was only seen for cells from mice fed the LF and SO diets. Macrophages from mice fed the LF diet had a higher proportion of arachidonic acid present in their lipids while those from mice fed the SO diet had a higher proportion of 18:2 n-6, the arachidonic acid precursor (table 2.3). This suggests that changes in the amounts of n-6 present in the environment of cells, or changes in the n-6:n-3 ration of fatty acids present when they are stimulated with LPS may modulate the production of inflammatory mediators. Cells from mice fed the LF and SO diets cultured with the FCS (which is most likely higher in n-3 fatty acids than the AP from mice fed these two diets) had an increased production of IL-1β and TNF-α compared to the cells from

mice fed these two diets cultured with AP. It may be that lowering the ratio of n-6: n-3 fatty acids present in the cellular environment might be important in modulating inflammatory responses. Jeffery *et al.*, (1996) found that lymphocytes from rats fed a diet with a lower ratio of n-6:n-3 fatty acids proliferated less well than lymphocytes from rats fed a diet with a high n-6:n-3 fatty acid ratio. Lymphocyte proliferation is dependent on the cytokine IL-2 produced by Th-1 cells (Abbas, 1994). The changes in lymphocyte proliferation imply that IL-2 production may have been modulated. The study by Jeffery *et al.*, (1996) therefore suggests that the production of IL-2 can be influenced by the n-6:n-3 ratio of fatty acids in the diet/cellular environment. It is possible therefore that such modulation may exist for other cytokines, such as IL-1β and TNF-α. With regard to IL-1β production by stimulated macrophages, the results of the current study which show an increase when cells are cultured with FCS agree with a study by Baldie *et al.*, (1993) which showed an increase in the release of IL-1 when both a macrophage like cell line and human mononuclear cells were incubated with arachidonic acid, which is present in significant quantities in the FCS.

Substantial differences were seen in PGE_2 production when the cells were cultured with FCS compared to AP. PGE_2 levels were significantly higher in the supernatants of cells from mice fed the LF diet compared to the high fat diets, and in contrast to the effect on TNF-α and IL-1β production by cells from mice fed this diet, these were not affected by culture conditions. PGE_2 production by macrophages from mice fed the FO diet was also not affected by culture conditions. N-3 fatty acids are present in high concentrations in both FCS and AP from the FO fed mice, and thus little effect of serum vs. plasma was seen. In the presence of FCS, PGE_2 production then decreased with increasing unsaturation of the diet. The pattern of PGE_2 production seen when cells were cultured in the presence of FCS was not observed when the macrophages from the mice fed the different diets were cultured in AP. PGE_2 production decreased when cells from mice fed the CO, OO and SO diets were cultured in AP rather than FCS. There were no differences in PGE_2 production between cells from mice fed the high fat diets when the cells were cultured with AP. Cells from mice fed the FO diet however produced similar amounts of PGE_2 regardless of culture conditions. The AA present in the FCS may be increasing the substrate available for PGE_2 synthesis, and

FO feeding seems to overcome this, perhaps by supplying a greater proportion of n-3 fatty acids. It may be that the ratio of n-6:n-3 fatty acids present in the cellular environment in the macrophages from mice fed this diet and cultured with FCS does not change as dramatically between the two culture conditions as for macrophages from mice fed the other diets.

The lack of an effect of diet on PGE_2 production seen when the cells from the high fat-fed animals were cultured along with AP may be due in part to the overall concentration of total fat present in the culture media. A study by Tappia *et al.* (1995) examined the effect of culturing rat peritoneal macrophages with individual fatty acids and then stimulating them with LPS. This study found that PGE_2 levels were unaffected when the concentrations of fatty acids in the serum were less than 50 μM. In the current study, the level of total fat present in the AP can be estimated by the TAG and NEFA concentrations of the serum (see table 4.2). The LF- and FO- fed mice had lower NEFA concentrations in their sera and the FO- fed mice also had the lowest TAG concentrations. It might be possible therefore that differences in the effects of the AP on PGE_2 production by macrophages are due in part to differences not only in the type of fatty acids present, but also the amount of fatty acid present in the serum.

4.6 Conclusion

The type of serum or plasma used in *ex vivo* cell culture can result in different patterns of production of inflammatory mediators by macrophages and can mask or enhance the effects of dietary manipulation. However, macrophages from mice fed a FO- rich diet appear to be resistant to the modulating effect of the serum/plasma present, at least with respect to the production of PGE_2, IL-1β and TNF-α. The results of this study have implications for the future design of studies investigating the effect of dietary fatty acid manipulation on the production of inflammatory mediators by LPS-stimulated macrophages. The choice of serum/plasma used in future investigations must be taken into account when planning experiments and interpreting the results obtained.

Chapter 5: Dietary fatty acids modulate the production of Th-1 but not Th-2 cytokines.

5.1 Introduction

5.1.1 Cytokine production by T- lymphocytes

The CD4+ T-helper cell is one of the most important cells involved in cell-mediated immunity. The products it secretes are essential for the differentiation of immature B-lymphocytes into antibody producing cells and also for the activation of cytotoxic T cell, monocytes and macrophages and natural killer cells. CD4+ T helper cells are classified into two subtypes (Th-1 and Th-2) depending upon the cytokines that they produce (Mossman, 1986; see section 1.2.7). CD4+ T cells which have yet to be directed towards a Th-1 or Th-2 subtype are called Th-0 cells, which are cells capable of producing at low levels, all cytokines found to be secreted by either Th-1 or Th-2 cells. These are the T-cells that are predominantly encountered in human immune responses. They can be shifted towards either a Th-1 response or a Th-2 response depending on the cytokine environment which is present when they encounter activation. For example, Th-0 cells encountering IL-12 produced by macrophages at the site of inflammation would be shifted towards a Th-1 response and away from a Th-2 type response.

Th-1 cytokines include IL-2 and IFN-γ. These cytokines have two key functions: they stimulate macrophages and enhance their microbial actions, and they stimulate the production of IgG antibodies by B cells. IgG antibodies bind to Fcγ receptors and complement proteins and are primarily involved in the phagocytosis of microbes such as bacteria. In addition, Th-1 cytokines can promote the activity of natural killer (NK) cells and the differentiation of CD8+ cells into active cytotoxic cells (Robey & Allison, 1995). The Th-1 cytokine IL-2 is also responsible for the proliferation of lymphocytes. Thus, the primary function of Th-1 cells is to direct phagocyte-mediated defence against bacterial and viral infections. Th-1 dominant responses are often associated with inflammation and tissue injury because IFN-γ recruits and activates inflammatory leukocytes. Overproduction of Th-1 cytokines has been implicated in the

progression of some autoimmune diseases such as rheumatoid arthritis, a disease in which chronically activated macrophages cause inflammation in the joints.

Th-2 cells synthesise IL-4, IL-5, and IL-10, but not IL-2 or IFN-γ. IL-4 is predominantly involved in B-cell isotope switching to IgE antibody production, while IL-5 has a role in activating eosinophils involved in the elimination of extracellular pathogens (especially in parasitic infections). A number of Th-2 associated cytokines have anti-inflammatory actions as well. IL-4 antagonises the macrophage activating action of IFN-γ, while IL-10 suppresses numerous macrophage responses (Abbas *et al.*, 1999). Consequentially, the net result of Th-2 cytokine actions is to suppress both acute and chronic inflammation. It is therefore possible that Th-2 cells are predominantly operating not as effectors but as regulators of the immune response (Robey & Allison, 1995). Inappropriate production of IgE is known to be involved in the pathology of allergic inflammation (Abbas *et al.*, 1994) Thus, Th-2 mediators, such as IL-4, are implicated in diseases such as chronic asthma and eczema (Black & Sharpe, 1997).

5.1.2 n-3 Fatty acids can affect T-lymphocyte function

A number of T-lymphocyte functions, such as lymphocyte proliferation in response to mitogens, have been shown to be altered following the ingestion of a diet rich in n-3 fatty acids (see chapter 1). One of the earliest animal studies showed that feeding rats on a linseed oil-rich diet suppressed spleen T lymphocyte proliferation compared to feeding a coconut oil-rich diet (Marshall & Johnston, 1985), while Kelley *et al.* (1988) found that the proliferation of rabbit spleen T lymphocytes and of peripheral blood T and B lymphocytes was suppressed following a fish oil (FO)-rich diet compared with feeding diets rich in hydrogenated coconut, safflower or linseed oils. A number of other animal studies have followed these and almost all have found that feeding a linseed oil or FO- rich diet to a variety of animals (mice, rats, rabbits, chickens, monkeys) causes a suppression of lymphocyte proliferation compared to diets rich in other oils (see chapter 1 section 1.3 for references).

There are a number of ways in which FO could be exerting its effects on lymphocyte proliferation, one of which is through the modulation of the production of T lymphocyte-derived cytokines particularly IL-2. A decrease in IL-2 production by n-3

fatty acids would explain the decreased lymphocyte proliferation observed in most of the studies noted above. However there have been only a few studies on the effects of n-3 fatty acids on lymphocyte derived cytokines, and not many have measured cytokines other than IL-2 (see chapter 1, section 1.3). A number of animal studies and have investigated the effect of n-3 fatty acids on IL-2 production (Fernandes *et al.*, 1994; Yaqoob & Calder, 1995b; Wu *et al.*, 1996; Jolly *et al.*, 1997). However, some of these studies have found an increase in IL-2 production following FO feeding (Fernandes *et al.*, 1994) while others have found no effect (Yaqoob & Calder, 1995a; Wu *et al.*, 1996) or a decrease (Jolly *et al.*, 1997). FO supplementation of the diet of healthy humans has been shown to decrease the production of IL-2 by blood lymphocytes (Meydani *et al.*, 1991; Endres *et al.*, 1993). The effect of n-3 fatty acid feeding on the *ex vivo* production of IL-2, and also on the production of other Th-1 cytokines such as IFN-γ, needs to be clarified. No human studies have reported Th-2 cytokine production after dietary lipid manipulation, and at the time the experiments presented in this chapter were carried out only one animal study had been reported (Yaqoob & Calder, 1995a). This study compared the effect of feeding weanling mice for 8 weeks on a FO-rich diet with feeding with feeding diets containing other types of fatty acids. FO feeding had no effect on IL-2, IL-4 or IL-10 production by the spleen lymphocytes. It is also important to note that cellular interactions between antigen presenting cells and T-cells involving the co-stimulatory molecules B7 and CD28 are also important in stimulatating IL-2 transcription and the proliferation of T cells (Abbas *et al.* 1994). B7 expression on some antigen presenting cells may be up-regulated by a variety of stimuli and therefore immune responses may be in part dependent on these stimuli. N-3 fatty acids have been shown to affect the level of expression of some surface molecules (Jolly *et al.*, 1998) and so it is possible that fatty acids may have affect the expression of other surface molecules such as CD28, and influence the ability of lymphocytes to proliferate through decreasing the transcription of IL-2. The effect of dietary fat on the expression of CD28 or the binding of CD28 to B7 on antigen presenting cells has not been previously investigated.

5.1.3 Possible effects of n-3 fatty acids on cytokines

The modulation of Th-1 cytokines by FO feeding may have implications with regard to the modulation of a number of disease states. If FO does decrease IL-2 and IFN-γ production then it might act to shift the balance between Th-1 and Th-2 cytokines towards a more Th-2 type response (see figure 1.2.7 for Th-1 vs. Th-2 balance). Certain diseases are characterised by a predominance of either Th-1 or Th-2 type responses (see section 1.2.7). For example, the shifting away from a Th-1 type response, characteristic of a disease such as rheumatoid arthritis, towards a Th-2 type response might be beneficial in this disease and lead to the relief of some symptoms. Indeed, diets high in FO have been shown to have benefit in ameliorating some of the symptoms of rheumatoid arthritis (see Belch & Muir, 1998 for a review). However Th-2 mediated diseases such as asthma and some forms of allergy, might be exacerbated by diets high in FO. This point however is open to debate as different fatty acids clearly exhibit different effects in atopic diseases (Calder & Miles, 2000). This requires further investigation.

5.2. Objective

The objective of this study was to examine the effect of diets high in n-3 fatty acids on spleen lymphocyte fatty acid composition, Th-1 and Th-2 cytokine production and proliferation after Con-A stimulation.

5.3 Methods

5.3.1 Animals and Diets

Male C57/BL6 mice weighing between 20-25 grams were obtained from Charles River, Margate, UK. They were housed in individual cages in a climate-controlled animal house at the School of Biological Sciences, University of Southampton; a 12-hour light/dark cycle was used. Mice were allowed access *ad libitum* to water and to one of five experimental diets (prepared by ICN Biomedicals, High Wycombe, Bucks, UK) for a seven-week period. The diets were identical to those previously described (see chapter 2). After 7 weeks on the diets the mice were sacrificed by CO_2 inhalation and spleens were collected into sterile RPMI 1640.

5.3.2 Preparation of Lymphocytes

In sterile conditions the spleens were gently mashed through wire mesh into petrie dishes and 10 ml of culture medium (consisting of RPMI 1640, 2 mM glutamine, 10% heat inactivated FCS and 50 μg/ml 1:1 mixture of streptomycin and penicillin) was added. The cells were then collected by centrifugation (5 min, 1500 rpm). The cells were re-suspended in 1 ml of medium and then layered over histopaque; a mixture of ficoll and sodium metrizoate with a density of 1.077g/ml. They were then centrifuged for 20 minutes at 2000 rpm in order to separate the lymphocytes from other cell types. The lymphocyte layer was collected, gently centrifuged, resuspended in 1 ml culture medium, counted and diluted to 2×10^6 cells/ml.

5.3.3 Lymphocytes Culture for Mediator Production

One ml of lymphocytes (2×10^6 cells) was placed in the wells of a 24 well tissue culture plate and 800 μl culture medium (described above) and 200 μl of 25 μg/ml Con-A added; unstimulated cells received RPMI instead of Con-A. The cells were cultured at 37°C in an atmosphere of 19:1 air:CO_2. After 24 hours the plate was centrifuged for 5 minutes at 1500 rpm. The supernatants were collected and aliquoted into sterile eppendorf tubes and frozen for later measurements of cytokine concentrations using ELISA kits purchased from Biosource International (Camarillo, USA).

5.3.4 Lymphocyte Proliferation

One hundred and eighty μl of cells (1×10^5 cells) were added along with 20 μl of 25 μg/ml Con-A (to give a concentration in the well of 2.5 μg/ml) to the wells of a 96 well microtitre plate; control wells received 20 μl RPMI instead of Con-A. The cells were cultured for 48 hours at a temperature of 37°C in an air/CO_2 (19:1) atmosphere. Then, 20 μl of tritiated thymidine (0.2 μCi/well) was added to each well and the cells were cultured for a further 18 hours. The cells were then harvested onto glass filter papers and washed and dried using a Skatron Cell Harvester. Radioactive thymidine incorporation was determined by liquid scintillation counting. The stimulation index (SI) was calculated as:

$$SI = \frac{\text{Incorporation of tritiated thymidine in the presence of Con-A}}{\text{Incorporation of tritiated thymidine in the absence of Con-A}}$$

5.3.5 Fatty Acid Analysis by Gas Chromatography

The fatty acid composition of lymphocytes was analysed in an identical manner to that of macrophages (see section 2.3.12)

5.3.6 Statistical Analysis

Statistical analysis was performed using SPSS version 6.0 (SPSS Inc., Chicago, IL, USA). Means and standard errors are reported. Differences between groups were determined by one-way ANOVA followed by the least significant post hoc test. A value of $P < 0.05$ was taken to indicate statistical significance.

5.4. Results

5.4.1. Lymphocyte Fatty Acid Composition

Table 5.1 shows the fatty acid composition of spleen lymphocytes from mice fed the various diets. In general, the fatty acid composition of the diets was reflected in the spleen lymphocyte fatty acid composition. Mice fed the LF diet had the greatest proportion of arachidonic acid (20:4n-6) in their lymphocytes. Lymphocytes from mice fed the CO diet had significantly higher levels of 14:0 than lymphocytes from mice fed the other diets. Lymphocytes from mice fed the OO diet had a significantly higher proportion of 18:1n-9 present compared to those from mice fed the SO, FO and LF diets. Lymphocytes from mice fed the SO diet had significantly elevated levels of 18:0 compared to those fed the OO and FO diets. The proportions of 20:3n-6 and 18:2n-6 were highest in the lymphocytes from mice fed the SO diet (although not significant). Lymphocytes from mice fed the FO diet had significantly more 20:5n-3, 22:5n-3 and 22:6n-3 than lymphocytes from mice fed the other diets. This appeared to replace 20:4n-6 as the proportion of this fatty acid was decreased in the lymphocytes from mice fed FO.

Table 5.1 Fatty acid composition of spleen lymphocytes from mice fed the different diets

Values are means and standard errors (se) of 4-6 animals per diet. Values not sharing the same letter across a row are significantly different.

Fatty Acid	*LF*	*Se*	*CO*	*Se*	*OO*	*Se*	*SO*	*Se*	*FO*	*Se*
8:0	0	0	4.9	0	0	0	0	0	0	0
10:0	0	0	3.3^{b}	0	1.0^{b}	0.1	4.0^{a}	0.4	0	0
12:0	3.3^{b}	0.8	4.0^{b}	0.7	1.1^{a}	0.1	3.1^{b}	0.1	3.3^{b}	0.1
14:0	2.8	0.7	7.3^{a}	1.0	3.6^{b}	1.5	3.2^{b}	0.6	3.1^{b}	0.4
16:0	32.4^{ab}	0.5	30.6^{ab}	1.3	29.0^{ab}	1.6	25.8^{b}	3.4	33.6^{a}	2.4
16:1 n-7	2.9^{b}	0.7	3.3^{b}	0.1	2.3^{b}	0.2	3.0^{b}	0.3	4.9^{a}	0.5
16:2 n-7	2.3^{b}	0	2.0^{b}	0	6.1^{a}	0	2.8^{b}	0	1.8^{b}	0.3
18:0	16.6^{ab}	1.2	15.5^{ab}	0.8	14.9^{b}	0.6	21.9^{a}	3.8	16.5^{ab}	2.1
18:1 n-9	11.4^{b}	0.6	10.9^{b}	0.3	15.7^{a}	0.5	7.0^{c}	0.5	9.5^{bc}	2.3
18.2 n-6	6.7^{a}	0.5	5.6^{a}	0.6	6.0^{a}	0.3	6.9^{a}	2.7	4.1^{a}	0.2
20:2 n-6	2.8^{bc}	0.9	4.3^{b}	0.8	1.6^{c}	0.4	4.7^{a}	0.5	2.2^{c}	0.7
20:3 n-6	1.9	0	2.1	0	2.4	0.5	3.3	0	1.0	0
20:4 n-6	16.3^{b}	0.3	13.2^{b}	0.4	17.4^{b}	0.6	8.1^{a}	3.0	7.6^{a}	1.2
20:5 n-3	0	0	0	0	0	0	0	0	4.9	0.7
24:0	4.0	0.6	2.5	0.4	4.3	0.2	0	0	0	0
22:5 n-3	0	0	0	0	0	0	0	0	4.0	0.6
22:6 n-3	0	0	0	0	3.0^{b}	0.7	2.5^{b}	0.2	8.8^{a}	1.5
n-3/n-6	0	0	0	0	0.1^{b}	0.1	0.2^{b}	0.1	1.6^{a}	0.3

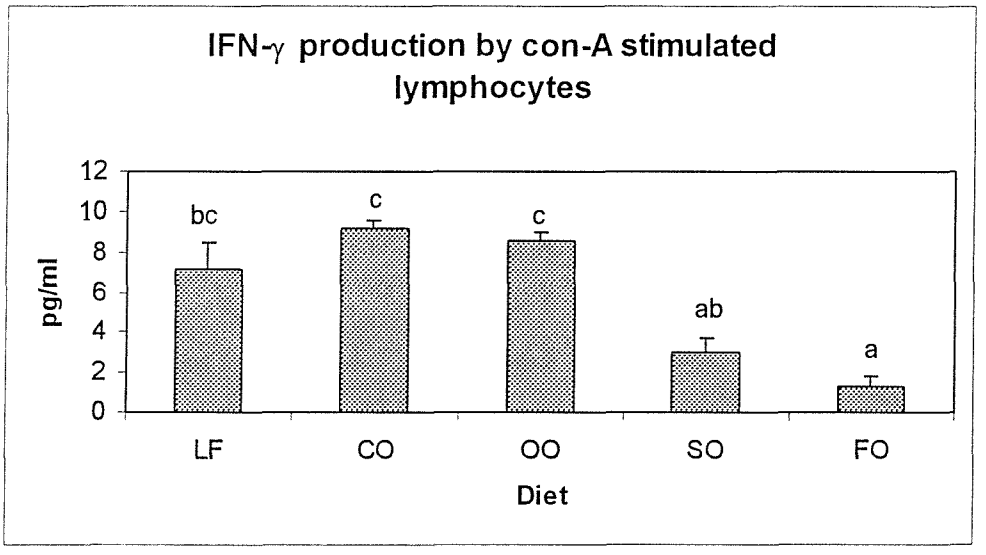
5.4.2. Cytokine production by lymphocytes

5.4.2.1 IFN-γ Production

Lymphocytes from mice fed the FO and SO diets produced significantly lower ($p<0.05$) amounts of IFN-γ in response to Con-A stimulation, compared with those from mice fed the OO, CO and LF diets (Figure 5.1).

Figure 5.1 IFN-γ production by murine spleen lymphocytes stimulated with con-A.

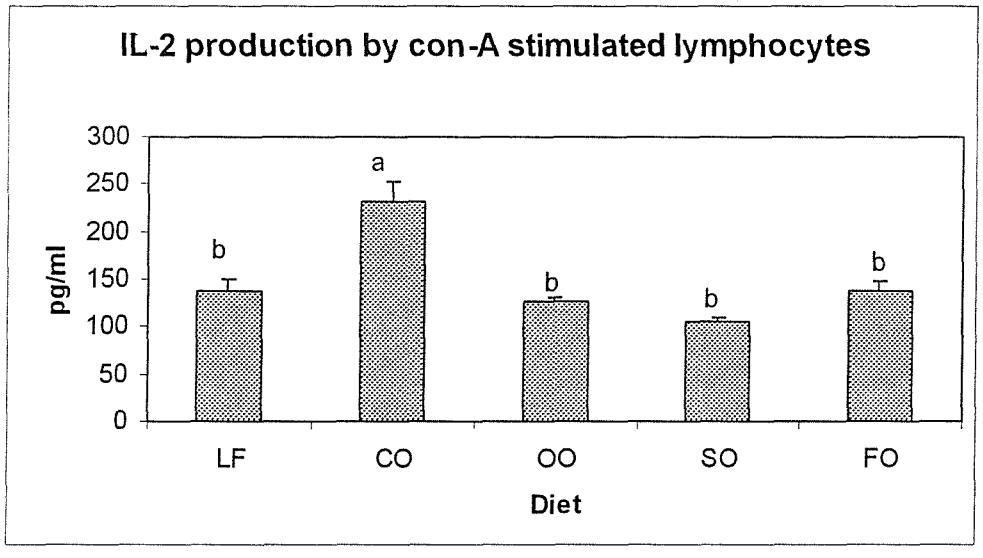
Spleen lymphocytes were cultured for 24 hours in the presence of Con-A and concentrations of IFN-γ present in the cell supernatants were then measured by ELISA. Values presented are means +/- SEM from 6 mice/diet. Columns not sharing the same letter are significantly different.



5.4.2.2 IL-2 production

Lymphocytes from mice fed CO produced a significantly higher concentration ($p<0.05$) of IL-2 compared to those from mice fed each of the other diets (Figure 5.2).

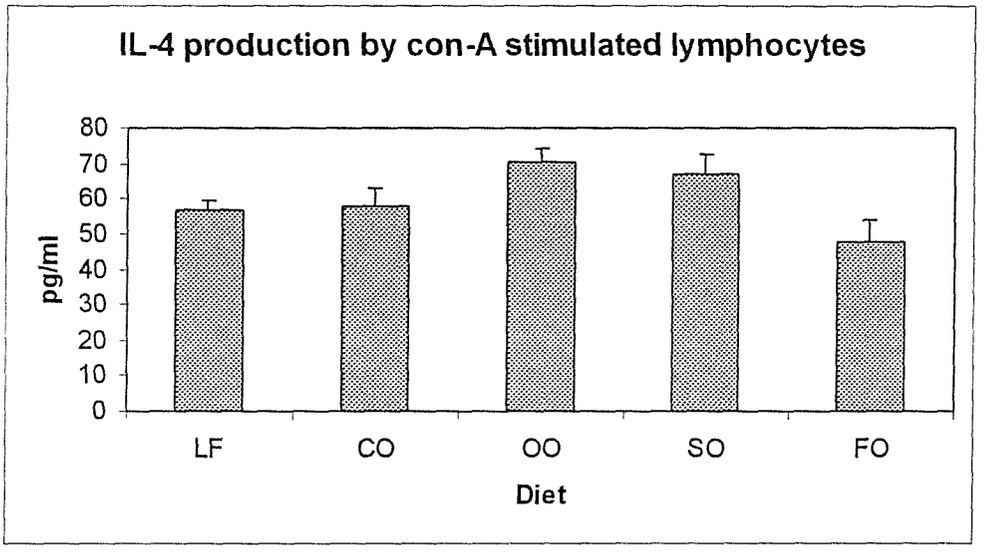
Figure 5.2. IL-2 Production by Murine Spleen Lymphocytes Stimulated with Con-A.
Spleen lymphocytes were cultured for 24 hours in the presence of Con-A and concentrations of IL-2 present in the cell supernatants were then measured by ELISA. Values presented are means +/- SEM for 6 mice/diet. Columns not sharing the same letter are significantly different.



5.4.2.3 IL-4 production

There were no differences between IL-4 production between lymphocytes from mice fed the different diets, although lymphocytes from mice fed the FO diet produced the lowest amount (Figure 5.3).

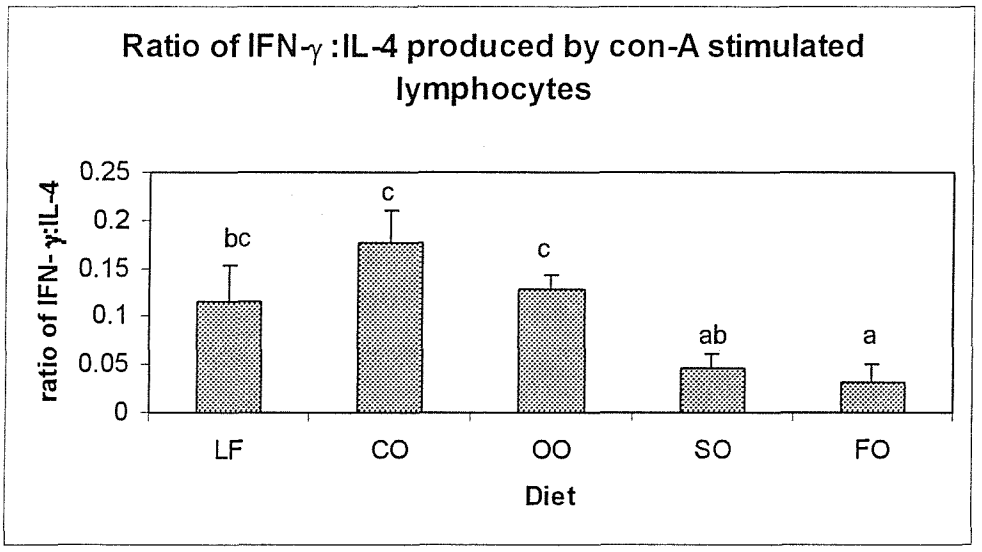
Figure 5.3. IL-4 production by murine spleen lymphocytes stimulated with con-A.. Spleen lymphocytes were cultured for 24 hours in the presence of Con-A and the concentrations of cytokines measured by ELISA. Values presented are means +/- SEM for 4-6 mice/diet.



5.4.2.4 IL-10. Spleen lymphocytes did not produce detectable levels of IL-10 after stimulation with Con-A.

5.4.5 The ratio of IFN-γ to IL-4 produced by lymphocytes can be altered by dietary fatty acids. FO feeding produced significantly lower IFN-γ:IL-4 ratios than feeding the LF, OO or CO diets (see figure 5.4). Lymphocytes from mice fed the SO diet had significantly lower IFN-γ: IL-4 ratios than those from mice fed the OO and CO diets.

Figure 5.4 Ratio of IFN-γ to IL-4 produced by lymphocytes.
Spleen lymphocytes were cultured for 24 hours in the presence of Con-A and the concentrations of cytokines measured by ELISA. Values presented are means +/- SEM for 4-6 mice/diet. Columns not sharing the same letter are significantly different.



5.4.3 Lymphocyte Proliferation

There were no significant differences in thymidine incorporation into unstimulated lymphocytes between the dietary groups (see table 5.2). However, in the presence of 2.5 μg/ml Con-A, lymphocytes from mice fed the CO diet showed significantly greater thymidine incorporation than lymphocytes from mice fed the OO, SO and FO diets (table 5.2). Lymphocytes from mice fed the FO diet had the lowest incorporation of thymidine. When expressed as SI, there were no significant effects of diet, although the SI was highest for the CO-fed mice and lowest for cells from FO-fed mice (table 5.2).

Table 5.2 Proliferation of Murine Spleen Lymphocytes

Lymphocyte proliferation by unstimulated and Con-A (2.5μg/ml) stimulated murine spleen lymphocytes as measured by incorporation of tritiated thymidine (cpm/well) or as a maximum stimulation index. Data are mean (se) for 4-6 mice per diet. Values within a column not sharing the same letter are significantly different.

Diet	*Without Con A (cpm/well)*	*Se*	*With Con A (cpm/well)*	*Se*	*Maximum SI*	*Se*
LF	190	69	19599[ab]	6427	103	15
CO	266	141	34631[b]	652	206	72
OO	109	51	12438[a]	938	188	42
SO	247	199	16438[a]	6795	177	39
FO	115	9	10402[a]	1499	92	13

5.5 Discussion

5.5.1 Fatty Acid Composition

The fatty acid composition of the diet was reflected in the spleen lymphocyte fatty acid profiles. In particular mice fed the FO diet had a significantly higher proportions of n-3 fatty acids in their spleen lymphocytes than did mice fed the other diets. Mice fed the diet high in n-6 fatty acids (i.e. SO) did not have a significantly greater amount of AA (20:4n-6) present in their spleen lymphocytes. This is surprising as 18:2n-6 is the precursor fatty acid to AA and it might be expected that a diet high in the precursor fatty acid would promote AA production. Macrophages from mice fed this diet showed an increased proportion of AA (see chapter 2). This increase does not appear to occur in lymphocytes, and this may relate to the functional capacity of the cells to use this fatty acid. Macrophages have the ability to convert AA into a range of lipid mediators, while lymphocytes lack some of the key enzymes such as cyclooxygenase needed to produce some of these mediators (Goldyne, 1988).

The proportion of 20:4 n-6 present in the lymphocytes from mice fed the OO, LF and CO diets (while higher than the proportion present in the lymphocytes from mice fed the SO and FO diets) was lower than the amount of AA present in the macrophages from mice fed those diets (see chapter 2). The fact that the proportion of AA present in the spleen lymphocytes decreased when the mice were fed SO, and that the proportion of AA in these cells is similar to the proportion of AA present in the spleen lymphocytes from mice fed FO is an unexpected result, and may suggest that very high intakes of 18:2 n-6 might inhibit the Δ6 desaturase enzyme. This contrasts with the findings of Brouard and Pascaud (1990), who fed rats for 6 weeks on diets providing either 3.5% of calories (5-6 % by diet weight) as corn oil (high in 18:2n-6), or as a corn oil linseed oil mix, or as a corn oil fish oil mix. They found that AA was increased in spleen lymphocytes from rats fed the corn oil diet compared to those fed the other two diets. The findings of the current study also contrast with those of Yaqoob *et al.* (1995) in which diets identical to the ones used in the current study were fed to rats for 6 weeks and the proportion of AA in phospholipids of spleen lymphocytes was increased

after SO feeding. However, there were no changes in the proportion of AA present in the spleen neutral lipids from rats fed the SO diet (Yaqoob *et al.*, 1995). The differences in results between this study and the current study may relate to species differences between mice and rats, and/or to the fact that the current study examined total lipid composition which measures neutral lipids and phospholipids together, while the changes in the proportions of AA observed by Yaqoob *et al.* (1995) occurred only in phospholipids. If the current study had examined the fatty acid composition of the neutral lipid fraction and the phospholipid fraction of the spleen lymphocytes separately, then the changes seen by Yaqoob *et al.* (1995) may have been observed. In the current study the level of fat in the diet was much greater (40% of calories – typical of the British diet; 20% by weight) than used by Brouard & Pascaud (1990). This may be one reason why elongation of 18:2 n-6 was not as prevalent in these cells. Whelan *et al.* (1993) have shown that the conversion of linoleic acid to AA appears to be highly regulated and that increasing the dietary levels of linoleic acid to above 3% of calories is poorly correlated with tissue AA content. In the current study, linoleic acid contributed approximately 24% of calories in the SO diet. It is also possible that certain tissues or cells are more sensitive to the inhibitory effects of high dietary linoleic acid. This may explain why the lymphocytes in the present study do not contain an increased proportion of AA despite having an abundant supply of its precursor fatty acid. In contrast, macrophages do increase their content of AA as linoleic acid supply increases. Macrophages are cells with extremely high rates of membrane turnover, and which are capable of producing a larger range of lipid mediators derived from membrane AA than are lymphocytes (Chilton *et al.*, 1996). Macrophages need a supply of AA to perform their immunologic functions, such as the generation of eicosanoids, and thus can use their membrane supply of AA fairly quickly. During an immunologic challenge, this AA would need to be generated in order to replace the AA lost via the generation of eicosanoids. It may be therefore that the regulatory mechanisms induced by high dietary LA may not occur in macrophages, and might only occur in cells or tissues that do not produce such a vast range of lipid mediators, and whose membranes are more quiescent. Nevertheless, dietary fatty acids do influence the fatty acid composition of lymphocyte lipids. Modulation of the fatty acid composition of the membrane might be one mechanism through which the effects of dietary fatty acids are exerted. Dietary fatty acids that are incorporated into cell membranes may affect how

certain proteins such as receptors, enzymes and ion channels sit in the membranes and may affect signalling events within the cell (Murphy, 1990). Consumption of unsaturated fatty acids, especially FO may also modulate membrane fluidity (Tappia *et al.*, 1997; Calder *et al.*, 1994) which may have effects on cellular function.

5.5.2 Cytokine Production and Proliferation

As mentioned previously, helper T lymphocytes can be divided functionally into different subsets (Th-1 and Th-2), depending on the types of cytokines produced upon stimulation (see chapter 1). Previous *in vitro* studies have shown when n-3 PUFAs are included in the culture medium of rat lymphocytes (Calder & Newsholme, 1992b) and human lymphocytes (Calder & Newsholme 1992a) they can decrease the production of IL-2. Little is known about the effect of fatty acids on the production of other Th-1 type cytokines (e.g. IFN-γ) or Th-2 cytokines (IL-4, IL-10). This was addressed in the current study. Diet had an effect on the production of the Th-1 cytokines IFN-γ and IL-2, but did not have an effect on the production of the Th-2 cytokine IL-4; the Th-2 cytokine IL-10 was not made at detectable levels by these cells under the conditions used. FO and SO feeding significantly lowered IFN-γ production by murine lymphocytes compared to the LF, OO and CO diets. This agrees with the results of a recent *in vivo* study by Byleveld *et al.* (1999) which reported that FO feeding significantly reduced IFN-γ production in the lung lymph nodes of mice given an intranasal challenge of influenza virus. It also agrees with Fritche *et al.* (1999) which showed that IFN-γ production by spleen lymphocytes was lower in mice given a challenge of *Listeria monocytogenes* after being fed a FO-rich diet.

In the current study, the significantly higher ratio of Th-1:Th-2 cytokines produced by spleen lymphocytes from mice fed the LF, OO and CO diets compared to the FO diet indicates that this ratio is amenable to dietary manipulation and that FO (and SO) feeding may shift the balance between Th-1:Th-2 type responses towards a Th-2 response. This may have consequences for naïve T-cells encountering a site of inflammation. Low concentrations of IL-4 and high concentrations of IFN-γ in the environment would lead to a situation that would promote the development of a Th-1 type response. Promotion of a Th-2 type response would occur by Th-0 cells

encountering a cytokine environment with a low Th-1:Th-2 ratio (high concentrations of IL-4 and low concentrations of IFN-γ). This implies that diets high in FO and SO might promote a Th-2 type of response. This interpretation agrees with a recent study by Kleemann *et al.* (1998) who studied the ratio between the mRNA for the Th-1 cytokine IFN-γ and mRNA for the Th-2 cytokine IL-10 in the Peyer's Patches of diabetic rats: FO feeding shifted the ratio towards a Th-2 mediated response. IL-10 production was not detected in the current study. However, in an earlier study using a similar in protocol to the current study, IL-10 production (which was also produced at very low concentrations) by spleen lymphocytes was not affected by the type of fatty acid present in the murine diet (Yaqoob & Calder, 1995a). An *in vivo* study by Sadeghi *et al.* (1999) which used diets identical to the ones in the present study, found that IL-10 levels in the plasma were increased in response to a non-lethal dose of *Escherichia coli* LPS in mice fed the CO diet compared to those fed the OO and LF diets. There was no effect of feeding SO or FO on plasma concentration of IL-10. IL-10 is produced by lymphocytes during the later stages of an inflammatory response in order to modulate the production of various pro-inflammatory cytokines. It may be that if the lymphocytes in the current study had been cultured for 48 or 72 hours rather than for 24 hours, IL-10 may have been present at detectable levels in the cell culture medium.

It has been shown previously that feeding diets rich in FO to rabbits, chickens, mice and rats results in suppressed proliferation of lymphocytes compared to feeding CO, SO, corn oil or lard diets (Alexander & Smythe, 1988; Kelley *et al.*, 1988; Fritche *et al.*, 1991; Yaqoob *et al.*, 1994; Yaqoob & Calder, 1995a; Sanderson *et al.*, 1995) (see chapter 1). The results of the current study agree with this. As well as decreasing IL-2 production (at least compared with the CO diet), FO feeding decreased lymphocyte proliferation (expressed as thymidine incorporation). Lymphocytes from mice fed the CO diet had significantly higher lymphocyte proliferation in response to Con-A stimulation, and produced significantly higher levels of IL-2 than cells from mice that had been fed an unsaturated fatty acid-rich oil as their fat source. In the current study there was a weak positive correlation between IL-2 production and lymphocyte proliferation when expressed as the incorporation of tritiated thymidine (r^2 =0.77). It must be remembered however that lymphocyte proliferation was measured 3 days after

the Con-A challenge and that the cytokines to which this data is correlated were measured after 1 day of culture. It is therefore possible that the weak association may have become stronger if it had been possible to assess the two measurements at the same time. Most *in vitro* studies agree that saturated fatty acids cause less inhibition of lymphocyte proliferation than unsaturated fatty acids (Rotondo *et al.*, 1994, Calder *et al.*, 1992a,b), and that the degree of inhibition by unsaturated fatty acids in part correlates with the degree of unsaturation (Calder, 1995). The present study agrees with an *in vitro* study by Calder & Newsholme (1992a) which reported that unsaturated fatty acids caused a decrease in the concentration of IL-2 present in the medium of cultured lymphocytes by 45%, while saturated fatty acids (such as those found in high doses in CO) had no effect on IL-2 production. It may be considered therefore that the amount of IL-2 produced by the CO fed mice might represent the 'maximal' amount of IL-2 that might be produced by these cells under these culture conditions. If the results of this study are considered in this way, then all of the unsaturated fatty acid-rich diets caused a decrease in IL-2 production compared to the high CO diet. In the current study, no difference was observed between the FO and SO groups in the production of IL-2 when the cells were cultured for 24 hours with Con-A. In fact lymphocytes from mice fed the SO diet actually produced less (although not significantly different) IL-2 in response to Con-A stimulation than did the lymphocytes from mice fed the FO diet. There was also no difference in the production of IL-2 by lymphocytes between the FO and the LF groups. However, the amount of IL-2 produced by lymphocytes from mice fed both of these diets was significantly different from the amount produced by the lymphocytes from the CO-fed group. It is also possible that dietary fatty acids might affect the expression of certain co-stimulatory molecules on T-cells, such as CD28 which is known to be important in stimulating T-cell proliferation, and known to drive T-cells to a more Th-1 type response. This possiblity has not been investigated in the current study.

5.4.3 Possible mechanisms of action of dietary fatty acids on lymphocyte function

The modulation of cytokine production by unsaturated fatty acids may be occurring in part through modulation the production of PGE_2 by surrounding macrophages. PGE_2 is known to inhibit T-cell activation (Rappaport & Dodge, 1982), but seems to have a stronger modulatory activity on the Th-1 subset rather than Th-2 subset. A study by

Gold *et al.* (1994) found that relatively high concentrations of PGE_2 completely inhibited IL-2 and IFN-γ production *in vitro*, while IL-4 production was unaffected. Although lymphocytes lack the COX enzyme and therefore do not possess the ability to make PGE_2 (Goldyne, 1988), it is made in appreciable quantities by macrophages (see chapter 2). Macrophages are present in the red pulp of the spleen and may be present in some degree in the lymphocyte cell preparation. It is likely that spleen lymphocyte preparations will have small numbers of contaminating macrophages capable of producing physiologically significant amounts of PGE_2, which might in turn have an effect on cytokine production by the spleen lymphocytes. The proportions of AA present in the lymphocytes from the SO and FO fed mice were lower compared to the amount of AA present in the lymphocytes from mice fed the other diets. It is also interesting that lymphocytes from both of these dietary groups had the lowest production of IFN-γ, suggesting that the production of an AA derived mediator may modulate the ability of the lymphocytes to produce IFN-γ. FO feeding has been shown to decrease the production of PGE_2 by peritoneal macrophages (see section 3.4.5) and it is likely to cause a decrease in the production of PGE_2 by the macrophages present in the spleen. This should remove the inhibitory effect of PGE_2 on Th-1 cytokine secretion and perhaps cause an increase in production of these cytokines. This effect does not seem to occur, as IFN-γ production was lowest in the FO group. The reason for this may possibly lie in the n-3 analogue of PGE_2, PGE_3. PGE_3 has been shown to have a suppressive effect upon lymphocyte proliferation and therefore may possibly be acting through modulation of the cytokine IL-2. Shapiro *et al.* (1993) have examined the *in vitro* effects of addition of PGE_2 and PGE_3 on lymphocyte proliferation, and found that while both eicosanoids suppress lymphocyte proliferation, PGE_3 is the more potent of the two eicosanoids. This may explain the decreased proliferation in the FO group. The effects of PGE_3 on cytokine production by lymphocytes have not been widely investigated, but recent studies from our group have shown that PGE_3 is a potent inhibitor of IFN-γ production (L.M. Aston, E.A. Miles & P.C. Calder, unpublished observations). In the current study, IFN-γ production was lowest by lymphocytes from mice fed the FO diet, the diet that would promote the production of PGE_3 by macrophages. The lack of effect of diet on IL-4 production by lymphocytes agrees with the Gold *et al.* (1994) which showed no effect of PGE_2 on the secretion of

Th-2 cytokines from lymphocytes. The effects of PGE_3 on cytokine production by immune cells however need to be examined in more detail and are under investigation by our group.

It is known that dietary fatty acids can exert their effects on T-lymphocytes through eicosanoid-independent mechanisms as well as through the modulation of eicosanoids. This was first shown by Santoli & Zurier (1989) who showed in an *in vitro* system that AA, DGLA and EPA could inhibit the production of IL-2 by T lymphocytes and that the inclusion of indomethacin (a COX inhibitor) did not affect this. Calder & Newsholme (1992a,b) have shown that culture of Con-A stimulated blood lymphocytes with oleic acid, linoleic acid, α-linolenic acid (ALNA), AA, EPA or DHA results in lower IL-2 concentrations in the culture medium than if the cells are cultured in the absence of fatty acids or in the presence of saturated fatty acids. This would agree with the current study as lymphocytes from mice fed the unsaturated fatty acid diets produced less IL-2 and proliferated less well in response to Con-A than did lymphocytes from mice fed the CO diet. An *in vitro* study by Purasiri *et al.* (1997) has recently confirmed that EPA and DHA inhibit IL-2 production by human peripheral blood mononuclear cells. This study also examined the effect of unsaturated fatty acids on IFN-γ production by human peripheral blood mononuclear cells and found that low levels of DHA enhanced production while EPA and GLA were without an effect (Purasiri *et al.*, 1997). It is also notable that inhibitors of PLA_2 and LOX when included in the medium of stimulated lymphocyte cultures in *in vitro* experiments also do not remove the inhibitory effect of unsaturated fatty acids (Calder *et al.*, 1992; Soyland *et al.*, 1993; Kumar *et al.*, 1992). This indicates that mechanisms occur in addition to and independent of modulation of eicosanoid production that are modulating the effects of unsaturated fatty acids on lymphocyte function (Calder *et al.*, 1992; Soyland *et al.*, 1993; Rotondo *et al.*, 1994; Khalfoun *et al.*, 1996). In the situation encountered by the lymphocytes *in vivo* however, it is likely that both eicosanoid modulated, and eicosanoid-independent effects might occur.

Studies by Jolly *et al.* (1997, 1998) have examined the effect of feeding C57Bl/6 mice (the strain of mice used in the current study) a diet composed of 3% total fat. The diets were 3% safflower oil, 2% safflower oil + 1% arachidonic acid, 2% safflower oil + 1%

EPA and 2% safflower oil + 1% DHA. Mice in this study were fed for 10 days and then spleen lymphocytes were collected, stimulated with Con-A and then IL-2 and IL-2 R mRNA expression were determined. IL-2 secretion, measured 48 post stimulation was significantly decreased by the EPA and DHA rich diets compared to the SO diet (Jolly *et al.*, 1997). IL-2 mRNA levels were not affected by dietary EPA or DHA, but the expression of mRNA for the α-subunit of the receptor for IL-2 was suppressed following a diet rich in EPA or DHA (Jolly *et al.*, 1998). The receptor for IL-2 consists of three chains α, β and γ (Mills *et al.*, 1993). Inhibition of the receptor, or one of its specific signalling chains may represent another way in which n-3 fatty acids might influence lymphocyte proliferation. The α chain of the IL-2 receptor contributes essentially to the high affinity IL-2R complex, which upon binding IL-2, signals driving the T lymphocytes to progress through their cell cycle (Mills *et al.*, 1993). The β chain of the IL-2R is also very important since it plays a role in intracellular signalling (Minami *et al.*, 1993), and it is unfortunate that the effects of EPA and DHA feeding on the IL-2R β chain were not assessed by Jolly *et al.* (1998). The level of expression of IL-2R was not examined in the current study. However, if the level of expression was suppressed as a result of the high intake of n-3 fatty acids present in the FO diet, it would explain the lowered (although not significant) proliferation from lymphocytes from mice fed this diet compared to the lymphocytes from mice fed the LF and SO diets high in n-6 fatty acids, as lowered expression of IL-2R might render the cells less responsive to IL-2. A recent *in vitro* study by Jenski *et al.* (1999) has also investigated the effect of DHA on IL-2R expression in spleen lymphocytes. This study found that DHA had no effect on the expression of this receptor, but that the proliferation of lymphocytes in response to Con-A was decreased. It is possible that the effects of n-3 fatty acids on lymphocyte proliferation may be exerted through both the production of the cytokine and the expression of its receptor, but this requires further investigation.

In contrast to the findings of Jolly *et al.* (1997), Fernandes *et al.* (1994) found that FO feeding caused an increase in IL-2 mRNA production in spleen lymphocytes from NZB/NZW (autoimmune-disease prone) mice in response to Con-A stimulation compared to a corn oil diet. Mice fed the FO diet also had increased lymphocyte

proliferation in response to Con-A compared to the corn oil-fed mice, and had decreased expression of IL-4 mRNA, indicating that a shift towards the increased production of Th-1 cytokines had taken place in response to diet. In the current study, this shift did not occur. In fact there was a shift towards the production of Th-2 type cytokines when the ratio of the IFN-γ:IL-4 present in the lymphocyte culture media was assessed (see figure 5.4) There was however no significant change in the ratio of IL-2:IL-4 present in the lymphocyte culture media (data not shown).

The difference between these studies illustrates the importance of the length of time of culture of the lymphocytes when making comparisons between results, and also the possible influence of the strain of mouse/type of animal used in the investigations. In the current study, the level of fat in the diet was much higher (20%, by weight compared to 3% by weight), and the length of feeding was much longer (6 weeks rather than 10 days), than in the Jolly *et al.* (1997) study. Also the feeding duration in the current study, was much shorter than the Fernandes *et al.* (1994) study (6.5 months). This might account for the differences observed in the production of IL-2 by stimulated lymphocytes. The contrasting effects of FO feeding between the Jolly *et al.* (1997) and the Fernandes *et al.* (1994) studies might also relate to the strain of mice used. The NZB/NZW mice used by Fernandes *et al.* (1994) spontaneously develop a murine form of the disease SLE. As such their immune system may be sensitised to environmental changes such as diet-induced changes in fatty acid composition of cellular membranes. The study by Jolly *et al.* (1997) used a C57Bl/6 strain, which is an inbred strain of mouse which does not develop autoimmune disease, but is prone to atherosclerosis and obesity. This was the type of mouse used in the current study.

It is possible that the effects of FO feeding on cytokine production by lymphocytes might be regulated at the cellular level by modulation of the levels of the intracellular second messenger, cyclic adenosine monophosphate (cAMP) via either modulating the production of eicosanoids, or modulation of the enzyme GTPase through alterations in membrane fluidity (Ross & Gilman, 1990). The mechanism of inhibition of PGE_2 on lymphocyte proliferation appears to be through increasing levels of cAMP that binds to its receptor, protein kinase A (PKA) (Kammer, 1988). Novak & Rothenberg (1990) reported that intracellular cAMP levels could regulate cytokine mRNA production in

lymphocytes. Increases in cAMP levels resulted in a suppression of IL-2 mRNA, but IL-4 mRNA was unaffected. IL-2 and IL-2R gene expression are both targets of PGE_2 induced suppression, possibly through the early events in T-cell signalling that include calcium influx and phosphatidylinositol breakdown (Anastassiou *et al.*, 1992; Alava *et al.*, 1993). PGE_2 is also known to inhibit the DNA binding activity of NFκB to the IL-2 transcripitional start site, thereby blocking formation of IL-2 (Chen & Rothenberg, 1994). A major mechanism of signal transduction in cells is mediated by receptor coupling to GTP-binding proteins, resulting in the activation of adenylate cyclase leading to an increase in intracellular cAMP. Unsaturated fatty acid feeding has been shown to modulate the generation of cAMP in macrophages (Tappia *et al.*, 1997). The effects of diets high in different unsaturated fatty acids on cAMP levels in lymphocytes has not been examined however. In the study of Tappia *et al.* (1997), the activity of GTPase and intracellular cAMP levels in rat peritoneal macrophages after feeding a variety of high fat diets for 4 or 8 weeks was studied. Intracellular cAMP content was elevated after 8 weeks of feeding rats a corn or coconut oil diet compared to a FO diet. GTPase activity after 4 weeks of feeding was also elevated in the macrophages from rats fed a CO and butter diet compared to FO, OO or LF (chow) diets. While this study examined macrophages rather than lymphocytes, if similar effects of diet occur in lymphocytes, by the reasoning presented by Tappia *et al.* (1997), a CO-rich diet should lead to increased GTPase activity, leading to increased cAMP levels and a suppression of IL-2. This is not the case as stimulated lymphocytes from mice fed the CO-rich diet had increased IL-2 and increased proliferation compared to lymphocytes from mice fed the other diets. The mechanisms by which dietary fatty acids exert their actions on specific cell types therefore requires further investigation. Modulation of the amount of substrate present for eicosanoid synthesis, the modulation of a number of enzymes involved in the release of AA from membrane phospholipids and the modulation of enzymes generating other intracellular messengers such as PKC (May *et al.*, 1993), and PLC-γ1 (Sanderson & Calder, 1998) may all be possible contributing factors to the observed effects of dietary fatty acids on lymphocyte cytokine production. FO feeding has also been shown to decrease the level of expression of some adhesion molecules (CD2, ICAM-1, LFA-1) on spleen lymphocytes (Sanderson *et al.*, 1995). These molecules play an important role in T-cell signalling and activation, and it is possible

that the effect of FO feeding on lymphocyte proliferation might be due to the modulation of these cellular molecules.

It is important to note that the cytokine concentrations measured in the medium represent the balance between the production of the cytokine and its utilisation in culture. It is possible that it is not the production that is different between the cells, but the increased utilisation of the cytokine which is responsible for the decreased levels seen in the medium after 24 hours of culture. This possibility has not been examined in this study. Enzymes present in the culture medium that are secreted from the cells themselves can also degrade cytokines. This has not been examined previously, but EPA has been shown to affect the levels of matrix metalloproteins secreted by some cells (McCabe *et al.*, 1999), and so it is theoretically possible that FO feeding may affect the production of other proteases as well. It is possible therefore that dietary fat is exerting its effects by modulating both the production and the utilisation of the cytokines by the cells or by affecting the activities of the extra-cellular enzymes secreted by the cells. It is possible that FO feeding causes an increase in cellular uptake of certain cytokines. This has never been investigated.

5.5.4 Implications

Several studies show beneficial effects of FO in diseases characterised by an inappropriate Th-1 response such as rheumatoid arthritis (Watson *et al.*, 1990; Esperson *et al.*, 1992; Geusens *et al.*, 1994; Kremer *et al.*, 1990, 1995; for a review see James & Cleland, 1997; Belch & Muir, 1998). Rheumatoid arthritis is a common disease and it produces substantial morbidity as well as an increase in mortality (Pincus *et al.*, 1994; Wolfe *et al.*, 1994; Weinblatt *et al.*, 1985; Felson *et al.*, 1990). Although the causes of this disease are not fully understood, studies have shown that proinflammatory cytokines an important role in its pathology (Arend & Dayer, 1995; Brennan & Feldman, 1992). The trials investigating the effects of FO on rheumatoid arthritis have primarily focused on monitoring clinical outcomes. However, some of the studies have also investigated the production of the macrophage-derived pro-inflammatory cytokines TNF-α and IL-1β, because these cytokines are involved in the pathology of the joint destruction that occurs in this disease (Firestein & Zvaifler, 1997). Indeed anti-TNF-α and anti-TNF-α receptor therapy as a treatment for arthritis

have been under investigation for the last few years (Moreland *et al.*, 1997) with some success. Both TNF-α and IL-1β are able to cause leukocyte migration and T cell differentiation which is dependent on IL-2, and so up-regulate the production of this cytokine by activated CD4+ cells. It has been suggested that treatment with the anti-inflammatory cytokines IL-4 and IL-10, which are known to down regulate the production of the pro-inflammatory cytokines such as TNF-α and IL-1β as well as regulating the production of IL-2 might be of use in the treatment of rheumatoid arthritis (Kawakami *et al.*, 1997; Firestein & Zvaifler, 1997). It is possible therefore that the mechanism of the beneficial effects of FO in the treatment of this disease might relate to the promotion of a Th-2 type response in these patients, thus increasing the production of IL-4 and IL-10 and resulting in the lower levels of TNF-α and IL-1β observed in these studies (Esperson *et al.*, 1992). The modulation of IL-4 and IL-10 production by FO supplementation of the diet has not been previously studied in this patient group or in healthy humans.

In the last two decades there has been an increase in the prevalence of asthma, eczema, and allergies in developed countries (Burr *et al.*,1989). Asthma may affect up to 5% of the Western population and is the most common chronic condition of childhood, with between 20% and 25 % of all children experiencing wheezing at some point in their lives (Flemming & Crombie, 1987). Some researchers have suggested that dietary fatty acids, especially those from the n-6 fatty family which are the precursors of the 4-series leukotrienes may aggravate the symptoms of asthma (Black & Sharpe, 1997). This has prompted some research groups to suggest that replacement of n-6 fatty acids with n-3 fatty acids, may have a beneficial effects on diseases modulated by leukotrienes. EPA, present in FO may be converted via the 5-lipoxygenase pathway to LTB_5 and LTC_5, LTD_5 and LTE_5. EPA is a slightly better substrate than AA for 5-lipoxygenase (Soberman *et al*, 1985), which may promote the formation of the less proinflammatory 5-series of leukotrienes and lessen the effects of the proinflammatory 4-series leukotrienes implicated in asthma. Indeed replacement in the diet of n-6 fatty acids with n-3 fatty acids has shown to improve the symptoms of asthma (Broughton *et al.*, 1997) and promote the production of the less pro-inflammatory 5-series leukotrienes. The possibility that the beneficial effects of n-3 fatty acids in asthma may also be

occurring through the modulation of T cell cytokines has not previously been investigated. The effects of dietary n-3 fatty acids on the production of Th-2 cytokines by lymphocytes that are associated with the progression of asthma are unknown. PGE_2 acts on T cells to decrease the formation of IFN-γ without affecting the formation of IL-4 (Gold *et al.*, 1994). This may lead to the development of allergic sensitisation since IL-4 promotes the synthesis of IgE, whereas IFN-γ has the opposite effect. n-3 fatty acids however have been shown in a number of animal and human studies to decrease the production of Th-1 cytokines (see chapter 1), and so in theory, they should promote the production of Th-2 cytokines and thus promote asthma. Therefore, before recommendations to asthmatics to increase n-3 fatty acid consumption in order to decrease 4-series leukotriene production can be made, the influence of n-3 fatty acids on the production of Th-2 cytokines needs to be determined in these patients.

5.6 Conclusions

Compared to saturated fatty acids, unsaturated fatty acids, especially those present in FO, cause a decrease in the proliferation of spleen lymphocytes. In agreement with this, feeding unsaturated fatty acids caused a decrease in IL-2 production compared to feeding the CO diet. IFN-γ levels in the culture medium were potently reduced by FO and SO feeding. The secretion of the Th-2 cytokine IL-4 by murine lymphocytes was not affected by dietary fatty acids. The Th-2 cytokine IL-10 was not made at detectable levels by these cells. In conclusion, compared to diets high in saturated fatty acids, unsaturated fatty acids, especially the highly unsaturated fatty acids found in FO, decrease the production of Th-1 cytokines and have no effect on Th-2 cytokine production. This results in changes in the Th-1/Th-2 cytokine balance in the cellular environment. These effects may be occurring through the fatty acid modulation of eicosanoid levels in cellular milieu, which in turn may modulate intracellular cAMP activity leading to decreased cytokine production. The exact mechanisms however require further investigation.

Chapter 6: Dietary DHA modulates the function of lymphocytes in stressed and unstressed mice.

This chapter will describe studies carried out in Professor Tom Hamazaki's laboratory at Toyama Medical and Pharmaceutical University in Toyama, Japan during my stay on a Monbusho/British Council Research Fellowship for Young Foreign Researchers (REFYFR).

6.1 Introduction

6.1.1 Dietary DHA and immune function

In chapter 5 it was shown that feeding fish oil (FO) affected the production of Th-1 but not Th-2 cytokines. It is postulated that this effect may be due to modulation in the production of the eicosanoid PGE_2 (Gold *et al.*, 1994) by macrophages in the cellular preparation. FO contains a variable mixture of EPA and DHA (see table 1.1.3), and only EPA is a substrate for eicosanoid synthesis (see section 1.1.9). It is estimated that 9% of the DHA in a rodent diet and 1.4% of the DHA in a human diet can be retroconverted to EPA (Brossard *et al.*, 1996). Thus, DHA supplementation of the diet could elicit changes in eicosanoid metabolism via its conversion to EPA. This has been illustrated by in a study by Peterson *et al.* (1998) which found that PGE_2 production by spleen lymphocytes in response to Con-A stimulation was reduced after feeding weanling rats a DHA-rich diet. The effect of DHA supplementation of the diet on prostaglandin production has also been investigated recently in humans. Kelley *et al.* (1999) showed DHA supplementation of the diet at a level of 6 g/day decreased the ability of blood mononuclear cells to produce PGE_2 and LTB_4 in response to LPS. In addition, the effect of a high DHA diet on cytokine production by murine spleen lymphocytes has recently been examined by Jolly *et al.* (1997; 1998; 1999). These studies found that DHA decreased IL-2 production and spleen lymphocyte proliferation, and also suppressed IL-2 receptor α-subunit mRNA levels. The effects of dietary DHA in the absence of EPA, on the production of T cell cytokines other than IL-2 has not been examined. This was addressed in the current study for the first time. Studies with DHA are important because it is the major n-3 fatty acid in neural tissues,

is the predominant structural fatty acid in the grey matter of the brain and retinal tissues in mammals, and the body tends to conserve more of it than EPA (Bazan, 1990; Horrocks & Yeo, 1999). DHA is accrued in these tissues during the last trimester of pregnancy and for this reason in recent years there has been a great deal of interest in increasing the supply of DHA in the maternal and premature and term infant diet in order to aid neural development (British Nutrition Foundation, 1999). Because FO feeding has been shown to suppress some aspects of immune cell function (see chapters 2-5) and because infants have naïve immune systems, it is important the immunomodulatory effects of DHA be known before recommendations to increase DHA intake in these populations can be made.

Some animal studies in recent years have found that n-6 dietary fatty acids can increase the production of glucocorticoids (Tannenbaum *et al.*, 1997; Engler *et al.*, 1999). Some stress hormones (i.e. cortisol and adrenaline) have been shown to be decreased following DHA supplementation of the human diet (Hamazaki *et al.*, 1999; Sawakaki *et al.*, 1999). Glucocorticoids are potent modulators of the immune response and have anti-inflammatory and immunosuppressive actions (Buckingham *et al.*, 1996). This is attributed to their powerful actions on the growth, differentiation, distribution and function of monocytes, macrophages, polymorphonuclear cells and lymphocytes (Buckingham *et al.*, 1996). High levels of glucocorticoids have also been shown to induce apoptosis in some lymphocytes (Telford *et al.*, 1991). Glucocorticoids also exert a profound inhibitory effect on the synthesis of many of the cytokines vital to immune cell function (Buckingham *et al.*, 1996). For instance, glucocorticoids have been shown to shift the production of certain lymphocyte cytokines away from a Th-1 type response towards more of a Th-2 type response (Ramirez *et al.*, 1996; Agarwal & Marshall, 1998). Dietary FO has also been shown to have immunomodulatory properties (see chapters 1-5). The interaction between dietary FO and glucocorticoids has not been thoroughly investigated. Yaqoob & Calder (1996) however, have shown that thymic lymphocytes from FO-fed rats are significantly more sensitive to the inhibitory effects of glucocorticoids *in vitro* than those from rats fed other high fat diets. As FO has also been shown to decrease the Th-1 response (see chapter 5), it is possible that dietary n-3 fatty acids might augment the immunosuppressive effects of glucocorticoids. The effect of dietary DHA on circulating concentrations of

corticosterone, the major murine glucocorticoid, has not previously been examined. As DHA supplementation has been shown to be associated with decreased circulating levels of cortisol in humans (Hamazaki *et al.*, 1996), the effect of DHA supplementation of a murine diet on this hormone was examined in the current study for the first time.

6.1.2 Stress, diet and immunity

An adverse relationship between stress and human disease is a common clinical observation (Cohen *et al.*, 1991; McEwen & Stellae, 1993; Hibbeln, 1998). Environmental stimuli can induce behavioral changes that can have tremendous effects on the physiology of an organism. Some research over the last two decades with human subjects has focused on the impact of stressful stimuli on immune function. For example, the stressful experience of first year medical school examinations has been correlated with a decrease in natural killer cell activity (Kiecolt-Glaser *et al.*, 1986) and alterations in the balance of Th-1/Th-2 cytokines (Marshall *et al.*, 1998). Decreased immunocompetence has also been associated with a high degree of loneliness and/or a high frequency of stressful life events in medical students and psychiatric patients (Glaser *et al.*, 1986; Kiecolt-Glaser *et al.*, 1986). Academic stress has been shown to decrease the concentration of IL-1 receptor antagonist, IL-6 receptor, and soluble glycoprotein 120 in the circulation, and the number of circulating CD8 positive cells (Song *et al.*, 1999). Taken together these results suggest that psychological stress induces immune-inflammatory changes, the mechanisms of which are likely to involve stress hormones such as cortisol, noradrenaline and adrenaline or ratios between them (Sawazaki *et al.*, 1999). Reciprocal communication between the brain-neuroendocrine system and the immune system is critical to host defense (Khansari, 1990; Olff, 1999). It provides a means whereby the central nervous system can detect alterations in immune status and initiate responses (behavioral, physiological, and immuno-regulatory) which are designed to protect the host and thus to restore homeostasis (Buckingham *et al.*, 1996). The two systems often use similar or identical cytokines to communicate with each other. Hibbeln *et al.* (1997) proposed that deficiency of very long chain n-3 fatty acids is an important factor underlying increased susceptibility to clinical depression and to hostile and aggressive behavior. Dietary DHA is crucial for proper brain function as it is an important component of synaptic membranes that are

involved in signal transmission through the nervous system. Decreases of DHA in the brain are associated with cognitive decline during ageing and with the onset of Alzheimer's disease (Horrocks & Yeo, 1999). Epidemiological studies show an inverse association between fish consumption and the prevalence of major depression around the world (Hibbeln *et al.*, 1998). Low intakes of n-3 fatty acids have also been associated with behavioral abnormalities in children (Stevens *et al.*, 1996). Manipulation of DHA intake has been shown to modulate behavior in rodents and primates (Nakashima *et al.*, 1993; Reisbick *et al.*, 1994; Wainwright *et al.*, 1997; Hamazaki *et al.*, 1999) and humans (Hamazaki *et al.*, 1996; 1999; Sawazaki *et al.*, 1999), and in recent years increasing the intake of dietary DHA has been suggested to be of possible use in modulating certain affective disorders such as depression (Hibbeln & Salem, 1995; Maes *et al.*, 1996; Hibbeln *et al.*, 1997; Edwards *et al.*, 1998), aggressive behavior in humans placed in stressful situations (Hamazaki *et al.*, 1996; 1999), and even schizophrenia (Laugharne *et al.*, 1996; Mellor *et al.*, 1996; Horrobin, 1999). Researchers in this area propose the mechanism of action of DHA in these diseases is through changes in serotonin handling in the brain. Modulation by dietary DHA of the overproduction or imbalance in the production of cytokines such as IL-1, IL-2, IL-6 and TNF-α which have been shown to be implicated in some of these psychological diseases might also be a mechanism involved (Miller *et al.*, 1999). This is a very new area of research and at present the mechanism of action of DHA on brain function cognition and learning remains unclear (British Nutrition Foundation, 1999). Many of these psychological diseases and conditions are associated with elevated levels of stress hormones (Osran *et al.*, 1993; Murphy, 1997; Sapse, 1997; MuckSeler *et al.*, 1999) which are themselves associated with immunosuppression (Dobbs *et al.*, 1993; Buckingham *et al.*, 1996; Agarwal & Marshall; 1998). Both stress and dietary fat have been shown to suppress immune function (see Khansari *et al.*, 1990 for a review on stress and immunity; Calder, 1995 for a review on dietary fat and immunity). As with FO feeding, the immunomodulatory effects of DHA feeding may not be solely modulated through changes in the production of eicosanoids. The mechanisms of the immunosuppressive action of FO, and its component fatty acids, on lymphocytes have yet to be elucidated, and may be involved in the modulation of a number of systems. Some of the possible mechanisms are modulation of eicosanoid production, modulation of signal transduction, modulation of the activity of various enzymes involved in

signaling pathways, modulation of receptor expression, and last but not least, modulation of inflammatory mediator production, (Calder, 1995). DHA has been shown to modulate this last factor. However, none of the trials investigating the effect DHA on indices of stress and behavior have examined effects on immunity. The study by Kelley *et al.*, (1998) which examined the effects of DHA-enriched diets (6 g DHA /day) on human immune function did monitor cortisol, T3 and T4 levels and reported no effect of DHA feeding on the levels of these hormones. This study however used healthy volunteers living in a metabolic ward, and not individuals under stressful conditions. There is a concern that DHA supplementation of the diet in an attempt to normalize mood or behavior may augment the immunosuppression in these conditions by adding to the immosuppression caused in part by the increased production of glucocorticoids associated with some of these conditions. This study attempted to investigate this for the first time using a standard animal model of stress which is known to raise stress hormone levels (Lysle *et al.*, 1987; Friedman *et al.*, 1999). This involved exposing mice fed experimental diets to a stressful conditioned aversive stimulus involving mild electric footshocks given while in an isolation box (Lysle *et al.*, 1987; Lysle *et al.*, 1988; Zhou *et al.*, 1993; Lysle & Perez, 1996).

6.2 Objective

The purposes of this study were:

1) To examine the effect of dietary DHA, in the absence of EPA, on the responses of murine lymphocytes.
2) To examine whether DHA supplementation of the diet would have an additive effect on the immunosuppression associated with acute stress.

6.3 Methods

6.3.1 Animals and diets

Male C57/BL6 mice (n=16 per diet and n=8 per treatment group within each diet) were purchased from Japan SLC Ltd (Shizuoka, Japan). They were fed for four weeks on diets rich in either DHA or soybean oil. The diets consisted of 90% fat-free powder (Funabashi Farm, Chiba, Japan; see table 6.1 for composition) and 10% fat (8% lard plus 2% soybean oil given as triglycerides in the control group and 8% lard plus 2% DHA ethyl ester in the DHA group). The fatty acid composition of the diets is shown in table 6.2.

Mice were kept in the climate-controlled conditions at Toyama Medical and Pharmaceutical University, Toyama, Japan. A 12 hour light/dark cycle was used. Mice fed each diet were randomly allocated to either a non-stressed group or a stressed group. Non-stressed mice (n=8 per diet) received no further treatment before killing. Stressed mice (n=8 per diet) were exposed at 24 hours and 1 hour prior to killing to isolation boxes (Cage VC3002L, Jhara Co Ltd, Tokyo, Japan) in which they received a mild electric footshock for one hour according to the method described by Zhou *et al.*, (1993). Briefly, this consisted of a 1.6 mA scrambled electric footshock of 5 seconds duration delivered through 16 0.5-cm thick steel bars forming the grid floor of a plexiglass shock box. The number of footshocks that the mice would receive during each of the two sessions was 16, with a 4 minute interval between footshocks. The total time the mice were present in the shock box was 64 minutes. Footshock exposure was always given between 9 and 10 am. Mice were then killed by ether inhalation, and blood was collected into heparin by cardiac puncture. Spleens were collected into sterile culture medium.

Table 6.1. Composition of the fat-free powder used as a base for the diets

Nutrient	*Amount (g/100g)*
Casein	23
DL-methionine	0.3
Corn starch	61.7
Sucrose	5
Cellulose powder	5
Vitamin mixture	1
Mineral mixture	4

Table 6.2 Fatty acid composition of the diets used (g/100g fatty acid) Nd = not detected

Fatty acid	*Soybean oil (g/100g fatty acids)*	*DHA (g/100g fatty acids)*
14:0	2.4	2.1
16:0	17.9	18.3
16:1	1.3	1.7
18:0	10.2	10.6
18:1	33.5	28.9
18:2n-6	18.3	10.4
18:3n-3	1.9	0.9
20:5	Nd	0.9
22:6	Nd	19:1

6.3.2 Chemicals and Materials

All chemicals used were obtained from Sigma (St Louis, USA) unless otherwise stated. RPMI was obtained from Gibco (Rockville, USA). DHA ethyl ester (95% pure) was obtained from Sagami Chemical, Research Center, Kanagawa, Japan. WST-1 cell counting kits were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Feotal calf serum (FCS) was obtained from Takara Labs (Kyoto, Japan). Cytokine ELISA kits were obtained from Biosource International (Camarillo, USA). PGE_2 ELISA kits were obtained from Neogen (California, USA). The anti-mouse-CD3 (Fitc conjugated) and anti-mouse B220 (streptavadin RPE conjugated) antibodies were obtained from Pharmagen (San Diego, USA) and the anti-mouse MAC-1 antibody

(Fitc-conjugated) was obtained from Cedar Lane Labs (Hornby, Canada). Monoclonal antibodies were diluted to the concentrations given in table 6.3 prior to use.

6.3.3 Preparation of Culture Medium

Culture medium consisted of RPMI supplemented with 2 mM glutamine and antibiotics (a 50 μg/ml 1:1 streptomycin/penicillin mix added at a concentration of 1:1000). Heat inactivated FCS was added to the culture medium at a final concentration of 10%.

6.3.4 Lymphocyte Preparation and Determination of Total Cell Number

In sterile conditions, spleens were gently ground through sterile wire mesh into petrie dishes and the mesh washed through with sterile culture medium. The cells were centrifuged at 1500 rpm x 10 min and then resuspended in 1 ml of lysing solution (9 volumes 0.83 g/100 ml ammonium chloride + 1 volume 2.06 g/100 ml Tris) and left for 5 minutes to destroy any contaminating red blood cells. The lysis reaction was then stopped by the addition of 10 ml of culture medium and the cells collected by centrifugation at 1500 rpm x 10 minutes. The medium was removed and the cells were washed twice in culture medium and then counted on a haemocytometer using trypan blue exclusion to test for cell viability.

6.3.5 Lymphocyte Culture for Mediator Production

Spleen cells were counted and diluted to a concentration of 2 x 10^6 cells/ml. 1 ml of cells was placed in a well of a 24 well sterile tissue culture plate along with either 200μl of 25 μg/ml Con-A or RPMI and 800 μl culture medium. Cells were cultured at 37°C for 24 hours. After this time the plates were centrifuged at 1500 rpm x 5 minutes and the supernatants collected and frozen at -20°C until analysis.

6.3.6 Mediator Assays

Concentrations of cytokines and eicosanoids were determined by ELISAs in an identical manner to that described in section 5.3.3.

6.3.7 Lymphocyte Proliferation

Spleen lymphocytes were diluted to 2 x 10^6 cells/ml in culture medium and 100 µl of cells added to wells of a 96 well tissue culture plate along with 20 µl of Con-A at concentrations in the well of 2.5, 5 or 10 µg/ml. Unstimulated cells received 20 µl of RPMI instead of Con-A. A further 80 µl of culture medium was added to all wells. Cells were cultured at 37° C in an atmosphere of 19:1 air:CO_2 for four days. Then 20 µl WST-1 counting solution was added and the plate was incubated for a further 4 hours before the absorbance was read on a standard plate reader at 450 nm using an air blank.

6.3.8 Natural Killer Cell Assay

Natural killer (NK) cell assay was carried out on a Becton Dickinson flow cytometer using the method of Slezak & Horan (1989). Briefly, lymphocytes were incubated for 4 hours along with target cells (Yac-1 cells) at increasing concentrations of effectors:targets. After incubation the cells were doubly labeled with PKH-1, a flourochrome that fluoresces green, binds to the cytoplasmic membrane and does not leak or transfer. This was used to identify the target cell population. Propidium iodide that fluoresces red, was used to identify non viable cells. In this manner, the number of live vs. lysed target cells could be identified.

6.3.9 Determinations of Cell Type

200 µl of cell mixture (2 x 10^6 cells/ml) was added into the wells of a 96 well plate. The plate was centrifuged at 13000 rpm x 10 minutes and the supernatant aspirated. 50 µl of premixed antibody mixture were added to the cells (see table 6.3). Antibodies were diluted to the concentrations given in table 6.3. Between the first and second staining the cells were left on ice in the dark for 30 minutes and then the second stain was added. Once again cells were left for 30 minutes on ice in the dark. Then the cells were resuspended in PBS and analysed on a Becton Dickinson flow cytometer.

Table 6.3 antibody staining for cell markers

Well Number	*1st staining*	*2nd staining*
1	No antibody (PBS)	PBS
2	CD3 (Fitc)/B220 (RPE) (1/500 dilution of both)	PBS
3	No antibody (PBS)	Fitc (1/500 dilution)
4	Mac-1(Fitc) (1/100 dilution)	PBS

6.3.10 Preparation of Plasma and Determination of Corticosterone

Blood was obtained by cardiac puncture and collected into sterile tubes containing heparin. All samples were obtained between 9:30 and 10:30 am. The blood was allowed to sit for approximately 2 hours at 4°C and then plasma was separated by centrifugation at a 10000 rpm for 10 minutes. Plasma was collected into a new sterile tube and sent to SRL Hachiohji Labs (Tokyo, Japan) for corticosterone analysis by an ^{125}I-based radioimmunoassay (RIA).

6.3.11 Determination of Cell Phospholipid Fatty Acid Composition

Spleen cell phospholipid fatty acids were extracted by the method of Folch *et al.*, (1957). Fatty acid composition of the spleen phospholipids were then determined on the Hewlard Packard Gas Chromatography machine under the conditions described in chapter 2.

6.3.12 Plasma NEFA Concentrations

Plasma NEFA concentrations were determined by a commercially available enzymatic assay (Boehringer-Mannheim, Germany).

6.3.13 Statistical Analysis

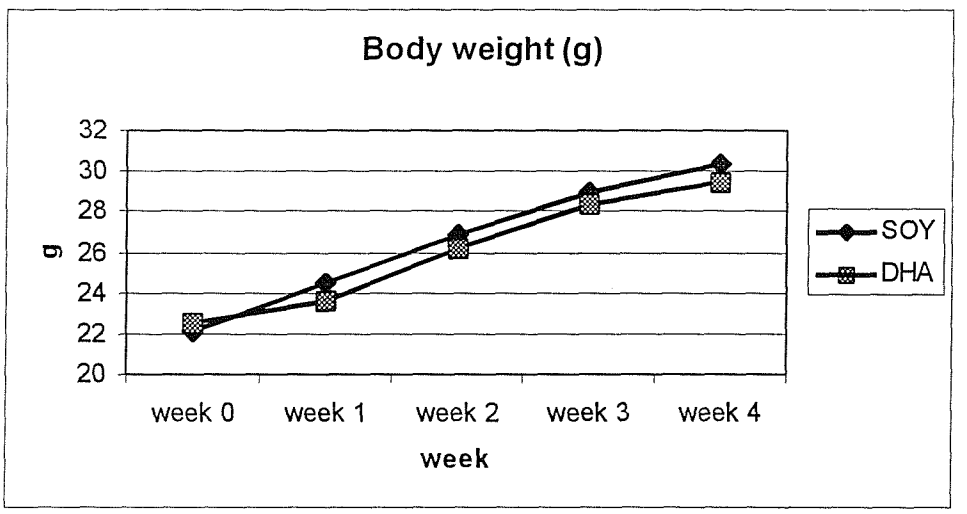
Statistical analysis was preformed using SPSS. Means and standard errors are reported. Significant differences were determined using independent samples t-tests for tests between diets under the different stress levels, a P value of < 0.05 was used to indicate significance.

6.4 Results

6.4.1 Mouse weights and food intakes

Mice fed the DHA diet weighed slightly less at the end of the study than the mice fed the soybean oil diet but this difference was not statistically significant (figure 6.1) Both groups of mice ate a similar amount of food (3.6 ± 0.07 g/day/mouse for soybean oil and 3.4 ± 0.12 g/day/mouse for DHA)

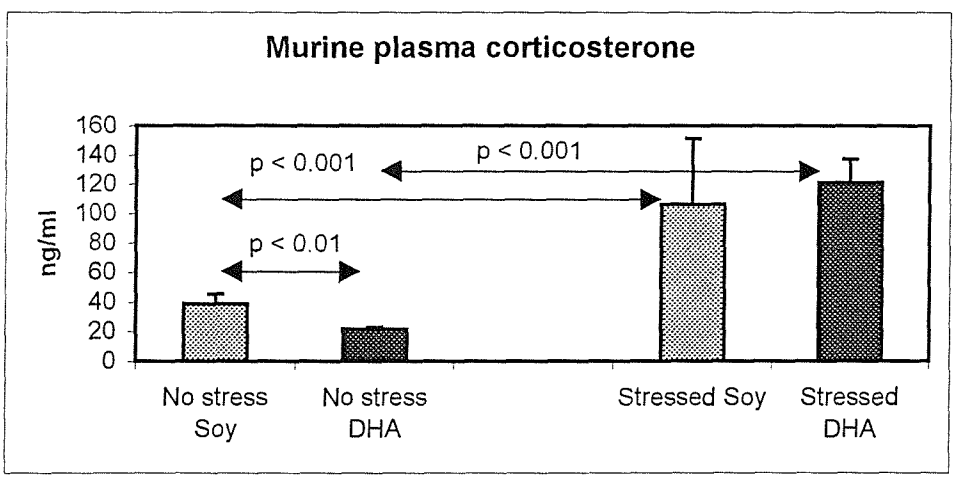
Figure 6.1 Body weights of mice fed the control or DHA-rich diets. Data are means ± se for 8 animals per diet and stress group.



6.4.2 Plasma corticosterone concentrations

Unstressed mice fed the DHA diet had a significantly lower plasma corticosterone concentration than unstressed mice fed the soybean oil diet ($p = 0.01$) (see figure 6.2). Stress dramatically increased the concentration of corticosterone in the plasma by an average of 273% in the Soybean oil group and 560% in the DHA group compared to unstressed conditions. There were no significant differences in the corticosterone concentration between the diets after stress.

Figure 6.2 Plasma corticosterone concentrations in stressed and unstressed mice fed the Soybean oil and DHA diets. Data are means ± se for 8 animals per diet and stress group.

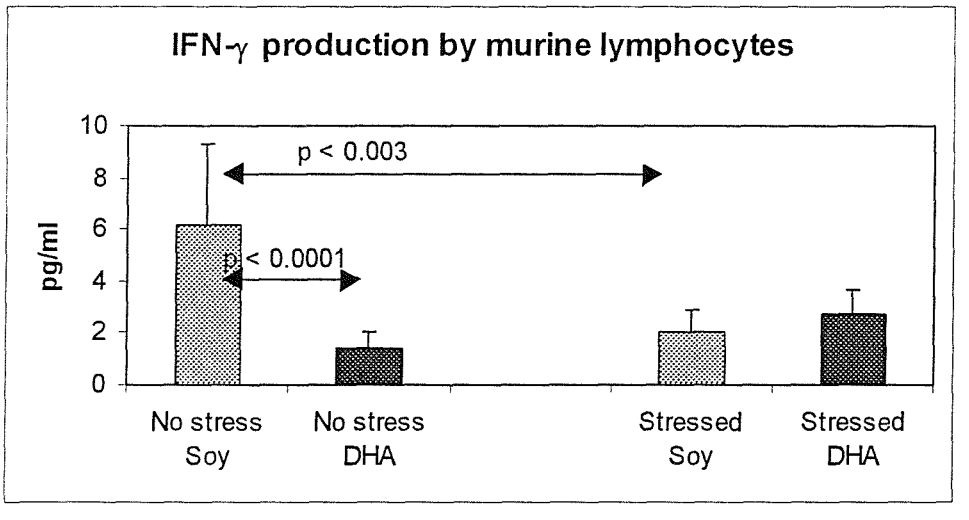


6.4.3 Th-1 cytokine production

6.4.3.1 IFN-γ production by lymphocytes from stressed or unstressed mice fed soybean oil or DHA diets.

Unstressed mice fed the DHA diet produced significantly less IFN-γ that unstressed mice fed the control soybean oil diet ($p = 0.0001$) (see figure 6.3). Stress conditions significantly decreased the amount of IFN-γ produced by lymphocytes from mice fed the soy diet ($p = 0.003$). There was no effect of stress on the production of IFN-γ by spleen lymphocytes from mice fed the DHA diet. After stress there was no difference in IFN-γ production by cells from mice fed the different diets.

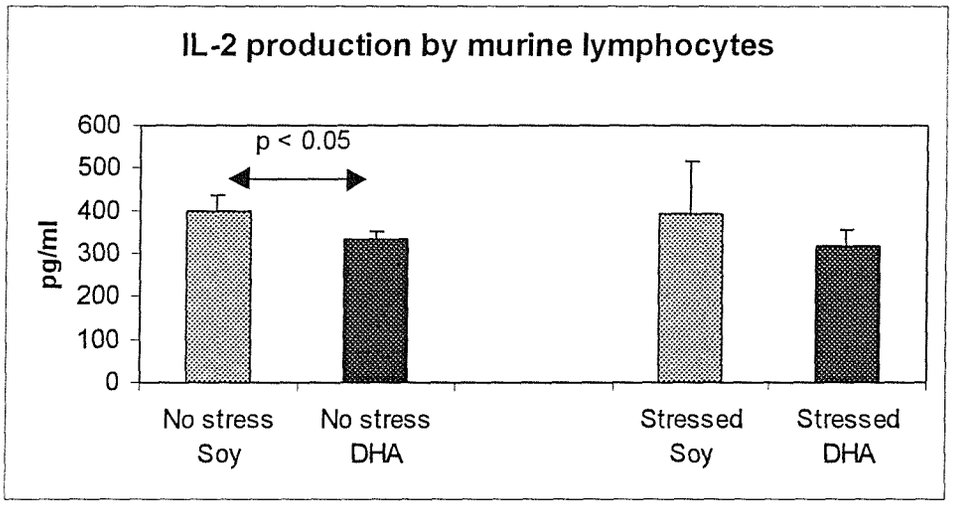
Figure 6.3 IFN-γ production by spleen lymphocytes from stressed or unstressed mice fed soybean oil or DHA diets. Data are means ± se for 8 animals per diet and stress group.



6.4.3.2 IL-2 production by lymphocytes from stressed or unstressed mice fed soybean oil or DHA diets.

IL-2 production was significantly lower in the unstressed DHA group compared to the unstressed soybean oil group ($p = 0.04$) (see figure 6.4). This difference between the two diets was not seen when the mice were stressed, although the DHA group still tended to have lower IL-2 production. Stress did not significantly affect the amount of IL-2 produced from mice fed either DHA or soy.

Figure 6.4 IL-2 production by murine spleen lymphocytes from stressed and unstressed mice fed soybean oil or DHA diets. Data are means ± se for 8 animals per diet and stress group.

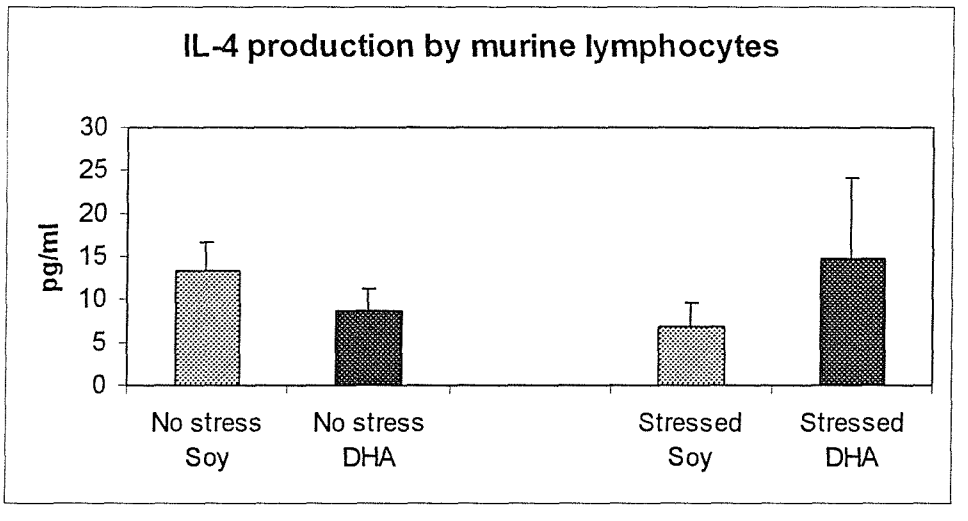


6.4.4 TH-2 cytokine production

6.4.4.1 IL-4 production by lymphocytes from stressed or unstressed mice fed soybean oil or DHA diets.

Diet had no significant effect on the amount of IL-4 produced by the mice in the non-stressed or stressed conditions (figure 6.5). Stress did not significantly affect the amount of IL-4 produced by the lymphocytes from mice fed either the soybean oil or DHA diets. However IL-4 production tended to be higher in stressed mice fed the DHA rich diet, then in those fed the soybean oil diet.

Figure 6.5 IL-4 production by murine lymphocytes from stressed and unstressed mice fed soy or DHA diets. Data are means ± se for 8 animals per diet and stress group.

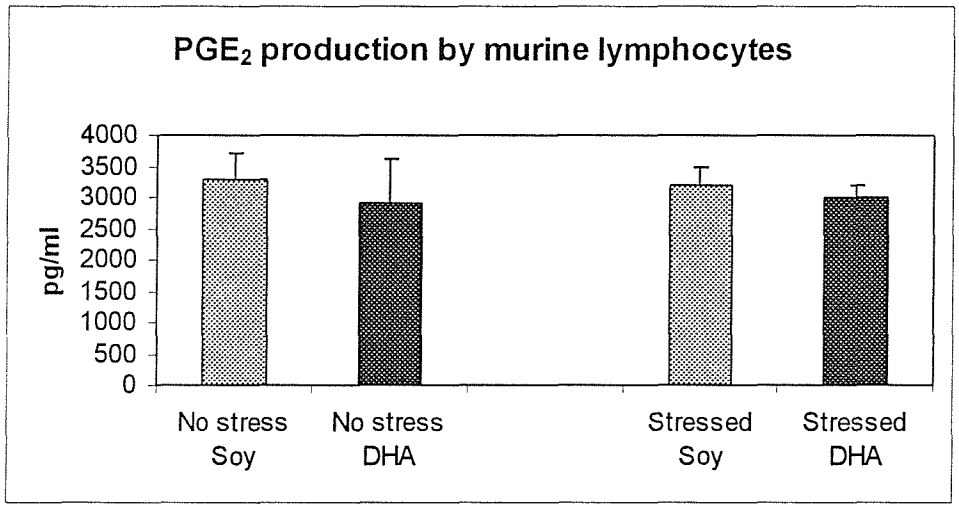


6.4.5 Eicosanoid production

6.4.5.1 PGE_2 production by lymphocytes from stressed or unstressed mice fed soybean oil or DHA diets.

DHA feeding had no effect on the amount of PGE_2 produced by spleen lymphocytes in either the non stressed or stressed groups when compared to the amount of PGE_2 produced by mice fed the soybean oil diet (see figure 6.6). Stress did not affect on the amount of PGE_2 produced by the soybean oil fed mice or the DHA fed mice.

Figure 6.6. PGE_2 production by lymphocytes from stressed or unstressed mice fed soybean oil or DHA diets. Data are means ± se for 8 animals per diet and stress group.



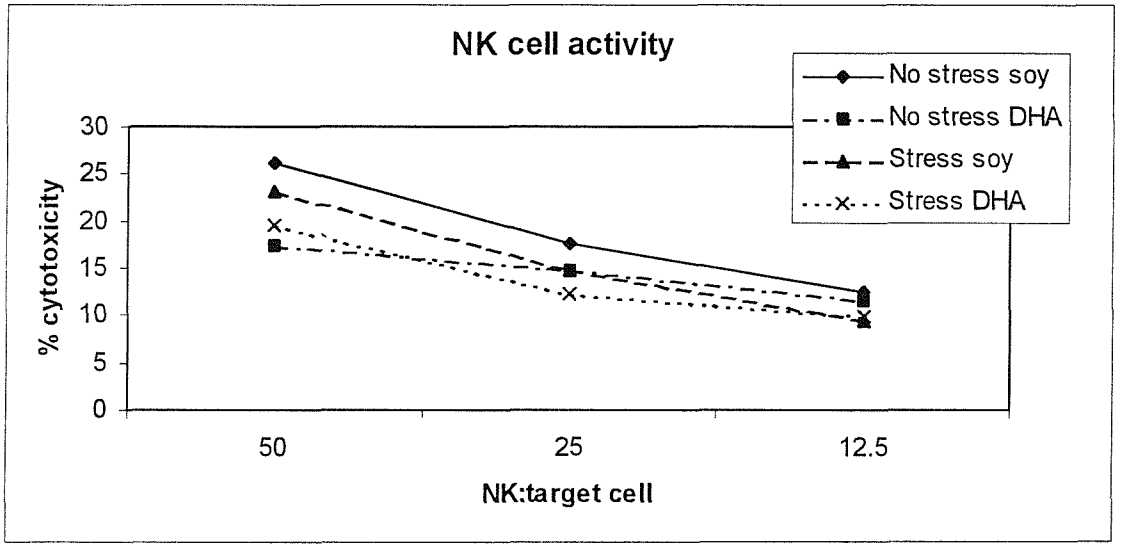
6.4.6 Cell function assays

In order to assess the ability of diet or stress to effect the functional capacity of spleen lymphocytes NK cell activity and lymphocyte proliferation assays were performed.

6.4.6.1 Natural killer cell activity

DHA feeding caused a significant decrease in the ability of NK cells to kill yac-1 target cells under non stressful conditions at all three NK:target cell ratios ($P < 0.001$ for ratio 50:1; $P < 0.03$ at ratio 25:1; $P < 0.004$) (see figure 6.7). Under stressful conditions however no significant effect of diet was observed at any effector:target ratio. Stress did not affect the ability of NK cells to kill yac-1 targets at any ratio in the soybean oil fed group ($p = 0.3$ for ratio 50:1; $p = 0.45$ for ratio 25:1; $p = 0.4$ for ratio 12.5:1) or DHA fed group ($p = 0.8$ for ratio 50:1; $p = 0.6$ for ratio 25:1; $p = 0.2$ for ratio 12.5:1).

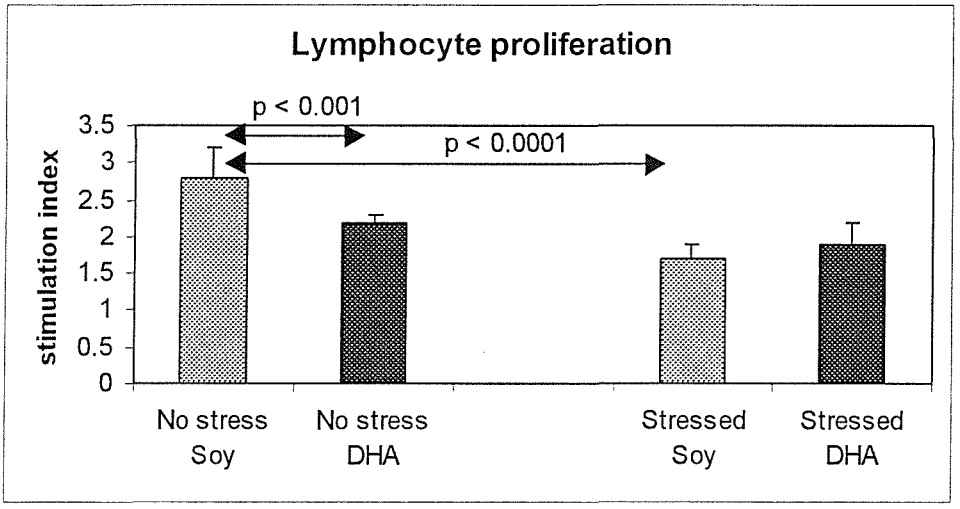
Figure 6.7 NK cell activity against yac-1 target cells. Data are mean se for 8 animals per diet and stress condition



6.4.6.2 Proliferation of lymphocytes from stressed and unstressed mice fed either soy or DHA diets in response to Con-A stimulation.

In non-stressed conditions DHA feeding caused a significant decrease in the ability of lymphocytes to respond to Con-A stimulation ($p = 0.001$) compared to soybean oil feeding (see figure 6.8). This effect of diet was not seen under conditions of stress. However, exposing lymphocytes from the soy fed mice to conditions of stress prior to obtaining spleen lymphocytes caused a significant decline in the ability of the lymphocytes to respond to Con-A stimulation ($p = 0.0001$). Stress conditions had no effect on the ability of lymphocytes from DHA fed mice to proliferate ($p = 0.3$).

Figure 6.8 Lymphocyte proliferation in response to Con-A stimulation.
Data are means ± se for 8 animals per diet and stress group.



6.4.7 Total Numbers of Cells and Characterisation of the Population of Gated Spleen Lymphocytes.

Table 6.4 shows the total number of viable cells in the spleen lymphocyte preparation from mice fed the different diets. Lymphocytes were gated for analysis. In the non-stressed conditions, spleens from mice fed the soybean oil diet contained significantly more cells than spleens from mice fed the DHA diet. This effect of diet was also seen for the spleens from mice under the stressful conditions, although the difference was not significant. In general the spleens of the stressed mice contained fewer cells than those from unstressed mice. This effect of stress was significant for mice fed the soybean oil diet. Spleens from non stressed mice fed the DHA diet contained a significantly greater percentage of CD3 positive cells compared to the stressed mice fed this diet (CD3 is a marker for T-lymphocytes). Although the levels were very low, spleens from mice fed the DHA diet also tended to have a greater proportion of MAC-1 positive staining cells compared to spleens from mice fed the soybean oil diet, indicating a greater number of contaminating macrophages within the population of gated lymphocytes. This reached significance in the stressed group. There were no significant differences in the number of B lymphocytes (B220 positive cells) in the spleens from mice fed the two diets under either condition.

Table 6.4. Cellular population of the spleen lymphocytes (gated lymphocytes) Data are means ± se for 8 animals per diet and stress group.

	No Stress Soy Diet	*Se*	*No Stress DHA Diet*	*Se*	*Stressed Soy Diet*	*Se*	*Stressed DHA Diet*	*Se*
Total cell # (million)	46.8[ab]	4.7	42.7	3.2	40.1	2.2	37.7	3.9
CD3 (% +ve)	31.4	0.9	31.1[c]	0.3	31.3	0.7	29.7	0.9
B220 (% +ve)	64.2	2.5	66.3	2.1	61.4	2.3	59.3	2.4
MAC-1 (% +ve)	2.0	0.9	3.5	1.4	1.8[d]	0.3	3.5	1.8

a. significantly different from no stress DHA group ($P<0.02$)
b. significantly different from stressed Soy group ($P<0.005$)
c. significantly different from stressed DHA group ($P<0.03$)
d. significantly different from stressed DHA group ($P<0.004$)

6.4.8 Fatty Acid Composition of Spleen Lymphocytes

The spleen phospholipid fatty acid composition is presented in table 6.5. DHA feeding caused a significant increase in the proportions of 20:5 n-3 and 22:6 n-3 and a significant decrease in the amount of arachidonic acid compared with soybean oil feeding.

Table 6.5 Fatty Acid Composition of Spleen cell phospholipids from Mice fed either the Soybean oil or DHA Diet.

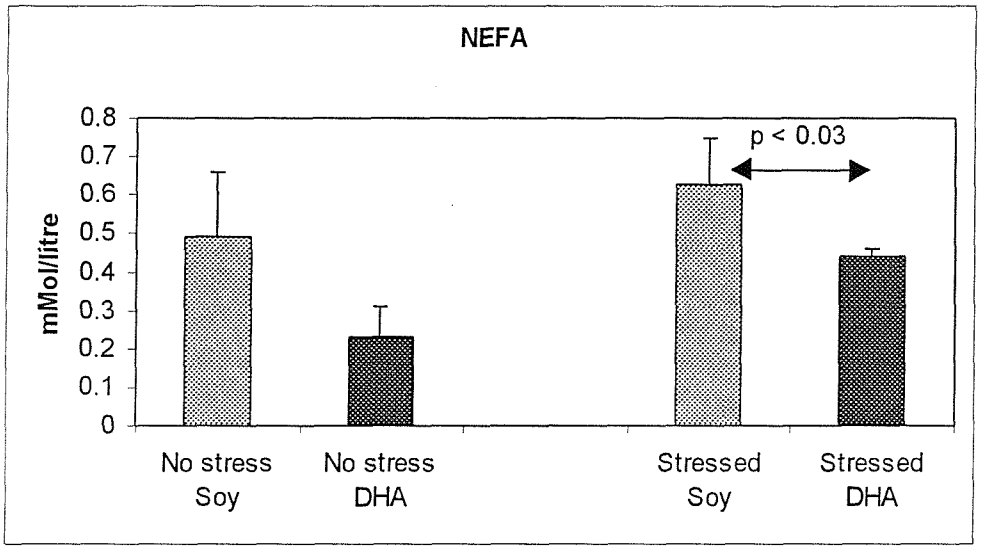
Gram/100g fatty acids. Data are means (se) (n=8 per diet). Numbers showing different alphabetic subscripts across a row are significantly different (P = 0.05).

Lipid	*SOY Diet*	*Se*	*DHA Diet*	*Se*
16:0	23.0	1.9	26.2	0.7
18:0	17.9	0.4	16.0	0.3
18:1 n-9	10.2	0.8	10.4	0.5
18:2 n-6	8.1	0.6	9.7	0.1
20:3 n-9	1.8	0.9	0.4	0.4
20:4 n-6	23.3^{a}	1.4	6.5^{b}	0.4
20:5 n-3	3.3^{a}	0.4	8.0^{b}	1.2
22:5 n-3	1.8	0.3	1.4	0.3
22:6 n-3	6.3^{a}	0.04	18.5^{b}	0.7

6.4.9 Plasma NEFA concentrations.

Plasma NEFA concentrations are shown in figure 6.8. Plasma NEFA concentrations were lower in the DHA-fed group compared to the soybean oil-fed group in both the stressed and non-stressed states. This was significantly lower after stress ($p = 0.03$) but not in the non-stressed group. Stress increased the concentrations of NEFA in both the soybean oil fed and the DHA fed groups; however these increases were not significant in either group.

Figure 6.8 Plasma NEFA concentrations from stressed or unstressed mice fed soybean oil or DHA diets. Data are mean and se of 8 animals per diet and stress group.



6.5 Discussion

6.5.1 Dietary DHA Can Modulate Basal Corticosterone Levels in Non-stressful Conditions

This study examined whether supplementation of the diet with DHA would lower the responses of spleen lymphocytes as previously reported for FO (see chapter 5). Spleen lymphocytes from mice fed the DHA diet had significantly lower production of IFN-γ and IL-2 and reduced proliferation and NK cell activity compared to those from mice fed the soybean oil diet. Plasma corticosterone concentrations were also significantly reduced following DHA feeding. Cells from the mice which had undergone the stress-inducing mild electric footshocks for one hour 24 hours and 1 hours before being killed showed a decrease in the production of IFN-γ if the they were from mice fed soybean oil, but not if they had been fed fish oil. No effects of stress were seen with the other mediators. Within each dietary group, NK cell activity was not affected by stress. Lymphocyte proliferation was decreased as a result of stress in the soybean oil-fed group only.

Interest in the use of dietary lipids to prevent or control human diseases has gained scientific support from numerous studies that have uncovered beneficial effects of providing increased amounts of n-3 PUFAs. These have included cardiovascular disease, inflammatory diseases, autoimmune diseases and graft rejection following transplantation (see chapter 1). A more conventional treatment for some of these conditions is the administration of glucocorticoids. Endogenously the glucocorticoids cortisol (in humans) or corticosterone (in rodents), are synthesised from cholesterol in cells of the zona faciculata of the adrenal cortex. This process is driven by the pituitary hormone, adrenocorticotrophin (ACTH), which acts via specific membrane receptors to activate the rate limiting enzyme in the steroidogenic pathway and thereby increase the conversion of the precursor cholesterol to pregnenolone (Genuthi, 1998). Glucocorticoids exert widespread actions in the body which are essential for the maintenance of homeostasis and thus permit the organism to withstand physical and emotional stress. They are powerful catabolic agents and promote the breakdown of

carbohydrate, lipid and protein, thereby serving as physiological antagonists of insulin. Increasing evidence suggests that the hypothalamic-pituitary-adrenal (HPA) axis plays a pivotal role in the regulation of immune function and that disturbances in its activity may compromise the body's defence mechanisms (Agarwal & Marshall, 1998). In accord with this concept, sustained elevations in circulating glucocorticoids, which may result either from primary or secondary (e.g. depression, alcoholism) disorders of the axis or from the administration of exogenous steroids are frequently linked to a decline in immunocompetence (Khansari *et al.*, 1990).

The current study attempted to relate the effects of dietary DHA on immune cell function and on the stress hormone corticosterone in non-stressful and stressful conditions. It was found that dietary DHA caused a decrease in the concentration of circulating corticosterone in unstressed mice compared to mice fed a soybean oil diet. This agrees with the trend seen in a recent study in which DHA was fed to rats (Engler *et al.*, 1999) and compared to n-6 PUFA rich corn/soybean oil mix. However it may be that rather than DHA causing a reduction in corticosterone, soybean oil is causing an increase in circulating corticosterone concentration. Widmaier *et al.*, (1992) showed that rats infused with Intralipid (a 10% lipid emulsion composed of soybean oil) had a 20-fold increase in corticosterone concentration 120 minutes after infusion began compared to saline-infused controls. This difference was still apparent an hour later. This effect was not apparent in rats pre-treated with the synthetic corticosteroid dexamethasone and given the Intralipid infusion. The authors suggest that, as this effect was not seen in rats with pre-existing high levels of glucocorticoids, the action of Intralipid may be at a site within the HPA axis. The authors of this study suggested that high circulating NEFA levels activate the HPA axis in rats. They also suggested that since stress activates glucocorticoid production and increases NEFA levels due to lipolysis, it is possible that circulating levels of NEFA acting on the HPA axis may be involved in a previously unrecognised positive feedback loop. NEFA levels have long been known to increase in a great variety of situations which are considered stressful in humans (Dimsdale & Herd, 1982) and in rodents (Mallov & Witt, 1961; Barrett, 1964; Turner, 1975). The mechanism suggested by Widmaier *et al.*, (1992) has also been proposed by Tannenbaum *et al.* (1997) who suggest that high fat diets act as a background form of chronic stress which augments basal corticosterone levels and

enhance HPA responses to stress. Tannenbaum *et al.*, (1997) fed rats a high fat (20% by weight) or a low fat (4% by weight) diet with corn oil as the predominant fat source. The rats were maintained for either a short (7-21 days) or long (9-12 weeks) period on the diets and were subjected to restraint stress in order to elevate corticosterone levels. Rats fed the high corn oil diet showed significantly elevated NEFA levels before and after a 20 minute period of restraint stress which remained elevated after the restraint stress had ceased. Overall rats fed the high fat diet showed significant augmentation of corticosterone, ACTH, and NEFA levels, with no effect on the concentration of growth hormone. This suggests a mechanism of action on the HPA axis as well, with elevated circulating levels of NEFA possibly playing a part in the regulation of stress hormones. This study illustrates that high fat diets composed of corn oil as the fat source augment both ACTH and corticosterone responses to acute stress. In particular, basal levels of corticosterone were elevated in response to a high corn oil diet. In the current study the high corticosterone levels in unstressed mice fed the soybean oil diet compared to the mice fed the DHA diet, agree with both Tannenbaum *et al.* (1997) and Widmaier *et al.* (1992). The current study suggests that DHA prevents the n-6 PUFA increased elevation of corticosterone in the apparently unstressed state. It is also interesting that in the present study, concentrations of plasma NEFA were higher in the soybean oil fed group than in the DHA fed group in both the stressed and unstressed states. The mechanism of action of DHA needs to be more closely examined however to ascertain whether its mode of action is simply through replacing a proportion of dietary n-6 fatty acids, or whether it is having its own effects on glucocorticoid levels. The possible mechanisms through which circulating levels of NEFAs may signal to the HPA axis, the particular fatty acids responsible, and the implications of this effect to certain metabolic diseases characterised by high NEFA levels have not been previously examined and represent an area ripe for future research. Certain fatty acids such as DHA and AA have been recently shown to act as potential ligands for certain receptors and transcription factors, such as PPAR-γ, and lead to modulation of the activities of cells such as macrophages (Ricote *et al.*, 1999). PPAR-γ mRNA has also been shown to be expressed in the adrenal gland of rats (Braissant *et al.*, 1996). It may be possible therefore that cells of the adrenal gland may be sensitive to circulating NEFAs, which may act as precursors for prostaglandins

which act as ligands for the activation of PPAR-γ. The possibility that certain circulating NEFAs may effect the activity of glucocorticoid secreting cells is an exciting possibility and has not been investigated.

A recent study by Cooke (1999) has concluded that arachidonic acid and its lipoxygenase products are involved in the control of steroidogenisis via cAMP-mediated processes. It may be therefore that n-6 PUFA rich diets could raise circulating levels of steroids by increasing the content of arachidonic acid available for the regulation of steroidogenesis. A dietary intervention study investigating this possibility needs to be carried out.

In the study by Kelley *et al.* (1998) plasma concentrations of cortisol were unchanged in healthy men following a dietary intervention of 6 g DHA per day for 90 days. The lack of an effect of dietary DHA on plasma cortisol might possibly reflect the amount of DHA used. The volunteers in the Kelley *et al.* study (1998) received 2.8% of their energy from dietary DHA, whereas the mice in the present study received 5% of their energy from DHA. Alternatively, it may be that the modulation of corticosterone levels by dietary DHA might be specific to rodents. It is also possible that as cortisol levels are subject to seasonal variation and change with physiological state and time of day (Walker *et al.*, 1997), other environmental factors may play a role in the different effects of DHA feeding on glucocorticoid concentrations in humans and rodents. In the study by Kelley *et al.* (1998) blood samples were always obtained from the volunteers after an overnight fast and although the actual values of cortisol are not reported, it is possible that they might be higher than if they had been obtained in a fed state. In the present study blood was obtained from the mice in the fed state. Some of the differences in the effects/lack of effect of DHA feeding on cortisol/corticosterone levels in the stressed condition might be explained by differences in the physiological state of the mouse or human from which the plasma was obtained.

DHA supplementation of the diet has been recently shown to decrease the ratio of noradrenaline:adrenaline in the blood of medical students undergoing stressful exams (Sawazaki *et al.*, 1999). Due to the difficulty of obtaining resting levels of these particular stress hormones in animals, these were not examined in the present study;

however, it may be that DHA supplementation of the diet may be able to influence the levels of stress hormones other than cortisol in humans and animals.

The relationship between DHA feeding and corticosterone/cortisol is unlikely to be a simple one. Under conditions of stress the effect of diet on corticosterone levels was not observed and there was no significant difference in the concentration of corticosterone in the plasma between the two dietary groups. However, because of the lower levels of corticosterone present in the plasma of unstressed mice fed the DHA diet, in response to the stressful conditions plasma corticosterone increased by 560% in the DHA group and only 260% in the soybean oil-fed group. It may be that other hormones released as part of the stress response may override any modulating effects of diet, perhaps ACTH. To my knowledge, this interaction has not previously been examined.

In accordance with the effects of FO feeding reported in chapter 5, DHA feeding caused a decrease in the production of the Th-1 cytokines IFN-γ and IL-2 from spleen lymphocytes in response to Con-A, but had no significant effect on production of the Th-2 cytokine IL-4. It is possible that these effects are related to the production of various eicosanoids, as was discussed in chapter 5 to explain the effects of FO. However, PGE_2 production by the cell preparations, although tending to be lower for cells from mice fed the DHA diet, were not significantly different between the two diets studied. Lymphocytes are generally agreed to lack the COX enzyme necessary for producing prostaglandins (Goldyne, 1988), but it is possible that macrophages present in the cellular preparation in the DHA fed group might be producing less PGE_2 and more PGE_3. PGE_3 has been shown to strongly suppress lymphocyte proliferation (Shapiro *et al.*, 1993). The non-significant trend for decreased production of PGE_2 by macrophages following DHA feeding would agree with the results of Kelley *et al.*, (1999) which showed decreased production of PGE_2 and LTB_4 by blood mononuclear cells in response to LPS after volunteers had consumed a high DHA diet.

6.5.3 DHA feeding under conditions of stress does not decrease lymphocyte mediated immunity

Under conditions of stress there was no apparent effect of diet on the production of IL-2 by lymphocytes. There was however a significant effect of stress on the proliferation of lymphocytes from the soybean oil-fed mice. The decreased proliferation of the spleen cells of the soybean oil fed mice may be due to the modulation of LTB_4 production by the high circulating levels glucocorticoids. A study by Goodwin *et al.* (1986) has shown that glucocorticoids can inhibit lymphocyte proliferation by suppressing the production of LTB_4. Goodwin *et al.*, (1986) showed that mitogen stimulated cultures of T cells produced 5 nM LTB_4 in 24 hours, and that this production of LTB_4 (but not production of LTC_4 or LTD_4) seems to be necessary, but not sufficient for IL-2 production by these cells. This LTB_4 production was completely inhibited lipoxygenase inhibitors. The effects of the 5-series leukotrienes derived from n-3 fatty acids were not assessed in Goodwin's study. LTB_4 has been shown to influence T-cell mediated responses in a number of ways, including modulating IFN-γ and IL-2 levels (Rola-Pleszczynski, 1985), and Morita *et al.* (1999) have recently shown that a LTB_4 receptor antagonist inhibited T cell proliferation induced by Con-A, immobilised anti-CD3 monoclonal antibody, or IL-2. It also inhibited IL-2, IFN-γ and IL-4 production by anti-CD3 immobilised T- cells. Modulation of LTB_4 production, and/or modulation of LTB_4 receptor expression might represent one way in which stress-induced a decrease in lymphocyte proliferation from soybean oil fed mice but had little effect on proliferation of lymphocytes from the from DHA-fed mice. DHA-fed mice might be making less LTB_4 to begin with, as less arachidonic acid is present in spleen cell phospholipids from mice fed this diet (see table 6.5). This might also explain why spleen lymphocytes from the non-stressed DHA-fed mice exhibit lower IL-2 production and proliferation. It is possible that levels of LTB_4 produced by the lymphocytes in response to Con-A stimulation may be important in modulating the proliferation of these cells.

Glucocorticoids are known to modulate inflammation by inducing the biosynthesis of polypeptide inhibitors of phospholipase A_2, known as lipocortins. The promoter region of the lipocortin gene contains binding sites for the glucocorticoid receptor (Ambrose *et al.*, 1992). The action of glucocorticoids on eicosanoid formation can be blocked by a specific receptor antagonist RU486 and by inhibitors of RNA and protein synthesis (Duval & Freyss-Beguin, 1992). Lipocortin appears to bind directly to phospholipid

thereby inhibiting the release of arachidonic acid (Duval & Freyss-Beguin, 1992). Protein kinases and proteases process lipocortin that leads to enhanced phospholipid binding (Ambrose *et al.*, 1992). This represents one way in which glucocorticoids may affect arachidonic acid metabolism and decrease LTB_4 levels. Modulation of the amount of arachidonic acid present in cellular membranes by n-3 fatty acid feeding might therefore represent one way in which DHA could mimic the actions of glucocorticoids on the immune system. It is also possible that the high levels of glucocorticoids in the stressed mice may also modulate the activities of other enzymes involved in eicosanoid synthesis. For example, glucocorticoids are know to have an inhibitory effect on 5-lipoxygenase-activating protein (FLAP) (Vickers, 1995). FLAP is important in associating 5-lipoxygenase to the nuclear membrane in the cell. Specific membrane association of 5-lipoxygenase is critical for the cellular utilisation of AA. An inhibition of FLAP by glucocorticoids would lead to decreased leukotriene synthesis. Modulation of these enzymes along with modulation of PLA_2 activity, suggests a mechanism by which DHA could mimic the effects of glucocorticoids on the immune system.

Stress was induced in the animals by exposing them to two one-hour sessions of mild electric footshocks 1 day before and one hour before being sacrificed. Stress caused a substantial increase in the levels of circulating corticosterone. The electric footshock model has been used to examine the effects of 'physical' stress on the immune system (Lysle *et al.*, 1988; Zhou *et al.*, 1993). Electric footshocks themselves have been shown by Lysle *et al* (1987) to modulate lymphocyte reactivity to Con-A: suppression of lymphocyte proliferation was directly related to the number of footshocks the animal was exposed to. Spleen lymphocytes showed a complete recovery of proliferation 24 hours after exposure to shock. Laudenslager *et al.* (1983) suggested that shock-induced suppression of lymphocyte responsiveness is related to the animals perception of the shocks as uncontrollable. While there is some dispute over this theory, it does seem likely that induction of stress hormones induced by fear of the unfamiliar situation of the footshocks would act as a form of 'short term psychological' stress and has been shown to induce the production of stress hormones such as ACTH and corticosterone (Coover *et al.*, 1978) which have immunomodulatory properties.

Stress, distress and a variety of psychiatric illnesses, notably the affective disorders, are increasingly reported to be associated with immunosuppression (Khansari *et al.*, 1990). DHA has been used in recent years with some success in the treatment of affective disorders (depression, schizophrenia) and has been shown to modulate behaviour in animals (Wainwright *et al.*, 1994; Wainwright *et al.*, 1997; Kozac *et al.*, 1997; Hamazaki *et al.*, 1999) and humans (Hamazaki *et al.*, 1996). Some of these disorders, especially clinical depression, are associated with high circulating levels of glucocorticoids (Osran *et al.*, 1993). Glucocorticoids themselves cause immunosuppression (Buckingham *et al.*, 1996) and have been shown, like FO feeding, to alter the production of cytokines from a Th-1 to a Th-2 type response (Wallace *et al.*, 1999; Agarwal & Marshall, 1998). A concern arises whether dietary DHA supplementation provided as therapy for these conditions might have an additive effect on the immunosuppression associated with these diseases. This does not appear to be the case under short-term conditions of stress in a standard animal model. While dietary DHA will decrease some aspects of T-cell mediated immunity such as the production of some Th-1 cytokines and lymphocyte proliferation in response to mitogen under non-stressful conditions, the immunomodulatory effect of DHA does not appear to occur under stressful conditions. This suggests that stress overcomes the effects of DHA. The high concentration of corticosterone present in the murine plasma was associated with a slight decrease in some of the immune responses measured (IFN-γ, lymphocyte proliferation in soybean oil fed mice), but had no clear effect on other responses (IL-2 and IL-4 production). The ratio of Th-1/Th-2 cytokines however was not significantly changed under conditions of stress. Therefore, there was no significant skewing towards a Th-2 response by glucocorticoids as shown in other experiments (Agarwal & Marshall, 1998). This may be due to the length of time the stress was applied. The current study used short term acute stress and Agarwal & Marshall (1998) have shown in *in vitro* experiments that for maximum skewing of the immune system towards a Th-2 type response by glucocorticoids, a continuous high exposure of corticosterone is preferable in order to see maximum effects. In the present study however, under stressful conditions associated with high glucocorticoid levels, the immunomodulatory effects of DHA are not observed.

6.6 Conclusions

DHA had immunosuppressive properties under non-stressful conditions. It may therefore be of use in the treatment of some autoimmune diseases associated with overproduction of Th-1 cytokines. Under stressful conditions however, there were no significant differences between the two dietary groups in the concentration of corticosterone in the plasma, and in the production of cytokines. Lymphocyte proliferation, although slightly decreased under the stressful situations, was not significantly different between the two dietary groups. In conclusion, in an animal model of stress, DHA feeding did not augment the immunosuppression associated with high endogenous levels of glucocorticoids. This suggests that the provision of DHA as a therapeutic agent in an attempt to correct some lipid abnormalities and mood disturbances associated with some of the affective disorders does not appear to augment the T-lymphocyte immuosuppression associated with high glucocorticoid levels. Nevertheless, a human trial monitoring immune parameters in individuals with affective disorders such as schizophrenia or depression while undergoing DHA supplementation therapy would be of use in assessing any potential immunomodulatory side effects of the use of DHA in the treatment of these diseases in humans.

Chapter 7: A double-blind placebo controlled study of the effects of different doses of fish oil on human immune cell function and inflammatory mediator production

7.1 Introduction

Over the last thirty years there has been a great deal of interest in the effects of dietary fatty acids on the production of inflammatory mediators by the cells of the immune system. This is due to the fact that the n-6 fatty acid linoleic acid. (present in high proportions in corn and safflower oils) is a precursor of arachidonic acid, which itself is a precursor of the prostaglandins and leukotrienes. These are pro-inflammatory mediators which have important roles in the inflammatory response (see chapter 1). These compounds are known to strongly influence the production of other mediators, including cytokines (see chapter 1), and have important roles in the pathology of inflammatory diseases such as asthma and rheumatoid arthritis (Calder & Miles, 2000; Belch & Muir, 1998). The effects of n-6 fatty acids are believed to be antagonized by n-3 fatty acids, partly as the result of decreasing production of PG and LT from arachidonic acid. However, it is also possible that dietary fatty acids may influence cytokine production independent of their effects on the production of eicosanoids. The results obtained from animal feeding experiments clearly show that dietary fatty acids can affect the production of mediators by immune cells and that this in turn can affect immune responses or functions such as lymphocyte proliferation and macrophage cytotoxicity (see chapters 2-5). The relevance of this to the human immune system needs to be determined. The effects of dietary fatty acids on the production of cytokines by human immune cells has been studied for a number of years: *In vitro* studies on the influence of individual fatty acids added to cell cultures have yielded conflicting results, depending on cell type, assay method and type of fatty acid studied (see chapter 1). *In vivo* studies are limited but have included assessments of immunologically-mediated reactions such as graft rejection in organ transplant patients who have been supplemented with various fatty acids. Some of these studies have shown that dietary n-3 fatty acids decrease the incidence of transplant rejection (Homan Van der Heide *et al.* 1993; Bennett *et al.* 1995; Maachi *et al.* 1995). Most of

the studies which have been carried out in humans are *ex vivo* studies which have involved supplementing the diet of individuals and measuring the circulating levels of cytokines or the production of cytokines produced by isolated monocytes or lymphocytes in response to a immunological challenge such as a mitogen or endotoxin. These studies have shown that increasing the amount of n-3 fatty acids in the diet leads to decreased production of certain cytokines such as IL-2, IFN-γ TNF-α and IL-1 (Endres *et al.*, 1989; Meydani *et al.*, 1991; 1993; Gallai *et al.*, 1993; Kelley *et al.*, 1999; Caughey *et al.*, 1996), decreased lymphocyte proliferation (Molvig *et al.*, 1991; Meydani *et al.*, 1991), decreased natural killer cell activity (Kelley *et al.*, 1999), and decreased expression of some adhesion molecules on monocytes (Hughes *et al.*, 1996).

The studies referred to above have used differing amounts of n-3 fatty acids to elicit these effects, ranging from high doses such as 18 g FO/day (providing 4.6 g of EPA + DHA per day) in the Endres *et al.* (1989) study to low doses of 0.65 g FO/day (providing 0.55 g EPA + DHA per day) in a study by Schmidt *et al.* (1996). Also, as with animal experiments, some have used encapsulated FO with the DHA and EPA present in triglyceride, while others have used the fatty acids in an ethyl ester form. In these studies, the effects of the n-3 fatty acids have been compared to results obtained from unsupplemented subjects (Endres *et al.*, 1989), subjects with different characteristics also supplemented with fish oil (healthy vs. diseased; old vs. young) (Meydani *et al.*, 1991) or volunteers supplemented with a 'placebo' which is usually a different type of oil such as olive oil or an n-6 fatty acid-rich oil such as corn or safflower oil (which may have their own effects on the immune system; see chapters 2-5) (Caughey *et al.*, 1996) or a placebo of 'mixed' oils typical of those ingested in the diet of the local population (Yaqoob *et al.*, 2000). This makes the studies very difficult to compare. The exact amount of n-3 fatty acids needed to affect cytokine production by human immune cells is far from clear, as are the duration of supplementation needed to elicit a response, and the exact mechanisms through which the n-3 fatty acids are exerting their effects. The determination of these parameters is of crucial importance if n-3 fatty acids are to be used in therapeutic ways to treat diseases in which the overproduction of cytokines is part of the pathology.

7.2 Purpose

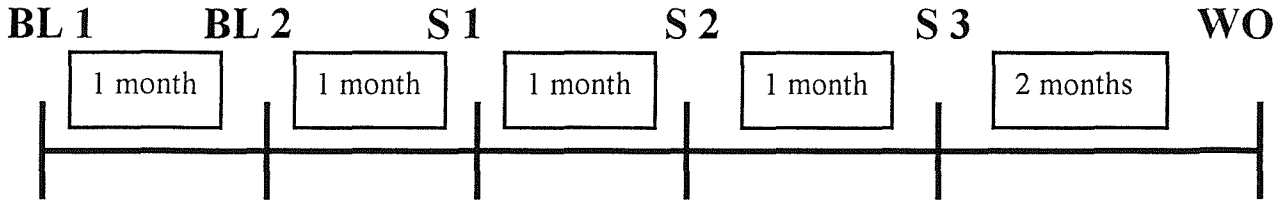
The purpose of this project was to investigate the effects of dietary n-3 fatty acids on the production of inflammatory mediators by human immune cells and to investigate if dietary n-3 fatty acids can modulate human immune cell functions. It was hoped that the results of this study would aid clarification some of the contradictions in the literature and allow for the determination of the optimum dose of fatty acids needed to modulate human immune cell function. This would have important consequences for the development of pharmacological interventions involving fatty acids and may have implications for the amount of fatty acids provided in novel functional foods.

7.3 Methods

7.3.1 Study design

The study was of 7 month duration and ran from November 1997 - June 1998. Subjects were required to attend the Institute of Human Nutrition at the University of Southampton. Subjects gave two blood samples one month apart to use for baseline measurements. The results of these two baseline measurements were averaged. Subjects then supplemented their diets with capsules for 3 months and gave monthly fasting blood samples during the supplementation period. One further blood sample was taken two months after the end of supplementation; this was termed the washout measurment. All blood samples were taken after an overnight fast. A diagram of the study design is shown in figure 7.1.

Figure 7.1 study plan for the supplementation study. BL1 = first baseline measurements. BL2 = second baseline measurements measurement. S1 = sample taken after one month supplementation, S2 = sample taken after two months supplementation. S3 = sample taken after three months supplementation. WO = washout sample (taken two months after supplementation had ceased).



7.3.2 Recruitment of the volunteers

Ethical approval for all the procedures involving the volunteers was obtained from the South and West Local Research Ethics Committee. Volunteers were recruited from the staff and student population at the University of Southampton. All volunteers completed a health and lifestyle questionnaire and doctor's consent for inclusion into the study was obtained. In total 48 subjects were recruited for the study, however 8 volunteers were not used in the final analysis of the study. This group was given a dose of 1.5 g FO/day but were not used in the final analysis of the study, as half of the members of this group encountered medical problems (not related to capsule use) during the course of the study which required them to take medications which could have affected mediator production by inflammatory cells. Of the 40 subjects used in the study, 36 completed all timepoints in the study.

7.3.3 Exclusion criteria

Volunteers were excluded if they had a history or a family history of autoimmune disease, were hypercholesterolemic, hypertriglyceridaemic, or diabetic, had high blood pressure, were taking any medication known to affect cytokine or eicosanoid production or blood lipid levels, had a body mass index (BMI) of greater than 25, were vegetarian, smoked more than 10 cigarettes a day, drank more than 10 units of alcohol per week, or consumed FO, or other vitamin supplements regularly.

7.3.4 Food diaries

At the beginning and end of the study each subject completed a five-day household measure food diary. At the time of the first diary, subjects were told to record their food intake in what was a going to be a week representative of their 'usual' intake and to avoid recording during the Christmas holidays where intake may not be representative of normal. The food diaries used contained pictures of representative portion sizes and were standard diaries used in studies previously supported by the Ministry of Agriculture, Fisheries and Food (MAFF; see appendix 2). Volunteers were asked to include at least one weekend day in their recording, due to the fact that food consumption patterns have been shown to differ between weekends and weekdays

(Willett, 1998). The food diaries were analyzed using FOODBASE (Institute of Brain Chemistry and Human Nutrition, London, UK), a program that allowed for the assessment of intakes of specific fatty acids. The study started in winter and ended in late spring, and as dietary intakes can be affected by seasonal variation (Willett, 1990), by having subjects complete two dietary records, one at the start and one at the end, of the study, any change in the habitual intake of the fatty acids over the study time period could be seen. It was also possible to assess any changes which might influence the results of the study such as any changes in the consumption of protein, energy, and antioxidants which have been shown to influence cytokine production (Grimble, 1996; Wu *et al.*, 1996; Yaqoob *et al.*, 2000). An index of habitual alcohol intake was also assessed in order to monitor compliance to the 10 units a week exclusion criteria.

7.3.5 Capsules

Subjects were randomly allocated to receive one of the five types of capsule in a double blind fashion. There were 8 subjects per treatment group. The capsules used were a gift from Scotia Pharmaceuticals (Stirling, UK). Subjects consumed 9 capsules per day, and each capsule contained 1 g of oil. Consumption of 9 capsules per day provided 3 g FO (25% FO containing 0.5 g EPA + DHA), 6 g FO (50% FO containing 1.3 g EPA + DHA) or 9 g FO (100% FO containing 2.3 g EPA + DHA) per day, or 9 g of linseed oil/day (providing 4 g α-linolenic acid per day) or 9 g of the placebo oil. The placebo oil consisted of 80:20 mix of palm oil and soybean oil. This was selected because it would contain a mixture of saturated, monounsaturated and polyunsaturated fatty acids with proportions of linoleic acid and α-linolenic acids and an n-6 to n-3 PUFA ratio similar to those in the current average UK diet. The fatty acid composition of the capsules used is shown in table 7.1. Capsules were supplied to subjects in plastic tubs. These contained more capsules than required for 4 weeks and were replaced every 4 weeks.

Table 7.1 Fatty acid components of the capsules used: g/100g fatty acid
Values are means (n=3) and SE.

Fatty acid	*Pla-cebo*	*Se*	*Lin-seed*	*Se*	*100% FO*	*Se*	*50% FO*	*Se*	*25% FO*	*Se*
14:0	2.1	0.6	0.4	0.4	6.0	1.0	4.9	1.7	5.1	1.4
16:0	34.9	1.7	7.0	0.3	22.8	1.7	29.6	2.1	30.3	2.0
16:1 n-7	2.0	1.0	0.2	0.2	5.3	2.0	4.3	0.5	3.3	0.8
18:0	3.7	0.1	6.5	2.0	6.7	1.7	5.0	0.1	4.5	0.6
18:1 n-9	33.8	2.7	18.9	1.4	15.5	1.4	25.2	4.7	27.3	1.8
18:2 n-6	18.9	0.5	16.9	0.7	2.4	0.7	12.0	1.5	14.4	1.8
α–18:3n-3	1.8	0.4	45.9	1.0	1.6	0.1	2.1	0.7	2.3	0.8
γ–18:3n-6	0	0	0	0	1.0	0.3	0	0	0	0
20:2n-6	0	0	2.0	1.1	0	0	Trace	-	Trace	Trace
20:4n-6	0	0	1.7	0.2	2.3	0.4	2.2	0.7	Trace	Trace
20:5n-3	0	0	0	0	6.4	1.1	3.3	0.8	1.9	0.6
22:5n-3	0	0	0	0	0.8	0.4	0	-	0	0
22:6n-3	0	0	0	0	18.5	1.9	8.7	1.1	3.7	0.4

7.3.6 Compliance

Compliance was measured by monitoring the weight of the tubs of capsules returned at each monthly visit and comparing this to the theoretical weight the tub should have been had all of the capsules been consumed. Compliance was also measured by monitoring the incorporation of the characteristic fatty acids of each supplement into plasma phospholipids.

7.3.7 Preparation of PBMC:

Twelve hour fasting blood samples were obtained from each volunteer between 8 and 10 am. Forty ml of blood was collected into 4 heparinized vacutainer tubes and then in sterile conditions 20 ml of blood was layered over an equal volume of lymphocyte separation medium (Sigma, Poole, UK)) and centrifuged at room temperature at 2000 rpm for 20 minutes. Plasma was collected off the top layer and frozen at –20°C for later analysis. Cells were collected from the interphase and resuspended in RPMI-1640 (Sigma, Poole, UK), and then pelleted by centrifugation at 1500 rpm for 10 minutes. This was repeated twice and then the cells were resuspended in 1 ml RPMI and counted on a Coulter cell counter. Cells were finally resuspended at a concentration of 2×10^6 cells/ml in RPMI medium containing 2 mmol/L glutamine (Gibco, Paisley,

UK), antibiotic (a 1:1 mix of penicillin and streptomycin (Sigma, Poole, UK) and 10% autologous plasma (AP).

7.3.8 Lymphocyte proliferations

100 µl of cell suspension was added to wells of a 96 well microtitre plate, along with 20 µl of either 75, 150, 250, 500, or 750 µg/ml Con-A and 80 µl of RPMI; the final concentrations of Con-A in the well were 7.5, 15, 25, 50 and 75 µg/ml. Unstimulated wells received RPMI instead of con-A. The final volume in the well was 200 µl. Proliferation was measured as the incorporation of tritiated thymidine over the final 18h of a 66-hour culture period. Thymidine incorporation values for the triplicate cultures were averaged prior to calculating the stimulation index.

7.3.9 Measurement of mediator production

For assessment of cytokine production 1 ml of cell suspension (2 x 10^6 cells/ml with 10% AP) was cultured along with 200 µl of 250 µg/ml Con-A (Sigma, Poole, UK) or 150 µg/ml LPS (Sigma, Poole, UK) and 800 µl RPMI for 24 hours at 37 °C (in 19:1 air/CO_2) The final concentrations of Con-A and LPS were 25 µg/ml and 15 µg/ml respectively. The plates were then centrifuged for 10 minutes at 1200 rpm and supernatants were collected and frozen at –20 °C until analysis of cytokine concentration by ELISA. Cytokine ELISA kits were purchased from Biosource International (Fleurus, Belgium). For all assays the manufacturers instructions were followed. The following cytokines were measured: TNF-α, IL-1β, IL-6 for LPS-stimulated PBMC and IL-2, IL-4, IL-10, and IFN-γ for Con-A stimulated PBMC. The concentrations of PGE_2 were also measured in the cell supernatants, using a PGE_2 ELISA kit from Neogen (Lexington, USA). Standard curves for the ELISA assays are given in appendix 3.

7.3.10 Measurement of PBMC fatty acid composition

Lipid was extracted from PBMC with chloroform:methanol (2:1 v/v). Fatty acids were prepared by a saponification reaction at 70°C in methanolic 0.5 M KOH. Samples were neutralized using concentrated sulfuric acid and fatty acids were extracted into ethyl acetate. After evaporation to dryness, fatty acids methyl esters were prepared by

reaction with an excess of diazomethane in ether. Fatty acid methyl esters (dissolved in hexane) were injected (1 μl) via a Hamiliton syringe into a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, Avondale, PA) fitted with a 30 m x 32 mm BPX70 capillary column, film thickness 0.25 μm. Helium at 2.0 ml/min was used as the carrier gas, and the split/splitless injector was used with a split:splitless ratio of 10:1. The separation was recorded with HP GC Chemstation software. Fatty acid methyl esters were identified by comparison with standards run previously.

7.3.11 Measurement of plasma phospholipid fatty acid composition

Lipids were extracted from plasma with chloroform:methanol (2:1 v/v) and then additional chloroform and water were added and the extraction was washed in 0.88 % KCL. The sample was centrifuged and the bottom layer transferred to a new tube. The sample was centrifuged under nitrogen. Then 1 ml of chloroform:methanol (2:1 v/v) was added. This was then layered onto the bottom of a thin layer chromatography (TLC) plate and the plate was placed into a tank containing a mixture of 90:30:1 hexane:diethylether:acetic acid, to separate the phospholipids from the neutral lipids. After 30 minutes in the tank the plate was removed left to dry for 5 minutes and then sprayed with Florocin (Sigma, Poole, UK). The plate was then examined under an ultraviolet light in order to visualize the phospholipid and other lipid bands. The phospholipid fraction was then scraped from the plate into glass tubes. Then phospholipid fatty acid methyl esters were prepared by incubation with 14% boron trifloride at 80°C for 15 min. Fatty acid methyl esters were isolated by solvent extraction, dried and separated by gas chromatography in a Hewlett-Packard 6890 gas chromatograph as described above.

7.3.12 Measurement of Plasma TBARS

Plasma thiobarbituric acid-reactive substances (TBARS) were measured by incubating 100 μl plasma with 1.2 ml of 3.35 mg/ml thiobarbituric acid dissolved in 100 g/l trichloroacetic acid for 15 min at 95°C and recording the absorbance at 535 nm after cooling; TBARS were calculated using an extinction coefficient of 1.56×10^5 (mmol L^{-1}) $^{-1}$/cm.

7.3.13 Measurement of plasma TAG concentrations

Plasma TAG concentrations were measured using a commercial kit from Sigma (Poole, UK; Procedure no 337). Triglycerides were first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol was then phosphorylated by ATP forming glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK). G-1-P was then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). A quinone dye is produced from the ensuing reaction and is detected on a spectrophotometer at 540 nm. The increase in absorbance was directly proportional to the triglyceride concentration of the sample.

7.3.14 Statistical analysis

Results are expressed as mean +/- SEM for six to eight subjects per treatment group. Statistical significance of treatment and time were determined using two-way ANOVA. The effects of treatment and of time were analysed further by one-way ANOVA using the *post hoc* least significant difference (LSD) test. A paired *t*-test was performed to determine if baseline and end of washout, and end of supplementation were statistically different. Statistical tests were performed using SPSS version 6.0 (SPSS Inc., Chicago, USA).

7.4 Results

7.4.1 Number who completed the study

Of the 48 subjects recruited, 40 were used in the final analysis of the study. Of these 40 subjects participating in the final study, 36 completed all timepoints. The four subjects who did not complete all timepoints all missed the final washout measurement. Table 7.2 shows the subject characteristics. Subjects were asked about any side effects experienced from the capsules at each appointment date. One subject reported problems swallowing the capsules. Eight subjects reported a fish after-taste after ingesting capsules. No other side effects were reported.

Table 7.2 Characteristics of the subjects.
Data are mean and SE of 8 subjects per group or 40 subjects (overall measurement)

	Age (y)	*Se*	*Height (cm)*	*Se*	*Weight (kg)*	*Se*	*BMI (kg/m^2)*	*Se*	*Smokers (no)*
Overall	24.1	0.9	180.9	1.1	74.8	1.7	22.9	0.4	8
Placebo	25.8	2.5	179.3	2.4	74.8	3.2	23.2	0.7	1
Linseed	22.7	2.0	189.3	2.4	80.8	5.0	22.5	1.1	1
3 g FO	24.8	1.4	178.7	2.1	75.2	3.1	23.5	0.9	3
6 g FO	25.1	2.5	178.9	2.1	70.1	3.1	22.3	0.8	3
9 g FO	22.0	1.2	178.4	1.7	72.5	4.3	22.8	1.3	0

7.4.2 Habitual nutrient intakes

Tables 7.3 and 7.4 show the habitual intakes of macronutrients, vitamins and minerals and specific fatty acids as assessed by averaging the results of the two food diaries. Food diaries which reported energy intakes at less than 70% of the daily reference value (DRV) were not used in the final analysis as this is an indicator of non compliance (M. Barker, personal communication). 4 diaries were discarded for this reason (1 diary from each of 4 different people). Differences in nutrient intakes between the two food diaries were assessed by paired *t*-tests. There were few significant changes in the intakes of most nutrients between the beginning and end of the study period, although there were significant changes in the intakes of vitamin B12 (P= 0.03), vitamin C (P= 0.04) and sulfur amino acids (P=0.003). These tended to be lower at the end of the study than at the beginning. There were no significant differences in the intakes of any fatty acids over the duration of the study. Because there was little difference in the intakes of nutrients between the two timepoints the two food diaries were averaged in order to give an assessment period of 10 days which is significantly long enough to get a reliable estimate of the habitual intakes of most nutrients (Willett, 1990). The mean intake of EPA + DHA at the beginning of the study was 0.20 g/day As a result of supplementation with the capsules, daily intakes of EPA + DHA increased to 0.7 g/day in the 3 g FO/day group, to 1.5 g/day in the 6 g FO/day and to 2.5 g/day in the 9 g FO/day and intakes of 18:3n-3 were increased from 1.25 g/day in the linseed oil group to 5.25 g/day.

Table 7.3 Habitual daily nutrient intakes of the subjects

Nutrient	*Mean*	*SE*	*SD*	*Range*	*Min*	*Max*	*DRV*
Energy (kJ)	10570	342	2265	10681	5371	16052	10600
Energy (kcal)	2579	72	480	2008	1818	3826	2550
% DRV	101	3	19	79	71	151	-----
Protein (energy %)	15	0.3	2	8	11	19	-----
Carbohydrate (energy %)	45	1	5	25	36	61	-----
Fat (energy %)	34	1	6	28	21	50	33
Alcohol (g)	25	4	24	91	0	91	-----
Vitamins							
B1 (mg)	3	0.4	3	13	1	14	0.9
B2 (mg)	2	0.1	1	3	1	4	1.7
B3 (mg)	30	2	10	41	18	59	17
B6 (mg)	3	0.1	1	4	1	5	1.4
B12 (μg)	5	0.2	1	5	3	8	1.5
C (mg)	94	9	62	276	8	285	40
Pantothenic acid (mg)	18	3	18	68	3	71	3-7
Folic acid (total) (mg)	336	17	110	467	145	612	200
Retinol equivalents (μg)	729	47	311	2071	152	2223	700
D (μg)	3	0.2	1	5.0	1	5	5
E (mg)	9	1	3	13	3	16	4
Minerals							
Ca (mg)	1061	35	230	1041	662	1703	700
P (mg)	1584	46	305	1262	981	2243	550
Na (mg)	3866	158	1047	4706	2622	7328	1600
Cl (mg)	5839	242	1603	7543	3629	11172	2500
Mg (mg)	363	16	106	545	130	671	300
K (mg)	3759	163	1083	4419	1727	6145	3500
Mn (mg)	4	0.2	1	5	1	6	
Cu (mg)	1	0.1	0.3	2	1	2	1.2
Fe (mg)	14	1	4	17	8	25	8.7
Se (μg)	71	4	24	130	30	160	75
Zn (mg)	94	9	62	276	8	285	9.5

Table 7.4 Average habitual intakes of specific fatty acids (mg/day)

Fatty acid	*Mean*	*SE*	*SD*	*Range*	*Min*	*Max*
10:0	654	47	313	1520	105	1625
12:0	1170	81	538	2640	175	2816
14:0	3424	201	1333	6344	672	7016
14:1	238	16	105	523	38	561
16:0	16471	635	4212	22077	6226	28308
16:1n-7	1190	55	365	1915	312	2227
18:0	7579	334	2214	10806	2736	13542
18:1n-9	23042	840	5573	24345	10196	34542
18:2n-6	12510	669	4435	18493	5223	23716
Ct18:2n-6	180	13	84	425	41	466
Tt18:2n-6	154	23	148	604	14	619
18:3n-3	1250	62	414	1503	644	2146
20:0	254	15	98	377	101	479
20:2n-6	29	3	20	85	0	85
20:3n-6	36	3	21	144	0	144
20:4n-6	162	13	84	477	10	486
20:5n-3	73	9	62	311	0	311
22:5n-3	65	5	34	204	0	204
22:6n3	126	16	104	579	14	593
24:0	88	8	56	307	9	316
Total n-6	13142	874	5595	19157	4869	24026
Total n-3	1526	72	481	1806	742	2548

7.4.3 Compliance over the study period

Table 7.5 shows subjects compliance throughout the supplementation period as assessed by the percentage of weight of the returned capsules compared to the theoretical weight that should have been returned. Compliance was fairly high throughout the study when assessed by this way and there were no significant differences between the capsule groups. Compliance was confirmed by an increase in the proportions of EPA and DHA in plasma phospholipids (section 7.4.5).

Table 7.5 Percent compliance based on capsule tub weights

	One month	*Two months*	*Three months*
Overall (n=35)	76.15 (1.42)	79.03 (3.97)	90.19 (2.62)
Placebo	75.71 (3.15)	77.25 (6.22)	84.81 (5.58)
Linseed	76.48 (3.53)	91.20 (7.91)	93.30 (5.23)
3 g	73.88 (3.48)	82.76 (10.01)	93.00 (6.60)
6 g	72.74 (10.56)	78.69 (2.30)	87.65 (7.13)
9 g	70.71 (9.33)	76.27 (3.64)	92.63 (6.17)

7.4.4 Blood pressure

Tables 7.6 and 7.7 show absolute values for systolic and diastolic blood pressure over the course of the study. There were no significant differences in systolic blood pressure between any of the groups at any point in the study. However, linseed oil supplementation caused a significant decrease in systolic blood pressure compared to baseline after two months supplementation, and supplementation with 9 g FO/day for one month caused a significant decrease in systolic blood pressure compared to baseline. Diastolic blood pressure was significantly higher in the group which was to be supplemented with 9g FO/day than the group which was to be supplemented with linseed oil. However after one month of supplementation with the capsules, the diastolic blood pressure of the group which had been given linseed oil was significantly lower than the group given 3 g FO/day, and the group which had been given 9 g FO/day had significantly lower diastolic blood pressure compared to baseline. The difference between the linseed oil and 3 g FO/day groups was also seen at three months supplementation. Tables 7.8 and 7.9 show the percent change in systolic and diastolic blood pressure in the different groups over the study period. When the data were analysed in this way, there were no significant changes in blood pressure over time; however, blood pressure (both systolic and diastolic) tended to decrease in the groups taking the linseed oil and the highest dose of FO compared to baseline values.

Table 7.6 Systolic blood pressure (mmHg). Data are means and se of 6-8 per group. Numbers not sharing the same alphabetic subscript within a column are significantly different. Numbers with a asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	123.9	3.3	118.9	5.4	124.3	4.2	124.5	4.4	119.8	2.6
Linseed oil	122.4	4.3	123.8	4.6	115.4*	3.8	117.8	6.4	120.3	3.6
3 g FO	125.1	5.1	122.8	7.2	121.9	5.6	124.9	4.4	123.9	6.8
6 g FO	120.1	4.3	118.6	4.9	120.9	5.0	121.9	5.2	118.7	5.1
9 g FO	129.3	2.0	121.5*	2.0	124.3	3.4	124.5	2.5	124.2	3.5

Table 7.7 Diastolic blood pressure (mmHg). Data are means and se of 6-8 per group. Numbers not sharing the same alphabetic subscript within a column are significantly different. Numbers with a asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	74.1^{ab}	2.0	72.8^{ab}	2.0	75.6	2.9	74.8^{ab}	1.8	74.6	2.4
Linseed oil	68.8^{b}	2.4	64.8^{b}	2.5	68.5	2.2	68.5^{b}	2.4	70.2	3.7
3 g FO	73.4^{ab}	3.2	73.9^{a}	4.5	73.3	3.2	75.1^{a}	3.0	72.6	3.6
6 g FO	70.7^{ab}	2.3	69.1^{ab}	3.6	70.0	4.1	71.9^{ab}	2.7	70.1	2.9
9 g FO	76.1^{a}	1.8	71.6^{ab}*	2.0	73.0	2.4	75.0^{ab}	2.0	74.5	2.9

Table 7.8 Percent change in systolic blood pressure (mmHg) compared to baseline Data are mean (se) for 8 subjects per group

	One month	*Two months*	*Three months*	*Wash out*
Placebo	-4.7 (3.6)	0.4 (2.5)	0.5 (2.1)	-0.3 (1.6)
Linseed	1.3 (2.5)	-5.6 (1.8)	-3.7 (4.2)	-0.5 (4.2)
3 g FO	-2.0 (3.6)	-2.5 (2.0)	0.4 (3.6)	- 2.5 (1.9)
6 g FO	-1.0 (3.1)	0.8 (2.7)	1.5 (2.7)	-0.5 (2.4)
9 g FO	-6.1 (2.1)	-3.8 (2.6)	-3.6 (2.2)	-4.0 (2.9)

Table 7.9 Percent change in diastolic blood pressure (mmHg) compared to baseline Data are mean (se) for 8 subjects per group

	One month	*Two months*	*Three months*	*Wash out*
Placebo	-1.6 (2.6)	2.25 (3.5)	1.3 (3.3)	2.6 (4.1)
Linseed	-5.7 (3.0)	-0.1 (3.0)	-0.6 (3.3)	4.3 (2.3)
3 g FO	0.4 (3.1)	0.1 (2.7)	2.6 (2.2)	-0.8 (5.1)
6 g FO	-2.2 (3.8)	-1.1 (4.4)	2.1 (3.9)	-0.2 (3.1)
9 g FO	-5.9 (1.8)	-4.1 (2.3)	-1.1 (3.7)	-0.7 (2.8)

7.4.5 Effect of supplementation on plasma phospholipids

7.4.5.1 Proportion of 16:0 into plasma phospholipids (Table 7.10)

At baseline, the groups which were to consume 3 g or 9 g FO per day had a significantly lower proportion of 16:0 present than did the group which was to consume 6 g FO/day. These differences between the groups were not apparent after one, two, and three months of supplementation or two months after the end of the supplementation period. Within the groups however, supplementation with linseed oil for two months resulted in a significantly decreased proportion of 16:0 in plasma phospholipids compared to the value at baseline for this group. The proportion of 16:0 present in the phospholipids of the linseed oil supplemented group had returned to baseline values after three months of supplementation. Supplementation with 6 g FO/day also resulted in a decreased proportion of 16:0 present in plasma phospholipids after one, two and three months supplementation compared to baseline values for this

group. Two months after the end of supplementation the proportion of 16:0 had returned to baseline values.

7.4.5.2 Proportion of 18:0 into plasma phospholipids (Table 7.11)

At baseline, the group which was to consume the linseed oil had a significantly lower proportion of 18:0 than did the group which was to consume 6 g FO per day. After one month of supplementation with the various oils, groups consuming the linseed oil and 6 g FO per day had significantly higher proportion of 18:0 present in plasma phospholipids compared to those volunteers who consumed the placebo oil and 3 g FO per day. The group consuming the 9 g FO per day also had significantly more 18:0 present in their plasma phospholipids than did the group consuming the placebo oil after one month of supplementation. After two months of supplementation with the oils, the volunteers in the group consuming 9 g FO per day had a significantly higher proportion of 18:0 in their plasma phospholipids than did volunteers from the other groups. After three months of supplementation with 9 g FO, this difference was only significant when compared to the proportion of 18:0 present in the phospholipids present in the group consuming 3 g FO per day. Two months after the end of supplementation (washout), there were no significant differences in the proprortion of 18:0 present in the plasma phospholipids from volunteers in any of the capsule groups.

One and two months of supplementation with linseed oil caused a significant increase in the proportion of 18:0 present in the volunteers plasma phospholipids compared to baseline values. Two months of supplementation with 9 g FO per day also caused a significant increase in the proportion of 18:0 present in plasma phospholipids compared to baseline values for this group.

Table 7.10 The proportion of 16:0 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	30.71^{ab}	0.63	30.75	0.47	30.1	0.69	30.40	1.2	30.93	0.53
Linseed oil	31.02^{ab}	0.47	30.90	0.33	28.78*	0.55	32.06	1.09	29.48	1.13
3 g FO	29.43^{b}	0.9	30.59	0.55	30.08	0.84	30.19	0.54	31.39	0.93
6 g FO	31.80^{a}	0.68	30.45*	0.52	29.51*	0.86	29.78*	0.33	30.43	1.28
9 g FO	29.94^{b}	0.37	30.32	0.54	30.96	1.15	31.26	0.9	30.76	0.47

Table 7.11 The proportion of 18:0 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	14.29^{ab}	0.38	13.66^{c}	0.38	14.78^{b}	0.22	14.09^{ab}	0.4	13.85	1.63
Linseed oil	13.79^{a}	0.25	15.50^{a}*	0.26	14.54^{b}*	0.16	14.80^{ab}	0.35	16.41	1.48
3 g FO	14.09^{ab}	0.36	14.10^{bc}	0.28	14.64^{b}	0.5	13.75^{b}	0.35	14.61	0.26
6 g FO	14.80^{b}	0.3	15.46^{a}	0.34	14.70^{b}	0.33	14.39^{ab}	0.27	15.41	0.82
9 g FO	14.67^{ab}	0.36	14.97^{ab}	0.38	15.87^{a}*	0.25	14.98^{a}	0.52	14.09	1.23

7.4.5.3 Proportion of 18:1n- 9 in plasma phospholipids (Table 7.12)

At baseline there was no significant differences in the proportions of 18:1n-9 present in the plasma phospholipids in any of the groups. One month of supplementation with 9 g FO/ day and the placebo oil mix caused a significant decrease in the proportion of 18:1n-9 present in the plasma phospholipids compared to the linseed oil supplemented group. This difference was not apparent after two or three months supplementation with the oils or after the two months washout period.

Supplementation with 3 g FO per day for one month caused a significant decrease in the proportion of 18:1n-9 present in plasma phospholipids compared to baseline. A significant decrease in the proportion of 18:1n-9 present in plasma phospholipids was also seen in the plasma of volunteers from this capsule group during the washout period. A decrease in the proportion of 18:1n-9 present in plasma phospholipids compared to baseline values was also seen in the washout period in the group that consumed the 9 g of FO per day.

7.4.5.4 Proportion of 18:2n-6 in plasma phospholipids (Table 7.13)

There were no significant differences in the proportion of 18:2n-6 present in plasma phospholipids of the different groups at baseline. After one month supplementation the proportion of 18:2n-6 present in the plasma phospholipids of the 9 g FO/day group was significantly lower than each of the other groups. The proportion of 18:2n-6 in the plasma phospholipids remained lowest for this group throughout the two month and three month supplementation time but was not significantly different from baseline values during the washout period. The group receiving the placebo oil had a significantly higher proportion of 18:2n-6 present in their plasma phospholipids compared to the 9 g and 6 g FO/day groups after two months supplementation and a significantly higher proportion of 18:2n-6 present in their phospholipids after three months supplementation compared to the 9 g FO/day group after three months supplementation. The group receiving the 9 g FO/day had a significantly lower proportion of 18:2n-6 present compared to baseline values after one and two months of supplementation. The group receiving 6 g FO/day had significantly lower proportion of 18:2n-6 present compared to baseline values after three months of supplementation.

Table 7.12The proportion of 18:1n-9 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	11.51	0.96	9.87^{b}	1.1	13.21	0.37	13.61	0.62	10.19	1.74
Linseed oil	12.8	0.97	12.64^{a}	0.74	12.93	0.58	12.38	0.5	8.73	0.61
3 g FO	12.28	0.57	9.39^{ab}*	0.98	13.24	0.64	12.31	0.6	9.67*	0.74
6 g FO	12.38	1.05	12.21^{ab}	0.35	12.78	0.99	12.97	0.54	8.67	0.37
9 g FO	11.31	0.67	8.94^{b}	0.7	12.22	0.23	11.95	0.47	8.37*	0.96

Table 7.13 The proportion of 18:2n-6 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	22.94	0.83	22.08^{b}	0.94	24.07^{c}	1	24.35^{b}	1.07	22.32	1.81
Linseed oil	23.55	0.49	23.39^{b}	0.58	23.17^{bc}	1.03	23.32^{ab}	0.44	24.05	0.65
3 g FO	24.35	1.05	22.64^{b}	0.67	23.41^{bc}	1.02	23.43^{ab}	1.26	22.87	0.53
6 g FO	23.8	1.02	22.02^{b}	0.88	21.34^{ab}	0.75	21.93^{ab}*	0.7	24.14	0.5
9 g FO	23.26	0.46	18.95^{a}*	1	19.16^{a}*	0.46	20.78^{a}	1.44	22.88	0.8

7.4.5.5 Proportion of 20:3n-6 in plasma phospholipids (Table 7.14)

There were no significant differences in the proportion of 20:3n-6 present in the plasma phospholipids of the different groups supplemented with the oils at baseline, after one month of supplementation, three months supplementation or after the end of supplementation. After two months of supplementation the group supplemented with 9 g FO/day had a significantly lower proportion of 20:3n-6 present in the plasma phospholipids as compared to the group supplemented with 6 g FO per day or with the placebo oil or linseed oil. After 3 months of supplementation the group supplemented with 9 g FO/day had the lowest proportion of 20:3n-6 present in their plasma phospholipids although this was not significantly different from the other groups.

Compared to baseline proportions of 20:3n-6 present in plasma phospholipids, the group supplemented with the 9 g FO/day was significantly lower after three months supplementation. The group supplemented with linseed oil was also significantly lower after two and three months of supplementation, and the group supplemented with 3 g FO/day was significantly lower after one, two and three months of supplementation compared to baseline values.

7.4.5.5 Proportion of 20:4n-6 into plasma phospholipids (Table 7.15)

At baseline the group which was to be supplemented with 3 g FO per day had a significantly greater proportion of 20:4n-6 present in their phospholipids than did the group which was to be supplemented with 6 g FO per day. After one month of the study, the group which had been supplemented with 6 g FO per day still had the lowest proportion of 20:4n-6 present in their phospholipids. This was significantly less than the proportion present in the group supplemented with 9 g FO per day. The groups supplemented with either linseed oil or 3 g FO per day had significantly decreased the proportion of 20:4n-6 present in their plasma phospholipids. After two months the group which had been supplemented with 6g FO/day had significantly less 20:4n-6 present compared to the group supplemented with linseed oil, although both the 6 g FO per day, linseed oil, and 9 g FO per day groups all had significantly decreased proportions of 20:4n-6 present in their plasma phospholipids compared to baseline

values. After three months supplementation, there were no significant differences between the groups in the proportions of 20:4n-6 present plasma phospholipids, although the groups supplemented with 3 g and 6 g FO/day were still significantly decreased compared to baseline. Two months after the end of supplementation, the groups supplemented with the 9 g FO/day had significantly increased 20:4n-6 compared to groups linseed oil or 6 g FO/day.

7.4.5.6 Proportion of 20:5n-3 in plasma phospholipids (Table 7.16)

At baseline there were no significant differences in the proportion of 20:5n-3 present in the plasma phospholipids of any of the groups. After one month of supplementation, the group supplemented with 9 g FO/day had significantly increased 20:5n-3 present in their plasma phospholipids compared to all other groups. The group supplemented with 6 g FO/day also had a greater proportion of 20:5n-3 present compared to placebo. All groups supplemented with FO had significantly increased 20:5n-3 present compared to baseline. After two months on the study, the groups supplemented with linseed oil and 9 g FO/day had a significantly higher 20:5n-3 present in their phospholipids as compared to the placebo supplemented group. All three groups which had been supplemented with FO had significantly more 20:5n-3 present compared to baseline. After 3 months on the study, all groups which had been supplemented with n-3 fatty acids had significantly greater proportion of 20:5n-3 present compared to baseline values, although there were no significant differences between the groups. There were also no significant differences in the proportion of 20:5n-3 present between the groups at washout. Washout values for 20:5n-3 were not significantly different from baseline values.

Table 7.14 The proportion of 20:3n-6 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	2.94	0.16	2.69	0.18	2.39^{b*}	0.14	1.68*	0.36	3.32	0.27
Linseed oil	2.10	0.48	2.31	0.22	2.27^{b}	0.18	1.15	0.37	3.02	0.28
3 g FO	2.84	0.22	2.33*	0.12	1.81^{ab*}	0.14	1.30*	0.27	2.53	0.18
6 g FO	2.24	0.55	2.36	0.22	2.36^{b}	0.32	1.20	0.41	3.22	0.46
9 g FO	2.57	0.39	2.12	0.23	1.37^{a}	0.34	0.82*	0.31	3.00	0.18

Table 7.15 The proportion of 20:4n-6 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	8.23^{ab}	0.52	8.04^{ab}	0.33	7.57^{ab}	0.34	8.41	0.53	8.93^{ab}	0.42
Linseed oil	8.76^{ab}	0.35	7.57^{ab*}	0.13	7.88^{a*}	0.28	7.03	1.04	8.16^{b}	0.21
3 g FO	9.19^{a}	0.53	7.50^{ab*}	0.27	7.43^{ab}	0.49	7.41*	0.39	8.65^{ab}	0.42
6 g FO	7.79^{b}	0.38	7.15^{b}	0.43	6.59^{b*}	0.34	6.97*	0.42	7.86^{b}	0.57
9 g FO	8.82^{ab}	0.43	8.26^{a}	0.36	7.40^{ab*}	0.35	8.24	0.74	9.89^{a}	0.71

7.4.5.7 Proportion of 22:5n-3 into plasma phospholipids (Table 7.17)

There were no significant differences in the proportion of 22:5n-3 present in the phospholipids between any of the capsule groups at baseline. After one month of supplementation, the proportion of 22:5n-3 present in the phospholipids had increased compared to baseline in all of the groups including the placebo. Those subject given 9 g FO/day had significantly more 22:5n-3 present in plasma phospholipids compared to the groups given 6 g FO/day or linseed oil. After two months of supplementation, the group given 6 g FO/day had a significantly greater proportion of 22:5n-3 present compared to the group supplemented with 3 g FO/day. The groups supplemented with linseed oil, 6 g FO and 9 g FO/day still had significantly greater proportion of 22:5n-3 present compared to baseline. After three months of supplementation however, there were no significant differences between the groups and only those supplemented with linseed oil had more 22:5n-3 present than at baseline. There were also no significant differences between the groups, but the groups which had been supplemented with linseed oil, 6 g FO and 9 g FO/day had a significantly increased proportions of 22:5n-3 present compared to baseline. There were no significant differences among the groups at washout, although they were still slightly higher than at baseline in the groups which had been supplemented with n-3 fatty acids.

7.4.5.8 Proportion of 22:6n-3 in plasma phospholipids (Table 7.18)

There were no significant differences in the amount of 22:6n-3 present among all groups at baseline. At one month of supplementation, the groups supplemented with FO had a significantly greater proportion of 22:6n-3 present compared to baseline, and a dose-response effect was seen. The group supplemented with linseed oil had a significant decrease in the amount of 22:6n-3 present in plasma phospholipids compared to baseline and the placebo and linseed oil supplemented groups had significantly decreased proportions of 22:6n-3 present compared to the FO supplemented groups. These effects were generally still observed after two and three months of supplementation. At washout, there were again no significant differences in the amount of 22:6n-3 present in plasma phospholipids between the groups or compared to baseline values.

7.4.5.9 Effect of supplementation on the n-6:n-3 ratio of plasma phospholipids (Table 7.19)

Table 7.19 and figure 7.2 show the ratio of n-6:n-3 (calculated as total n-6 fatty acids divided by total of n-3 fatty acids) over all of the study timepoints. There were no significant differences in the ratio of n-6:n-3 between any of the groups at baseline. After one month of supplementation with the capsules however, there was a significant decrease in the ratio of n-6/n-3 in the 6 g and 9 g FO/day groups compared to the linseed oil and placebo supplemented groups. There was also a significant decrease compared to baseline values in all the groups supplemented with n-3 fatty acids. The decrease compared to the placebo group and baseline values remained significant throughout the supplementation period for the groups supplemented with 6 g and 9 g FO/day. At washout however there were no significant differences between the groups.

Figure 7.2 The effects of dietary supplementation on the n-6:n-3 ratio of plasma phospholipids

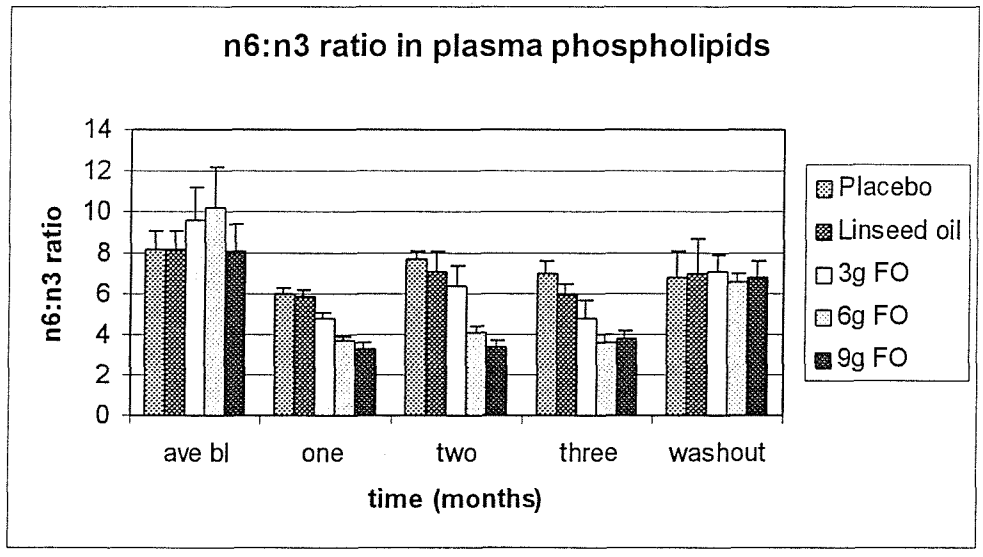


Table 7.16 The proportion of 20:5n-3 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	1.08	0.24	0.95^{c}	0.14	1.03^{b}	0.08	0.83	0.32	0.88	0.08
Linseed oil	0.96	0.46	1.36^{bc}	0.16	1.56^{a}	0.15	1.62*	0.54	1.33	0.26
3 g FO	0.54	0.39	1.20^{bc}*	0.10	1.16^{ab}*	0.11	1.54*	0.3	1.33	0.37
6 g FO	0.66	0.29	1.48^{b}*	0.10	1.38^{ab}*	0.09	1.67*	0.32	1.20	0.09
9 g FO	0.75	0.21	2.36^{a}*	0.26	1.55^{a}*	0.26	1.98*	0.57	0.98	0.10

Table 7.17 The proportion of 22:5n-3 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	1.33	0.19	2.64^{ab}*	0.16	1.79^{ab}	0.18	0.97	0.29	1.93	0.1
Linseed oil	0.81	0.27	2.40^{b}*	0.14	1.99^{ab}*	0.21	1.45*	0.23	1.98*	0.11
3 g FO	1.15	0.39	2.65^{ab}*	0.21	1.58^{b}	0.23	1.39	0.13	1.97	0.21
6 g FO	0.97	0.32	2.24^{b}*	0.13	2.38^{a}*	0.29	1.22	0.2	2.06*	0.08
9 g FO	1.3	0.19	2.96^{a}*	0.17	1.98^{ab}*	0.21	1.25	0.21	1.88*	0.16

Table 7.18 The proportion of 22:6n-3 in plasma phospholipids. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	3.15	0.13	2.79^{c}	0.23	2.21^{c*}	0.11	4.09^{b*}	0.3	2.82	0.22
Linseed oil	3.70	0.50	2.06^{c*}	0.19	2.66^{c*}	0.32	4.00^{b}	3.8	2.99	0.44
3 g FO	3.01	0.50	3.98^{b*}	0.21	3.39^{c}	0.39	6.60^{a*}	0.9	2.88	0.43
6 g FO	3.12	0.65	4.87^{b*}	0.48	4.71^{b}	0.45	7.84^{a*}	0.81	3.02	0.28
9 g FO	2.95	0.18	6.09^{a*}	0.54	5.97^{a*}	0.57	6.82^{a*}	0.48	3.37	0.47

Table 7.19 The plasma phospholipid n-6:n-3 fatty acid ratio over all study time points. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	8.2	0.9	6.0^{a}	0.3	7.7^{b}	0.4	7.0^{a}	0.6	6.8	1.3
Linseed oil	8.2	0.9	5.9^{a*}	0.3	7.1^{b}	1.0	6.0^{ab}	0.5	7.0	1.7
3 g FO	9.6	1.6	4.8^{ab*}	0.3	6.4^{b}	1.0	4.8^{bc*}	0.9	7.1	0.8
6 g FO	10.2	2.0	3.7^{c*}	0.2	4.1^{a*}	0.3	3.6^{c*}	0.4	6.6	0.4
9 g FO	8.1	1.3	3.3^{c*}	0.3	3.4^{a*}	0.3	3.8^{c*}	0.4	6.8	0.8

7.4.6 Fatty Acids Composition of PBMC lipids

7.4.6.1 Proportion of 16:0 in PBMC total lipids (Table 7.20)

At baseline, the group which was to be supplemented with 6 g FO per day had significantly less 16:0 present in their PBMCs than the groups which were to be supplemented with linseed oil or 3 g or 9 g FO/day. There were no significant differences in the proportion of 16:0 present in the PBMCs at any other time in the study. After 2 months supplementation with 9 g FO/day however there was a significant decrease in the amount of 16:0 present in PBMCs compared to baseline. This difference did not occur at any other time in the study.

7.4.6.2 Proportion of 18:0 in PBMC total lipids (Table 7.21)

There were no significant differences between the groups in the proportion of 18:0 present in PBMC lipids at any time in the study. The groups supplemented with 3 g and 6 g FO/day however showed a significant increase in the proportion of 18:0 present in PBMC lipids at washout. This difference did not occur for any of the other groups although 18:0 tended to increase in all groups towards the end of the study.

7.4.6.3 Proportion of 18:1n-9 in PBMC total lipids (Table 7.22)

There were no significant differences between the groups in the proportion of 18:1n-9 present in PBMC lipids at any time in the study. The group supplemented with 6 g FO/day however, did have a significantly greater proportion of 18:1n-9 present at washout compared to baseline values.

7.4.6.4 Proportions of 18:2n-6 in PBMC total lipids (Table 7.23)

There were no significant differences between the groups in the proportion of 18:2n-6 present in PBMCs at any time in the study. The group supplemented with 9 g FO/day however showed a significant increase in the proportion of 18:2n-6 present after one month of supplementation. This increase was not significant at any other time in the study. After two months of supplementation the group given 6 g FO/day showed a significant increase in the proportion of 18:2n-6 present compared to baseline values. This increase was not significant at any other time in the study. After 3 months of supplementation, the group supplemented with the linseed oil capsules showed a

significant increase in the proportion of 18:2n-6 present compared to baseline values. Again, this increase was not significant at any other time in the study.

7.4.6.5 Proportion of 20:3n-6 in PBMC total lipids (Table 7.24)

At baseline the subjects that were to be supplemented with 6g FO/day had a significantly a higher proportion of 20:3n-6 than each of the other groups. After two months of supplementation the group supplemented with linseed oil had a significantly greater proportion of 20:3n-6 compared to those group supplemented with FO. After three months of supplementation, the group supplemented with 6 g FO/day had significantly lower proportion of 20:3n-6 compared to the groups supplemented with linseed oil or 9 g FO/day. There were no significant differences between the groups at washout. When the data were compared to baseline values, the group supplemented with 3 g FO/day had a significantly higher proportion of 20:3n-6 present after one month supplementation. After two months on the study, the group supplemented with linseed oil had significantly higher proportion of 20:3n-6 compared to baseline. After three months on the study, the group supplemented with 6 g FO/day had a significantly lower proportion of 20:3n-6 present in PBMCs compared to baseline values.

7.4.6.6 Proportion of 20:4n-6 in PBMC total lipids (Table 7.25)

There were no significant differences in the proportion of 20:4n-6 present in the PBMC lipids between any of the subject groups at baseline or after one month of supplementation. After two months of supplementation however, the groups supplemented with 3 g or 9 g FO/day had significantly increased proportion of 20:4n-6 present compared to the group supplemented with 6 g FO/day. There were no significant differences between the groups after three months on the study. At washout however, the groups supplemented with 6 g and 9 g FO/day had a significantly decreased proportion of 20:4n-6 present compared to baseline values.

Table 7.20 The proportion of 16:0 in PBMCs. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with a asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	22.3[ab]	0.8	20.8	1.1	20.2	0.3	22.4	1.0	21.2	1.0
Linseed oil	25.2[a]	1.5	19.1	1.8	22.0	1.5	21.8	0.9	24.1	0.8
3 g FO	23.2[a]	1.0	18.8	1.1	23.0	1.4	23.1	1.3	20.7	0.5
6 g FO	18.9[b]	2.3	22.6	1.3	22.8	0.6	20.7	1.1	21.5	1.2
9 g FO	24.3[a]	0.2	19.1	1.1	22.3*	0.6	21.5	1.1	23.4	1.4

Table 7.21 The proportion of 18:0 in PBMCs. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	17.1	0.9	19.9	0.5	18.8	0.7	18.3	0.8	21.3	0.7
Linseed oil	17.1	0.7	17.6	1.0	16.2	1.0	18.8	1.0	20.8	1.4
3 g FO	17.1	0.5	17.3	1.4	16.5	1.8	19.4	0.6	20.2*	0.7
6 g FO	17.1	0.7	18.3	1.0	16.2	0.6	19.0	0.5	21.4*	0.7
9g FO	17.6	0.6	17.2	1.1	19.3	0.8	19.3	0.5	20.9	0.8

Table 7.22 The proportion of 18:1n-9 in PBMCs. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	15.6	0.7	16.6	1.0	16.1	0.5	17.16	0.5	16.8	0.7
Linseed oil	15.6	0.5	14.6	1.1	14.8	0.5	15.5	0.4	17.0	0.8
3 g FO	14.9	0.8	13.9	0.6	14.2	1.4	16.9	0.7	16.9	0.7
6 g FO	14.6	0.5	15.4	1.6	14.7	0.3	15.9	0.8	16.1*	0.5
9 g FO	16.6	1.3	14.6	0.7	16.1	0.7	16.4	1.1	15.3	0.7

Table 7.23 The proportion of 18:2n-6 in PBMCs. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	8.7	0.5	9.2	0.5	10.5	0	9.5	0.3	8.7	0.8
Linseed oil	8.7	0.6	10.7	0.5	9.3	1.0	10.4*	0.5	8.6	0.6
3 g FO	8.8	0.5	10.3	0.8	10.1	1.6	10.3	0.5	9.3	0.3
6 g FO	8.2	0.2	8.9	0.3	9.4*	0.3	9.3	0.4	8.6	0.4
9 g FO	9.1	0.4	10.3*	0.4	8.9	0.2	9.8	0.5	9.1	0.9

Table 7.24 The proportion of 20:3n-6 in PBMCs. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	2.7^{b}	0.5	4.2	1.0	3.5^{ab}	0.1	2.9^{ab}	0.4	3.3	0.3
Linseed oil	3.3^{b}	0.6	5.0	1.9	5.1^{a*}	0.6	3.0^{a}	0.5	2.0	0.8
3 g FO	3.0^{b}	0.6	5.9*	1.1	1.9^{b}	1.9	2.2^{ab}	0.5	2.9	0.2
6 g FO	4.9^{a}	0.9	3.3	1.0	0.8^{b}	0.5	1.6^{b*}	0.5	3.8	0.8
9 g FO	3.4^{b}	0.3	5.1	1.1	2.1^{b}	0.8	3.6^{a}	0.6	4.0	0.9

Table 7.25 The proportion of 20:4n-6 in PBMCs. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	18.0	1.2	17.4	0.7	16.4^{ab}	0.1	15.0	0.6	13.2	1.3
Linseed oil	15.7	1.0	15.9	1.4	13.8^{ab}	1.0	15.4	0.7	14.7	0.4
3 g FO	14.9	0.7	13.9	0.9	16.9^{a}	2.1	15.6	0.9	14.2	1.1
6 g FO	15.3	0.7	17.3	2.3	13.2^{b}	0.3	15.7	0.8	12.6*	0.4
9 g FO	16.9	0.5	14.7	1.1	16.4^{a}	1.1	16.7	0	14.3*	0.6

7.4.6.7 Proportion of 20:5n-3 in PBMC total lipids (Table 7.26)

At baseline the group that was to be supplemented with 6 g FO/day had a significantly increased proportion of 20:5n-3 compared to the groups that were to be supplemented with the placebo oil or linseed oil. There were no significant differences between the groups at any other time in the study. After one month of the study, the group supplemented with the placebo oil had significantly lower proportions of 20:5n-3 present and the group supplemented with 3 g FO/day had significantly higher proportions of 20:5n-3 present in PBMCs compared to baseline values. After two months on the study, the group supplemented with 9 g FO/day had significantly decreased proportions of 20:5n-3 present compared to baseline, which is quite suprising. This difference also occurred at washout. After three months on the study, the groups supplemented with 6 g FO/day had significantly lower proportions of 20:5n-3 present compared to baseline. This difference was also present at washout.

7.4.6.8 Proportion of 22:5n-3 in PBMC total lipids (Table 2.27)

At baseline the group which was to be supplemented with 6 g FO/day had a significantly increased proportion of 22:5n-3 present compared to the groups which were to be supplemented with 3 g and 9 g FO/day. After one month on the study, the group supplemented with 3 g FO/day had significantly increased 22:5n-3 present compared to 6 g FO/day, and the 6 g FO per day and 9 g FO/day had significantly decreased in the proportions of 22:5n-3 present compared to baseline. There were no significant differences between the groups after two months on the study, however, the group which had been supplemented with linseed oil and 6 g FO/day had significantly less 22:5n-3 compared to baseline. After three months on the study the group supplemented with linseed oil had significantly less 22:5n-3 present compared to the group supplemented with 6 g FO and 9 g FO/day. The groups supplemented with the placebo oil, linseed oil and 9 g FO/day had a significantly decreased proportion of 22:5n-3 compared to baseline. At washout, the group which had been supplemented with 6 g FO/day had significantly more 22:5n-3 present compared to the group which had been supplemented with 9 g FO/day. The proportion of 22:5n-3 present in both of these groups was significantly different from the amount present at baseline.

7.4.6.9 Proportion of 22:6n-3 in PBMC total lipids (Table 7.28)

There were no significant differences between the groups at baseline. After one month on the study however, the group supplemented with 9 g FO/day had a significantly increased proportion of 22:6n-3 present compared to the groups supplemented with 6 g FO/day, linseed oil or placebo oil. The proportion of 22:6 present in the groups supplemented with 9 g FO/day or 3 g FO/day was significantly increased from the baseline values. There were no significant differences in the proportions of 22:6 present in the PBMCs between the groups after two months on the study. There were also no significant differences in the proportions of 22:6n-3 present in PBMCs compared to baseline values. After three months on the study, the groups supplemented with 6 g FO and 9 g FO/day had significantly increased proportions of 22:6n-3 present in PBMCs compared to the placebo oil and 3g FO supplemented groups. There were no significant changes in 22:6n-3 compared to baseline however. At washout the groups which had been supplemented with linseed oil and 6 g FO/day were significantly different. Again there were no significant differences compared to baseline however.

7.4.6.10 Effect of supplementation on ratio of n-6 to n-3 fatty acids in PBMC total lipids (Table 7.29)

There were no significant differences between groups in the ratio of n-6 to n-3 fatty acids in the PBMCs at baseline. After one month on the study however, the group supplemented with the placebo oil was significantly different from the group supplemented with 3 g FO/day, and the group supplemented with 6 g FO/day had increased significantly compared to baseline values. After two months of supplementation however there were no significant differences between the groups and compared to baseline values. After three months of supplementation, the groups which had been supplemented with the placebo and linseed oils had significantly increased ratios of n-6:n-3 fatty acids present in their PBMCs compared to baseline values, although there were no significant differences between the groups. At washout there were also no significant differences in the ratio of n-6:n-3 fatty acids in the PBMCs compared to baseline values, although the group which had been supplemented with the linseed oil had a significantly greater ratio present compared to the group which had been supplemented with 6 g FO/day.

Table 7.26 The proportion of 20:5n-3 in PBMCs. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	3.1^{b}	0.5	2.3*	0	2.5	0	2.0	0.3	3.1	0.5
Linseed oil	3.2^{b}	0.2	3.2	1.6	3.2	0.3	2.0	0.2	2.0	0.1
3 g FO	3.0^{ab}	0.4	4.6*	1.3	3.0	1.0	1.8	0.2	2.4	0.3
6 g FO	4.2^{a}	0.4	3.9	1.5	2.3	0.5	2.2*	0.2	3.1*	0.4
9 g FO	3.2^{ab}	0.2	2.2	0.5	1.6*	0.2	2.0	0.3	2.1*	0.2

Table 7.27 The proportion of 22:5n-3 in PBMCs. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	2.8^{ab}	0.3	1.4^{ab}	0.5	0	0	0.6^{ab*}	0.4	2.5^{ab}	0.2
Linseed oil	3.0^{ab}	0.4	1.8^{ab}	0.2	1.1*	0.7	0.2^{b*}	0.2	2.3^{ab}	0.2
3 g FO	2.6^{b}	0.5	2.6^{a}	1.3	0.5	0.5	1.1^{ab}	0.4	2.5^{ab}	0.4
6 g FO	3.7^{a}	0.3	0.3^{b*}	0.3	0.1*	0	1.5^{a}	0.6	2.6^{a*}	0.3
9 g FO	2.6^{b}	0.2	0.9^{ab*}	0.4	1.3	0.5	1.6^{a*}	0.4	1.4^{b*}	0.6

Table 7.28 The proportion of 22:6n-3 in PBMCs. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	3.2	0.2	3.2^{b}	0.6	2.6	0.8	2.4^{b}	0.3	5.0^{ab}	0.6
Linseed oil	2.6	0.2	3.7^{b}	0.8	2.1	0.9	3.3^{ab}	0.2	3.3^{b}	0.5
3 g FO	2.6	0.5	4.8^{ab}*	0.5	2.7	0.1	2.5^{b}	0.2	4.2^{ab}	0.5
6 g FO	3.0	0.6	3.8^{b}	0.5	3.2	1.3	4.1^{a}	0.6	5.3^{a}	0.8
9 g FO	2.9	0.3	5.8^{a}*	0.6	2.2	0.3	4.3^{a}	0.7	4.6^{ab}	0.6

Table 7.29 The PBMC n-6:n-3 fatty acid ratio over all study time points. Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	3.1	0.4	4.0^{b}	0.5	5.3	0.7	5.1*	0.4	2.3^{ab}	0.4
Linseed oil	3.0	0.3	3.5^{ab}	0.6	4.3	0.9	4.9*	0.4	3.1^{a}	0.3
3 g FO	3.1	0.3	2.5^{a}	0.5	4.7	0.7	5.0	0.5	2.7^{ab}	0.4
6 g FO	2.4	0.3	3.7^{ab}*	0.5	4.9	0.8	3.7	0.7	2.1^{b}	0.2
9 g FO	3.1	0.2	3.0^{ab}	0.4	5.1	0.6	3.8	0.9	2.9^{ab}	0.3

7.4.7 The effects of n-3 supplementation on inflammatory mediator production by PBMC

7.4.7.1 TNF-α (Tables 7.30 & 7.31)

Supplementation with n-3 fatty acids had no significant effect on the production of TNF-α by PBMC either when the cells were stimulated with LPS (table 7.30) or when the cells were unstimulated (table 7.31). When the data were analysed as percent change from baseline there were no significant differences at any time point of the study, in either the LPS stimulated or unstimulated groups (data not shown).

7.4.7.2 IL-1β (Tables 7.32 & 7.33)

When the PBMC were stimulated with LPS, supplementation with 9 g FO/day caused an increase in IL-1β production compared to placebo values after one month of feeding. There was no significant difference in the production of IL-1β between the groups after two or three months supplementation, although after two months supplementation cell supernatants from the subjects in the 9 g FO/day group tended to have higher concentrations of IL-1β compared to the other groups, while after three months supplementation the cell supernatants from the 6 g and 9 g FO/day groups tended to have the lowest concentration of IL-1β. Two months after supplementation had stopped, cell supernatants from the group which had been given 9 g FO/day had significantly lower concentrations of IL-1β compared to all other groups. When IL-1β production was compared as percentage change from baseline, there were no differences between the groups at any time in the study.

There was no change in the amount of IL-1β in the supernatants of unstimulated cells at any time in the study (table 7.33). There was however a change in the amount of IL-1β produced during the washout period when the values were examined as a percent change from baseline. When the data were examined in this way then the cells from subjects given the placebo capsule produced significantly higher concentrations than all the other groups. There was no significant difference at any other time in the study.

Table 7.30 TNF-α production by LPS stimulated PBMC (absolute values in pg/ml). Data are means and se for n=6-8 samples per group. Numbers not sharing the same alphabetic subscript across a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	4648	906	5459	996	4540	328	5900	803	6929	1705
Linseed oil	5294	669	7500	704	4951	278	6600	770	6405	816
3 g FO	4429	320	6929	491	4551	469	5459	717	5701	620
6 g FO	5282	782	7865	1390	4522	793	5619	715	4822	1077
9 g FO	4809	690	5926	770	5786	469	6916	942	5860	1156

Table 7.31 TNF-α production by unstimulated PBMC (absolute values in pg/ml). Data are means and se for 6-8 samples per group. Numbers not sharing the same alphabetic subscript across a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	559	218	7*	6	368	308	157	74	112	69
Linseed	849	116	14*	7	67*	33	535	354	273	188
3 g FO	637	220	6*	6	85*	37	140	61	133*	73
6 g FO	842	298	41*	41	36*	22	57*	42	120*	59
9 g FO	602	197	0*	0	318	17	112	86	33	32

Table 7.32 IL-1β production by LPS stimulated PBMCs (absolute values in pg/ml). Data are mean and se of absolute values of 6-8 subjects per group. Groups not sharing the same alphabetic subscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	5938	1318	6084[a]	954	5923	1031	8274	970	7451[a]	2425
Linseed	8366	1389	7761[ab]	804	4964	647	7462	934	6899[a]	1067
3 g FO	5938	1317	7842[ab]	1344	5109	1300	8267	1923	6076[ab]	794
6 g FO	6893	720	8433[ab]	963	5900	721	6573	747	7566[a]	914
9g FO	6470	916	9733[b]	1274	6353	1344	6086	654	3332[b]*	419

Table 7.33 IL-1β production by unstimulated PBMCs (absolute values in pg/ml). Data are mean and se of absolute values of 6-8 volunteers per group. Groups not sharing the same alphabetic subscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	257	140	0	0	231	207	51	32	45	27
Linseed	413	121	103*	102	10	6	221	170	181	108
3 g FO	374	127	1*	1	12	9	37*	16	72*	66
6 g FO	448	144	4*	3	10*	5	17*	8	64	40
9 g FO	511	304	0*	1	28	14	30	25	5	5

7.4.7.3 IL-6 (Table 7.34 & Tables 7.35)

LPS-stimulated cells from subjects fed 6 g FO/day produced significantly lower concentrations of IL-6 after 3 months of supplementation. This was significantly different from the placebo, linseed and 3 g FO/day groups. LPS-stimulated PBMC from the 9 g FO/day group also produced lower concentrations of IL-6 although this decrease was not significantly different from the other groups, perhaps because of the large standard error in this group compared to the other groups. When the data were examined as a percentage change from baseline, it was found that after three months of supplementation, PBMC from subjects in the 3 g FO/day group produced significantly higher concentrations of IL-6 than PBMC compared with subjects fed the 6 g FO/day. There were no other significant changes at any other time in the study when the data were examined in this way.

Unstimulated PBMC from subjects given 9 g FO/day had significantly lower IL-6 production during the washout period compared to subjects given the linseed oil capsules. There were no significant changes in IL-6 production by cells from subjects in any of the other dietary groups, although unstimulated cells from subjects given 6 g FO/day for three months had tended to have decreased concentration compared to the other groups. There was no significant difference in the concentration of IL-6 produced by cells from subjects from any of the supplemented groups when the data were examined as percent change from baseline values of IL-6.

7.4.7.4 PGE_2 (Tables 7.36 & 7.37)

There were no significant differences between the groups in the concentration of PGE_2 present in the supernatants of LPS-stimulated PBMC at any time in the study. However, there were some significant changes in the production of PGE_2 compared to baseline values throughout the study. After two months of supplementation, the groups which had been given linseed oil or 9 g FO/day showed significantly lower PGE_2 production than at baseline. The decrease was still apparent after three months supplementation for these two groups and also for the 6 g FO/day group. At washout however, only the groups which had received the placebo, linseed oil, and 6 g FO/day were significantly lower than at baseline. There were no significant differences

between the groups in the concentration of PGE_2 present in the supernatants of unstimulated PBMC. However, one month after supplementation with the capsules the amount of PGE_2 produced by the unstimulated cells had decreased significantly compared to baseline in all the groups. After two and three months supplementation however, this significant decrease in PGE_2 production compared to baseline was only apparent in the linseed oil and 6 g FO/day groups. At washout the groups which had been supplemented with linseed oil, 3 g FO/day and 6 g FO/day were still significantly lower compared to baseline. However, when the data were analyzed as a percentage change from baseline values there were no significant differences between the groups.

7.4.7.5 IL-2 (Tables 7.38)

IL-2 production differed between the groups at baseline with cells from subjects in the group who were to receive the linseed oil supplement producing significantly higher concentrations than the cells from subjects who were to receive 6 g FO/day and 9 g FO/day. After 2 months supplementation, supernatants from stimulated cells from subjects receiving the two highest doses of fish oil had significantly lower concentration of IL-2 present compared to supernatants from stimulated cells from subjects receiving the other supplements, although the values for the 9 g FO/day and 6 g FO/day groups were not different from their baseline values. After 3 months supplementation, the supernatants from the stimulated cells from subjects receiving 9 g FO/day still had significantly lower concentrations of IL-2. This was significantly different from the amount of IL-2 in the supernatants from cells from subject receiving the linseed oil capsules although not different from baseline values. When the data were analyzed as percentage change from baseline, supernatants from cells from subjects receiving 9 g FO/day had significantly higher IL-2 concentrations compared to supernatants from cells from volunteers in the low dose fish oil group after three months supplementation. There were no other changes at any other time point. Unstimulated PBMCs did not make detectable levels of IL-2.

Table 7.34 IL-6 production by LPS stimulated PBMCs. Data are mean and se of absolute values of 6-8 samples per group. Groups not sharing the same alphabetic subscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	51089	4546	53082	8052	36066	4813	44913^{b}	5837	30273	10369
Linseed	59052	7314	61400	7719	42836*	4398	44984^{b}	8813	36648*	4955
3 g FO	52913	6001	59674	10808	40271	4952	49653^{b}	7572	37018	5556
6 g FO	49707	4782	49897	5749	31494*	5835	24644^{a}*	4501	38575*	5104
9 g FO	50661	7214	53923	5503	39965	4332	34617^{ab}	2553	29001	11840

Table 7.35 IL-6 production by unstimulated PBMCs. Data are mean and se of absolute values of 6-8 samples per group. Groups not sharing the same alphabetic subscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	2192	436	121*	34	1337	492	1364	503	1333^{ab}	590
Linseed	2352	247	273*	112	908*	313	1552	514	2301^{a}	946
3 g FO	2160	285	235	119	1080*	331	1327	413	1374^{ab}	316
6 g FO	2156	462	271*	229	937*	402	758	351	1082^{ab}*	329
9 g FO	2144	258	137	68	928	281	1279	399	466^{b}	125

Table 7.36 PGE_2 production by LPS- stimulated PBMCs (absolute values in ng/ml). Data are means and se of 6-8 per group. Groups not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	133	24	134	19	120	30	104	23	86*	41
Linseed	141	25	135	19	85*	15	68*	12	80*	20
3 g FO	132	21	153	29	93	14	94	16	87	14
6 g FO	112	21	111	26	91	22	63*	21	72*	16
9 g FO	158	34	119	31	83*	22	76*	22	86	26

Table 7.37 PGE_2 production by unstimulated PBMCs (absolute values in ng/ml). Data are means and se of 6-8 per group. Groups not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	13	8	5*	1	10	2	8	2	8	2
Linseed	16	2	6*	1	8*	2	9*	3	9*	3
3 g FO	19	5	5*	1	10	2	9	2	8*	1
6 g FO	14	3	3*	1	7*	2	6*	1	6*	1
9 g FO	14	3	4*	1	8	1	9	1	8	2

7.4.7.6 IFN-γ (Table 7.39)

There were no significant change in the concentration of IFN-γ in the cell supernatants from any time in the study. There was also no significant differences in the concentration of IFN-γ in the cells supernatants between the groups when the data were analyzed as a percent change from baseline IFN-γ production. There were significant changes however when the values were compared to baseline values. After one month of supplementation, IFN-γ production by lymphocytes from subjects taking FO capsules were significantly higher after one month supplementation compared to baseline. After two months of supplementation, however this increase was only apparent for cells from the 9 g FO/day group. The production of IFN-γ was significantly higher from the cells from the subjects given the placebo oil compared to baseline after two and three months of supplementation. At the washout, IFN-γ production was significantly higher compared to baseline only by cells from the subjects given 6 g FO/day. Unstimulated cells did not produced detectable amounts of IFN-γ.

7.4.7.7 IL-4 (Table 7.40)

There were no significant differences between any of the groups in the concentration of IL-4 present in the supernatants of Con-A stimulated PBMC at any time in the study. After one month supplementation however, the groups which had received 3g FO/day and 9g FO/day had significantly greater IL-4 concentration than at baseline. The amount produced by the 6g FO/day group had also doubled compared to baseline. However, there were no changes when the data were analyzed as percentage change from baseline. Unstimulated PBMCs did not produce detectable concentrations of IL-4.

7.4.7.8 IL-10 (Table 7.41)

There were no significant differences in the concentration of IL-10 present in the supernatants of Con-A stimulated lymphocytes from subjects in the various groups at any time in the study. There were also no changes when the data was analyzed as

percentage change from baseline values. Unstimulated cells did not produce detectable levels of IL-10.

7.4.8 Th-1:Th2 ratio

There were no significant changes in the ratios of IL-2/IL-4 or IL-2/IFN-γ or the ratio of (IL-2 +IFN-γ) / (IL-4 + IL-10) produced by the stimulated cells at any time point in the study.

7.4.9 Lymphocyte proliferation in response to Con-A (Tables 7.42 & 7.43)

Tables 7.42 and 7.43 show lymphocyte proliferation by PBMC expressed either as a ratio of the maximum stimulation index (determined over a range of con-A concentrations), or the absolute values of thymidine incorporation at 25 μg/ml con-A. When the data were expressed as the a maximum stimulation index (table 7.42), there were significant differences in proliferation between the groups at baseline, with the two high dose FO groups having lower stimulation indices compared to the other groups. There were no significant differences in the ability of con-A stimulated lymphocytes from subjects in the various groups to proliferate at any time during the supplementation period. After one month supplementation, cells from the subjects given 6 g or 9 g FO/day had a significantly higher MSI value compared to baseline. When the data were compared as percent changes from baseline values, after three months supplementation, the 9 g FO/day group was significantly higher than the placebo and linseed oil groups (data not shown). Table 7.43 shows the absolute values of thymidine incorporation for PBMC stimulated with 25 μg/ml con-A. When the data were expressed in this way, the groups which had been given the FO had significantly lower proliferation after three months supplementation and during washout compared to baseline. There were no significant differences between the groups at any point in the study except at washout where the group which had been given the placebo had significantly lower proliferation compared to the linseed oil and 9 g FO/day groups. This was also significantly lower compared to baseline.

Table 7.38 IL-2 production by con-A stimulated PBMCs (absolute values in international units/ml). Data are mean and Se of absolute values of 6-8 volunteers per group. Groups not sharing the same alphabetic subscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values. un-stimulated cells made no detectable levels of IL-2

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	10^{ab}	2	15	5	12^{ab}	4	13^{ab}	5	8	3
Linseed	12^{a}	2	17	2	14^{a}	3	14^{a}	2	12	2
3 g FO	11^{ab}	2	16*	3	8^{ab}*	1	13^{ab}	2	10	3
6 g FO	6^{b}	1	11*	2	6^{b}	1	8^{ab}*	1	6	1
9 g FO	7^{b}	1	11*	2	7^{b}	1	7^{b}	1	9	3

Table 7.39 IFN-γ production by con-A stimulated PBMC (absolute values in international units/ml). Data are mean and se of absolute values of 6-8 per group. Groups not sharing the same alphabetic subscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values. IFN-γ was not produced at detectable levels by unstimulated cells.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	173	66	252	79	271*	83	253*	89	225	62
Linseed	154	28	266	65	217	30	183	41	208	51
3 g FO	195	37	318*	77	207	55	197	60	243	57
6 g FO	160	50	261*	70	251	80	163	31	273*	72
9 g FO	149	52	278*	59	229*	61	199	43	213	60

Table 7.40 IL-4 production by con-A stimulated PBMCs (absolute values in pg/ml). Data are means and se of 6-8 per group.Numbers not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values. Unstimulated lymphocytes made no detectable levels of IL-4

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	11	2	15	5	12	3	9	3	11	6
Linseed	19	9	16	4	14	3	14	4	8	1
3 g FO	16	6	30*	10	22	8	18	6	16	6
6 g FO	9	2	18	7	14	3	11	4	10	4
9 g FO	11	4	21*	7	12	5	14	6	17	6

Table 7.41 IL-10 production by con-A stimulated PBMCs (absolute values in pg/ml). Data are mean and se of absolute values of 6-8 volunteers per group. Groups not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values. Un-stimulated lymphocytes made no detectable levels of IL-10

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	246	27	329	55	234	39	268	50	191	25
Linseed	284	73	290	58	286	60	329	100	295	76
3 g FO	349	187	493	80	360	42	271	40	317	48
6 g FO	260	55	332	62	335	71	299	62	247	37
9 g FO	309	45	445	100	343	88	412	104	353	103

Table 7.42 Proliferation of PBMCs (MSI) Data are means and se of a maximum stimulation index. Numbers represent 8-6 samples per group. Groups not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	139[a]	32	115	22	145	23	84	28	92	23
Linseed	154[a]	19	126	28	231	56	124	38	123	24
3 g FO	139[a]	32	146	30	191	30	148	26	181	63
6 g FO	85[b]	9	123*	16	146	30	91	14	67	13
9 g FO	98[b]	11	189*	35	173	65	167	49	108	16

Table 7.43 Thymidine incorporation (cpm/well) into PBMCs cultured with con-A (25 μg/ml) . Data are means and se of cpm. Numbers represent 6-8 samples per group. Groups not sharing the same alphabetic superscript within a column are significantly different. Numbers with an asterisk indicate significantly different from average baseline values.

	Average baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	41286	7277	49824	5470	44188	4919	32170	5058	24617[b]*	3655
Linseed	46757	5410	61444	4003	52716	5284	37720	2612	41075[a]	4664
3 g FO	54022	4647	59015	6251	53085	3180	37024*	1932	35395[ab]*	4857
6 g FO	47937	4049	55835	5950	50558	4630	29004*	4204	34688[ab]*	4250
9 g FO	50546	5379	54986	4976	48272	4951	33581*	7618	40593[a]*	4485

7.4.10 Plasma TBARS and Triacylglycerols

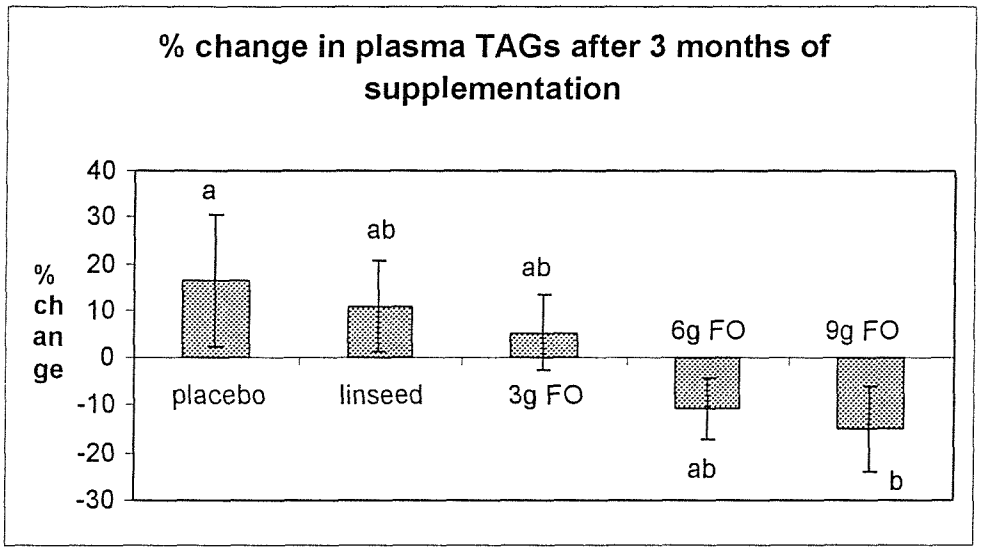
7.4.10.1 TBARS (Table 7.44)

Table 7.44 shows plasma TBARS concentrations over the study period. Plasma TBARs is an index of lipid peroxidation. After one month the group supplemented with 3 g FO/day had significantly lower plasma TBARs compared to the group supplemented with 6 g FO/day, and compared to their baseline. There was still a significant decrease compared to baseline measurements in the 3 g FO/day group after two months supplementation, although the differences in TBARs concentrations were no longer apparent between the different capsule groups. After 3 months of supplementation with the capsules there were no significant differences between the groups or compared to baseline measurements. At washout however, plasma TBARs were significantly lower in the groups that had been given 6 g or 9 g FO/day compared to the 3 g FO/day group, and also compared to baseline measurements. When the data were compared as percent change from baseline, the group supplemented with 3 g FO/day had significantly decreased TBARs compared to the groups supplemented with the placebo, linseed oil, or 6 g FO/day after one month of supplementation. There were no significant differences between any of the groups at any of the other study timepoints however.

7.4.10.2 Triacylglycerol (Table 7.45)

There were no significant differences in the concentrations of plasma TAGs in any of capsule groups at any time point in the study when the data were analysed as absolute concentration (table 7.45). However, when the data were analysed as percent change from baseline, supplementation of the diet with 6 g or 9 g FO/day for one month caused a significant decrease in the concentrations of TAGs present in the plasma compared to supplementation with the placebo oil. This decrease was still apparent after two months of supplementation, but was no longer significant. Three months of supplementation with 9 g FO/day however caused a significant decrease in plasma TAGs compared to the placebo oil group (figure 7.3). There was a clear dose-response relationship between the percentage decrease in plasma TAGs and the amount of FO given (see figure 7.3)

Figure 7.3 percent change in plasma TAGs after 3 months of supplementation. Data are means and standard errors for 8 samples per group. Columns not sharing the same alphabetic superscript are significantly different.



7.4.10.3 Ratio of TBARS:TAG (Table 7.46)

The concentration of TBARs in the plasma is strongly dependent on the overall concentrations of plasma lipids. For this reason, the ratio of plasma TBARs to plasma TAGs was calculated in order to examine whether the (non-significant) decline seen in plasma TBARs in the two high dose FO groups at the end of the study was due to decreased peroxidation or to declining concentrations of plasma lipids with these capsules. When the data were examined in this matter, there were no significant differences between the capsules groups at any time. There was also no significant difference from baseline measurements for any of the groups at any time in the study.

Table 7.44 Plasma TBARS (μmol/L). Data are means and se of 6-8 per group Numbers not sharing the same alphabetic subscript within a column are significantly different. Numbers with a asterisk indicate significantly different from average baseline values within a capsule group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	4.7	0.5	4.5^{ab}	0.4	4.5	0.5	4.3	0.3	4.1^{ab}	0.3
Linseed oil	4.9	0.4	5.2^{ab}	0.5	4.6	0.3	4.2	0.4	4.4^{ab}	0.3
3 g FO	5.7	0.3	4.1^{a}*	0.3	4.7*	0.3	4.8	0.4	4.9^{b}	0.4
6 g FO	4.8	0.5	5.4^{b}	0.4	4.7	0.3	4.2	0.4	3.7^{a}*	0.7
9 g FO	5.2	0.3	5.0^{ab}	0.3	4.8	0.5	4.2	0.4	3.9^{a}*	0.4

Table 7. 45 Plasma TAG concentrations (mmol/L). Data are means and se of 6-8 per group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	1.1	0.2	1.2	0.2	1.0	0.1	1.2	0.3	1.4	0.4
Linseed oil	1.0	0.2	1.1	0.2	0.9	0.1	1.2	0.4	0.9	0.2
3 g FO	1.1	0.2	1.1	0.2	0.9	0.1	1.2	0.3	1.1	0.3
6 g FO	1.3	0.4	1.2	0.3	1.1	0.3	1.1	0.3	1.0	0.2
9 g FO	1.0	0.1	1.0	0.2	0.9	0.2	0.9	0.4	1.0	0.1

Table 7.46 TBAR:TAG ratio (μmol/mmol). Data are means and se of 6-8 per group.

	Average Baseline	*Se*	*One month*	*Se*	*Two months*	*Se*	*Three months*	*Se*	*Washout*	*Se*
Placebo	5.3	1.1	4.8	1.1	4.6	1.0	4.8	1.2	3.5	0.5
Linseed oil	5.8	0.7	4.8	1.2	4.5	0	5.5	0.9	5.9	0.8
3 g FO	6.0	1.2	5.4	1.0	5.9	0.9	5.8	1.0	6.2	0
6 g FO	4.6	0.9	2.2	0.3	5.4	0.9	4.6	2.0	5.0	0.9
9 g FO	5.8	0.6	4.9	0.7	5.5	0.9	6.8	1.1	6.3	1.2

7.4.11 Correlations between cytokine production and specific fatty acids in PBMCs

Table 7.47 shows the significant correlations which occurred between PBMC cytokine production and the proportions of fatty acids present in the PBMC. In general, n-3 fatty acids correlated negatively with PGE_2 and IL-4 production, and positively with IFN-γ, IL-6 and IL-10 production. N-6 fatty acids correlated negatively with IL-10 and positively with IL-6 and IL-2. Monounsaturated fatty acids correlated negatively with IL-10 production by these cells. Lymphocyte proliferation expressed as a maximum stimulation index correlated negatively with the proportion of 18:0 present in PBMCs (r=-0.1, p=0.03).

Table 7.47 Spearman correlation coefficients between the proportion of particular fatty acids in PBMC and production of mediators by those cells.

Mediator	*Fatty acid*	*r*	*P*
PGE_2	22:5n-3	-0.22	0.002
PGE_2	22:6n-3	-0.15	0.04
TNF-α	18:2n-6	0.20	0.007
IFN-γ	22:6n-3	0.16	0.03
IL-2	20:4n-6	0.16	0.06
IL-4	22:6n-3	-0.14	0.05
IL-6	16:0	-0.18	0.02
IL-6	20:4n-6	0.15	0.04
IL-6	22:5n-3	0.26	0.000
IL-10	18:1n-9	-0.23	0.002
IL-10	20:4n-6	-0.15	0.04
IL-10	22:6n-3	0.16	0.03

7.5 Discussion

The current study involved planning and executing a double-blind dose-response study on the effects of fish oil supplements (3, 6, 9 g/day) given to healthy male volunteers on inflammatory mediator production and lymphocyte proliferation. Comparisons to a placebo (a mix of soybean/palm oil providing a mixture of fatty acids characteristic of the typical UK diet) and to linseed oil (4 g/day α-linolenic acid) were made. The duration of the study was 8 months. Subjects gave 6 blood samples, one per month. There were two baseline time points and a two month washout period at the end of the study. Subjects were asked to take their respective dietary supplements for 3 months and were monitored for frequency of illness, change in weight and BMI, blood pressure, exercise levels, and habitual nutrient intake. Blood mononuclear cells were stimulated with either LPS or Con-A or left unstimulated and the production of inflammatory mediators in the culture medium assessed after 24 hours. TNF-α, IL-1β, IL-6, PGE_2, IL-2, IL-4, IFN-γ, IL-10 were measured by ELISA. Fatty acid incorporation into the mononuclear cells and into plasma phospholipids was assessed by gas chromatography.

7.5.1 Subjects's habitual diets

The percentage DRV for energy reported in the subjects food diaries was 101%. This is a good indication that the subjects reported their food intakes in a reliable manner. This is also borne out by the fact that the nutrient intakes of most of the subjects were within the normal ranges of intakes for men of this age group (COMA, 1992) which indicates that the population of healthy men participating in the current study had diets which were representative of the general UK population. This was especially true for the fatty acids. For instance, the daily average intake of n-6 fatty acids was identical to the national average for men of this age groups (14.2 g/day) while the daily average intake of total n-3 fatty acids (1.5 g/day) was slightly lower than the national average for this age group (2 g/day national average) (British Nutrition Foundation, 1999). Assessment of nutrient intakes by food diaries showed that there were no significant changes in the subject's intakes of most nutrients between the beginning and end of the study period when assessed by paired t-tests. There were significant changes in the intakes of vitamin B12, vitamin C and intakes of sulfur amino acids. These tended to be lower at

the end of the study than at the beginning, which may reflect seasonal variations. In regard to changes in vitamin C intake, many volunteers reported eating an increased number of citrus fruits during the period of time the first food diary was recorded. This diary was done just prior to the Christmas holidays. There were no significant differences in the intakes of any fatty acids over the duration of the study, when average intakes were compared by t-tests between the beginning and end of the study. There were significant differences in the intakes of fatty acids between the groups. The group which was to be supplemented with 3 g FO/day had a significantly higher intake of total n-6 fatty acids compared to the group which was to be supplemented with 9 g FO/day (data not shown). These two groups were also significantly different in total intakes of n-3 fatty acids which was accounted for in differences in intakes of 18:3n-3. Subjects in the group which was to be supplemented with 9 g FO/day had a higher intake of conjugated linoleic acid compared to the group which was to be supplemented with 6 g FO/day. There were no significant differences in intakes of 20:5n-3 and 22:6n-3 between the groups.

7.5.2 Blood pressure

Blood pressure can be defined as the pulsating pressure of blood on the walls of the main arteries: systolic pressure, or highest pressure, occurs when the heart is contracting and diastolic, or lowest pressure, when it is relaxing. Systolic and diastolic blood pressure are directly related to the risk of coronary heart disease and stroke. An overview analysis of 9 prospective studies which together followed 420,000 adults (96% men) for an average of 10 years, showed the relationship between CHD and stroke incidence and diastolic blood pressure to be linear, positive and continuous across a range of pressures studied (MacMahon *et al.*, 1990). All subjects in the current study had blood pressure at baseline within a healthy range and the average blood pressure was 124/72.

A number of studies have shown that FO can affect blood pressure (de Deckere *et al.*, 1998). Previous studies have suggested that a minimum of 3 g of FO per day may be needed for a significant reduction in blood pressure (Appel *et al.*, 1993; Morris *et al.*, 1993). The ingestion of this amount from a normal Western diet would require the consumption of about 10 portions of oily fish a week (British Nutrition Foundation,

1999). This is an unrealistic dietary goal for the overall population but it could be realized via substantial supplementation of some at risk groups. In the current study, those groups, which had been supplemented with FO at al level of 3, 6 and 9 g per day, showed a decrease in both systolic and diastolic pressure compared to baseline values after one month of supplementation. Although this decrease was not statistically significant, it was most substantial in the group consuming 9 g FO/day.

The mechanism of action of n-3 fatty acids on blood pressure may be related to their influence on the balance of the eicosanoids which constrict and dilate blood vessels such as thromboxane A_2 (TXA_2; strongly constricts blood vessels). Thromboxane A_3 (TXA_3) derived from EPA found in FO does not have as strong an effect on blood vessel constriction as do the TXA_2 derived from arachidonic acid more commonly eaten in the diet. Prostacyclin I_3 (PGI_3) derived from EPA however has been shown to be just as potent in terms of blood vessel vasodilation as PGI_2 derived from arachidonic acid (British Nutrition Foundation, 1999). Plasma TXA_2 concentrations have been shown to decreased following FO supplementation of the diet of animals (Brouard & Pascaud, 1990), and so it is possible that dietary FO may modulate the production of certain eicosanoids important in the modulation of blood pressure. It is also possible that n-3 fatty acids may be exerting their blood pressure lowering effect through the modulation of Nitric Oxide (•NO). •NO, mainly produced by endothelial cells, is a potent relaxer of blood vessels (Harris, 1996). A number of animal experiments have shown •NO production by macrophages to be increased following consumption of FO (see chapter 1 for references). In chapter 2 it was shown that elicited macrophages from mice fed a FO-rich diet produced more •NO than did mice fed a saturated fatty acid rich diet. In the human study, there were no detectable levels of •NO present in the supernatants from the LPS-stimulated PBMC cells from the subjects (results not shown). However this may be due to differences in the state of activation/differentiation of the cells under investigation, (stimulated blood monocytes vs. the differentiated elicited macrophages used in the murine study in chapter 2). Macrophages present in tissues would be in a higher state of differentiation/activation than circulating blood monocytes and would have a increased capacity to make some inflammatory mediators, some of which may be subject to the modulating effects of

fish oil. Therefore, it is possible that FO may be modulating blood pressure through augmenting the production of this mediator in certain cells of the body. Endothelial cells are known to produce •NO (Snyder & Bredt, 1992), and Harris *et al.* (1997), have reported increased urinary excretion of •NO metabolites following FO supplementation in human volunteers. The design of the Harris et al. (1997) study however, does not allow conclusions to be drawn about where in the body the •NO was produced. A study investigating the effects of dietary fish oil on the production of •NO by endothelial cells needs to be performed.

7.5.3 Incorporation of dietary lipids into plasma phospholipids

A number of studies have shown that FO supplementation of the diet leads to significant changes in the proportions of fatty acids present in plasma phospholipids (Meydani *et al.*, 1991; Allard *et al.*, 1997; Yaqoob *et al.*, 2000; Thies *et al.*, 2000). These studies show that following FO supplementation of the diet, the proportions of EPA and DHA are increased at the expense of AA and DGLA. In the current study, supplementation of the diet with 3, 6 or 9 g FO/day caused significant increases, compared to baseline values, in the proportions of EPA and DHA present in plasma phospholipids (significant for all groups after 1 month of supplementation, and still significant after 3 months of supplementation). The proportion of DPA present in plasma phospholipids also increased compared to baseline values after one month of supplementation but had returned to baseline values at three months of supplementation. The group supplemented with linseed oil also had a significant increase in the proportion of DPA present in plasma phospholipids, and this remained elevated throughout the study. EPA also increased in the plasma phospholipids of the linseed oil supplemented group, however this did not increase significantly compared to baseline until after three months supplementation.

There was a decrease in the proportion of 20:4n-6 in the plasma phospholipids in the linseed oil and the FO-supplemented groups compared to baseline values after one month of supplementation. This reached significance in the 6 g and 9 g FO/per day groups and also in the linseed oil group after two months of supplementation. There was also a decrease in 20:3n-6 present in the phospholipids of the groups supplemented with FO. This reached significance in the groups supplemented with 3 and 9 g FO/day

after two months of supplementation. This agrees with another recent study by our group (Yaqoob *et al.*, 2000), in which 9 g FO/day was given to volunteers for 3 months and with previous studies in healthy subjects (Jensen *et al.*, 1988) and in patients with psoriasis (Bjorneboe *et al.*, 1988).

7.5.4 Incorporation of dietary lipids into PBMCs

In this study the incorporation of various fatty acids into the PBMCs was examined. Total lipids (measuring both the lipids present in phospholipids and as triacylglycerols within the cell) were measured rather than phospholipids in order to maximize the amount of lipids in the assay, as the cells assessed were the remaining cells after those needed for the proliferation and cytokine analysis were taken. The incorporation of dietary fatty acids into PBMCs was not as dramatic as was observed with the incorporation into plasma phospholipids. Dietary supplementation with FO had no effect on the proportions of 16:0, 18:0 or 18:1n-9 present in these cells. The proportion of 18:2n-6 present in PBMC cells however increased in the linseed oil group compared to baseline values after three months supplementation, and tended to increase over the supplementation period in most groups compared to baseline, although in most cases not significantly. The current study agrees with a study by Caughey *et al.* (1996) in which the proportion of 18:2n-6 present in the PBMCs increased following the ingestion of linseed oil for 8 weeks. This study also showed that supplementation of the diet with linseed oil increased the proportions of EPA and DHA. Kelley *et al.* (1991) has also investigated the effects of α-linolenic acid on PBMC fatty acid composition. When healthy men consumed 6.3 % of calories from linseed oil, it was found that the proportions of EPA and DHA in blood mononuclear cells were increased. The current study does not agree with this as supplementation of the diet with linseed oil did not affect the proportions of EPA and DHA present in PBMC total lipids. In the current study, α-linolenic acid was successful in increasing the concentrations of EPA and DPA in plasma phospholipids, but this effect was not seen in PBMCs. In another study by our group of a similar design to the current study, in which linseed oil providing about 2 g α-linolenic acid/day was given to elderly volunteers, the proportions of EPA and DHA present in PBMC phospholipids did not increase (Thies *et al.*, 2000). It is probable therefore that to get the changes in plasma phospholipid EPA composition

through supplementation with linseed oil, a relatively large dose is of linseed oil providing approximately 4g α-linolenic acid/day required for a duration of at least three months.

The proportions of EPA in PBMC total lipids did not increase in the FO supplemented groups in this study. This result differs from the study by Yaqoob *et al.* (2000) in which dietary supplementation with FO caused an increase in the proportion of EPA in PBMC cells. The FO capsules in the Yaqoob *et al.* (2000) study provided a greater proportion of their long chain n-3 fatty acids as EPA rather than DHA, while the opposite is true in the current study. This might be one reason why in the current study the proportion of DHA increased in plasma total lipids, without having an effect on EPA. It may be however that the lack of an effect of FO supplementation on PBMC EPA concentrations is in part due to slightly higher intakes of 18:2n-6 in the subject's. Cleland *et al.* (1992) have found that high intakes of 18:2n-6 inhibit the incorporation of EPA into neutrophil phospholipids. In the present study the proportion of 18:2n-6 was found to increase slightly during the supplementation period (although not significantly) and return to close to baseline values during the washout period. It also appears that the proportions of EPA in the volunteers PBMC lipids at the start of the current study were higher than the proportion of EPA present in the volunteers PBMCs in the Yaqoob *et al.* (2000) study. This might have prevented further incorporation of this fatty acid into the cells despite increased dietary consumption of this fatty acid in the n-3 supplemented groups.

7.5.5 Effects of n-3 supplementation on monocyte derived cytokines

In the current study, there was no effect on the production of TNF-α by the LPS-stimulated PBMCs at any time in the supplementation study. This result agrees with the study by Blok *et al.* (1997) which examined the production of this cytokine by LPS-stimulated peripheral blood cells from healthy volunteers who had been supplemented for one year with FO. It also agrees with the Schmidt *et al.* (1996) study, which also showed no effect of a low dose of a FO supplement for three months on the production of TNF-α by PBMCs. The current study agrees also with a study by Endres *et al.* (1989) in which 18 g FO/day was given to healthy volunteers. In this study it was

found that the concentrations of TNF-α produced by mononuclear cells were not effected during the supplementation period. They did however, decrease after the end of supplementation, which contrasts with the current study. In the Endres *et al.* (1989) study, IL-1β decreased over time and continued to decrease after the end of the FO supplementation. In the current study IL-1β production also decreased over time and the decrease was still apparent two months after the end of supplementation. The reason for this long lasting effect is unclear but may relate to changes in products of arachidonic acid metabolism. PGE_2 production by PBMC cells was decreased in the linseed oil, 6 g and 9 g FO/day groups after three months of supplementation, and this effect was seen at washout in the linseed oil, 6 g FO/day and placebo oil groups. It is possible that changes in PGE_2 production might be responsible for the decreased IL-1β production seen at washout. It is also possible that changes in LTB_4 production by PBMC might account for lowered IL-1β production. Adding exogenous LTB_4 to human mononuclear cells has been shown to enhance LPS stimulated production of IL-1β (Rola-Plesczynski *et al.*, 1985). Thus, a possible mechanism for decreased IL-1β production is decreased synthesis of LTB_4 and generation of biologically less active LTB_5. This was not measured in the current study. TXA_2 production has also been shown to be decreased following FO supplementation of the diet (Caughey *et al.*, 1996). It is been suggested that TXA_2 production is a facilitator of cytokine production by human monocytes (Caughey *et al.*, 1996; 1997). It is therefore possible that modulation in the production of this eicosanoid may be in part responsible for the changes observed in IL-1β production. TXA_2 was not examined in the current study, however.

The study here contrasts in some ways with the study by Meydani *et al.* (1991) which showed decreased production of TNF-α, IL-1β and IL-6 by PBMCs from young and old women fed FO for 3 months. The comparisons in that study were made with reference to baseline and there was no placebo group or unsupplemented group to use as a comparison. In the current study IL-6 production was decreased significantly in the group supplemented with 6 g FO/day for three months compared with baseline and the linseed oil, placebo oil and 3 g FO/day supplemented groups. The Meydani *et al.* (1991) study showed that the production of these cytokines decreased over time and

that the decrease in cytokine production was greater in the older women then in the younger women. However in the current study, the production of cytokines from mononuclear cells tended to decrease in all of the supplemented groups including the placebo group. While this was not always significant, there were definite decreases compared to baseline in almost all cytokines studied. This illustrates the danger of drawing conclusions about the effect of a supplement without having the proper control groups for comparison. It may be that the decreases in cytokine production over time in all of the groups might be due to an effect of increased fat consumption in the groups. Alternately, it may be due to the effects of seasonal variations in hormones. Certain hormones known to regulate cytokine production, such as cortisol, have been shown to have seasonal variation (Walker *et al.*, 1997). The current study began in winter (late November) and ended at the beginning of summer (early June), and thus it may be changes in the production of cytokines might be due in part to seasonal variation of hormones. This could have been compensated for by running the study with differing start dates throughout the year. However, due to logistics of carrying out the current study, this could not be done.

Another difference between the current study and a number of other studies which have shown a more dramatic effect of FO supplementation on cytokine production is that this study examined the concentrations of cytokines from LPS-stimulated cell supernatants. A number of studies have examined the concentrations of cytokines present in both the intra-and extracellular environments. For example, the Meydani *et al.* (1991) study used lysed cells in the assessment of the cytokines IL-1β and TNF-α and therefore both intracellular and secreted cytokines were measured. Molvig *et al.* (1991) found FO increased intracellular IL-1β but had no effect on secretion of IL-1β, TNF-α, PGE_2 or LTB_4. There was no effect of supplementation on the production of TNF-α in the current study and LTB_4 was not assessed. The decrease in the production of PGE_2 after three months supplementation with linseed oil, 6 g and 9g FO/day in the current study agrees with Molvig *et al.* (1991) and contrasts with the study by Meydani *et al.* (1991). In the Meydani *et al.* (1991) study, supplementation of the diet with FO did not affect PGE_2 production by cells from young women of a similar age to the young men used in the current study. The Meydani *et al.* (1991) used a

radioimmunoassay (RIA) to assess these cytokines while the current study used an ELISA, and so differences in methodology or assay specificity might account for differences between the results obtained in that study and those obtained in the current study.

There were no effects of linseed oil supplementation on the production of TNF-α and IL-1β by PBMCs compared to baseline measurements. The current study contrasts with the study by Caughey *et al.* (1996) which showed a decrease in the production of TNF-α and IL-1β by PBMC after consuming a dietary supplement of linseed oil compared to PBMCs from those volunteers fed a sunflower oil based diet. However in this study ELISAs were performed on cell supernatants from lysed cells which would have resulted in the measurement of both intra- and extracellular cytokines, while in the current study only extracellular cytokines were measured. The Caughey *et al.* (1996) study also used FCS in the cell culture media rather than AP from the volunteers. In chapter 4 it was shown that culture of murine cells from mice fed a n-6 fatty acid rich diet cultured with FCS showed increased TNF-α and IL-1β production compared to cells from these mice cultured in AP while cells from mice fed an n-3 fatty acid rich diet were resistant to changes in cytokine production induced by the presence of FCS in the culture media. It is possible that the increase in cytokine production by cells from volunteers fed the n-6 rich diet observed in the Caughey *et al.* (1996) study might be have been partly due to the influence of the type of serum used in the study on cells from the volunteers fed the n-6 rich diet, and so the differences in the production of IL-1β and TNF-α production between the PBMCs from the volunteers fed the n-6 and n-3 diets may have been less dramatic if AP had been used in the cell culture media. In the current study only AP from each individual subject was used when culturing the PBMCs. This assured that the fatty acids in the culture medium reflected the actual fatty acids that the cells would be exposed to *in vivo*, and that any increases/decreases observed in cytokine production could be attributed to the effects of diet and not the confounding effects of the addition of any different fatty acids present in the serum or plasma used.

7.5.6 Lymphocyte cytokines

As discussed in chapter 1 and chapter 5, FO supplementation of the diet has been shown in a number of studies to affect the production of lymphocyte-derived cytokines. The production of cytokines within individual is genetically determined according to polymorphisms in the promoter regions of the genome that control transcription of the cytokine gene, or in the cytokine gene itself. The cytokines measured in current study have all been described as polymorphisms in their promoter regions (Di Giovine *et al.*, 1992; Epplen *et al.*, 1994; Eskdale *et al.*, 1997; Wilson *et al.*, 1992; Pociot *et al.*, 1997). This results in individual variations in cytokine production changes of which might be partially compensated for by examining changes from baseline measurements. In the current study, the concentration of IL-2 present in cell supernatants decreased in the 6 g and 9 g FO/day groups compared to baseline values after two and three months of supplementation when a comparison was made to the linseed oil group. However, when the data were analyzed with reference to baseline values, supernatants from cells from subjects receiving 9 g FO/day were not different from baseline, and cells from subjects receiving 6 g FO/day had elevated levels of IL-2 compared to baseline. It could therefore be interpreted that FO supplementation with 9g FO/day had no effect on IL-2 production (if a comparison is made at three months supplementation from the change from baseline), or that it decreases cytokine production (if a comparison is made with other groups). The importance of including controls in dietary studies is illustrated by the results obtained for this cytokine. The danger of mis-interpretation of results if the proper controls are not in place is illustrated in the study by Meydani *et al.* (1991) which examined the effects of a FO supplement on the production of cytokines by PBMC from younger and older women. While there were significant differences in IL-2 production by lymphocytes from young and older women during fish oil supplementation, there were actually no differences compared with the initial baseline measurements for either group. Thus, fish oil supplementation did not affect the concentrations of IL-2 produced by lymphocytes from either young or older women as claimed by the authors. Another criticism of this study is a bioassay was used to measure concentration of IL-2 in the culture medium, which also contained 10% (v/v) AP. The type of serum or plasma in the culture medium has a significant effect on the IL-2 bioassay (Yaqoob & Calder, 1995a) but this was not accounted for in the Meydani *et al.* (1991) study. It is possible

that the apparent decrease in IL-2 production was in fact due to an effect of plasma from fish oil-supplemented subjects on the target cell line.

In the current study, there were no significant effects of FO supplementation on the production of IFN-γ between the groups at any time in the study, although there were changes in the groups with respect to baseline measurements. For instance, the groups supplemented with FO had significantly elevated levels compared to baseline measurements after one month of supplementation. After two months of supplementation, the groups supplemented with the placebo oil and 9 g FO/day had significantly elevated concentrations of IFN-γ compared to baseline. After three months of supplementation, only the group supplemented with the placebo oil had significantly higher levels of IFN-γ compared to baseline, and at the washout, only the group which had been supplemented with 6 g FO/day had higher concentrations of IFN-γ compared to baseline. Very few studies have investigated the effects of dietary FO supplementation on the production of IFN-γ although Gallai *et al.* (1993) have shown decreased production following supplementation for 24 weeks. This study examined IFN-γ production in a con-A stimulated whole blood cultures, and there was no unsupplemented or control oil group for comparison, so the decrease observed in the Gallai *et al.* (1993) study might be an effect of time. In agreement with the current study, a recent study by Yaqoob *et al.* (2000) has shown no effect of FO supplementation for twelve weeks on IFN-γ production by con-A stimulated PBMCs and whole blood cell cultures. Another very recent study by our group (Thies *et al.*, 2000) has also shown no effect on IFN-γ production following supplementation with 6g FO/day in elderly subjects.

In the current study there was also no effect of FO supplementation on the production of IL-10 by con-A stimulated PBMC. This also agrees with the results of Yaqoob *et al.* (2000). The effects of FO supplementation of the human diet on the production of IL-4 have never been examined previously. This was done in the current study for the first time. After one month of supplementation the groups given 3 g and 9 g FO/day did have significantly elevated concentrations compared to baseline values. This increase from baseline values was not seen in any of the other groups, or at any other point in

the study. Between the groups there were no differences in IL-4 production at any time in the study. The results obtained for IL-4 in the current study agree with the results obtained for IL-4 in the murine studies reported in chapter 5 and 6. Supplementation of the murine or human diet with n-3 fatty acids, either as FO or as DHA appears to have little overall effect on the production of IL-4 by con-A stimulated PBMC or lymphocytes.

7.5.7 Lymphocyte proliferation

The effects of dietary n-3 fatty acids on proliferation of con-A stimulated animal lymphocytes are reviewed extensively in chapters 1 and 5. A great number of human studies have examined the effect of dietary fish oil on the proliferation of con-A simulated PBMCs (Meydani *et al.*, 1991;Molvig *et al.*, 1991; Meydani *et al.*, 1993; Endres *et al.*, 1993; Kelley *et al.*, 1998; 1999; Yaqoob *et al.*, 2000). These studies have shown FO supplementation either decreases in proliferation or has no effect on proliferation (see table 1.17 for references). In the current study, no effect of FO supplementation was seen between the groups at any time in the study when the data were expressed as maximum stimulation index (MSI) (table 7.42). However, when the data were examined in relation to change from baseline, the groups supplemented with 6 g and 9 g FO/day had significantly increased proliferation compared to baseline after one month of supplementation. No effect was seen at any other time in the study. When the data was analysed as thymidine incorporation, there were no differences between any of the groups during the supplementation period. At washout, however, the group supplemented with 9 g FO and linseed oil had significantly higher thymidine incorporation compared to the placebo oil group. When the data were analysed in comparison to baseline values, three months supplementation with FO caused a significant decrease in lymphocyte proliferation. This affect was still seen during the washout period. It could be claimed therefore that FO supplementation for three months leads to decreased proliferation (thymidine incorporation compared to baseline values) or has no effect on proliferation (comparisons between groups using either MSI values or thymidine incorporation values). If the values are interpreted at one month of supplementation, then it could also be claimed that supplementation with 6 g or 9 g FO per day causes an increase in proliferation. Maximum stimulation indices are frequently used in reporting this type of data as they control for the ability of

unstimulated cells to incorporate thymidine. However, in examining the effects of the supplements over time, changes in the maximum stimulation index from baseline measurements are perhaps best used.

The mechanisms involved in the modulation of lymphocyte proliferation by FO are unclear. PGE_2 has been shown to decrease the production of IL-2 and decrease lymphocyte proliferation as has PGE_3 (Santoli & Zurier, 1989, Gold *et al.*, 1994, L.M Aston, E.A Miles and P.C. Calder, unpublished observations). Therefore modulation of eicosanoid production could be a possible mechanism. DHA and EPA have also been shown to decrease the expression of the IL-2 receptor in animal studies (Jolly *et al.*, 1998), which in theory would lead to decreased proliferation, so this could be a possible mechanism. This was not examined in the current study. It is also possible that n-3 fatty acids found in FO might effect the activities of, or expression of, certain transcription factors involved in cytokine synthesis, such as NFkB or NFAT. For example, EPA has recently been shown to inhibit the degradation of the inhibitory subunit of NFkB, IkB *in vitro* (Ross *et al.*, 1999). The effect of dietary fatty acids on NFkB or other transcription factors has not been previously examined.

7.5.8 Plasma oxidisibility

Polyunsaturated fatty acids are highly susceptible to peroxidation. This susceptibility to peroxidation in erythrocytes has been shown to increase in a dose-dependent manner in subjects given 2.5-7.7 g EPA plus DHA per day (Palozza *et al.*, 1998). The effect of increasing doses of FO on the oxidisability of PBMC is not known. In the current study, plasma TBARs (an index of lipid peroxidation) were found to be decreased in the FO groups towards the end of the study. However, as this index is dependent on the amount of lipid in the plasma, and as FO supplementation of the diet was found to have a TAG-lowering effect, this effect of FO on plasma TBARs may have been due to the fact that there was less lipid present. For this reason a ratio of TBARs:TAG was calculated (see table 7.46). There were no significant differences between the groups at any of the timepoints in the study or compared to baseline. Yaqoob *et al.* (2000) have shown that supplementation of the diet of healthy humans with 9 g FO/day for 12 weeks did not affect plasma TBARs. The results of this study and the current study would suggest that supplementation of the diet with 9 g FO/day is safe and does not

cause an increase in lipid peroxidation, despite an increased consumption of highly unsaturated fatty acids. It has been suggested that this may not hold true however for older individuals as Meydani *et al.* (1991) have shown a slightly increased plasma MDA (another index of peroxidation) concentrations in older women supplemented with FO. A recent study however by Thies *et al.* (2000) in healthy elderly subjects supplemented with 4 g FO/day has shown that FO supplementation of the diet had no effect on the plasma concentration of TBARs.

It is possible that increased consumption of α-tocopherol might protect cells against any deleterious effects, such as increased lipid peroxidation, caused by increased consumption of highly unsaturated fatty acids. A study by Wu *et al.* (1996) has shown that enrichment of the primate diet with EPA and DHA decreased PGE_2 production. This agrees with the current study in which PGE_2 tended to decrease in the groups taking the n-3 supplements. In the Wu *et al.* (1996) study, the authors interpreted their results as being due to the fact that the monkeys had been fed variable concentrations of α-tocopherol and so were better able to maintain their antioxidant defenses in the face of different dietary PUFA levels. This suggests that n-3 PUFA may exert at least some of their inhibitory effects by inducing some oxidative damage in the cells. In the current study, the supplements contained the same amount of α-tocopherol despite the fact that they contained differing concentrations of long chain n-3 fatty acids that would need to be protected. A recent study by Wander *et al.* (1997) has shown that increasing the n-3 PUFA content of the diet of dogs increased the levels of lipid peroxidation products in the plasma, and while this did not occur in the present study, it is clear that the precise interactive effects between lipid peroxidation products, α-tochopherol and FO consumption on cytokine production by immune cells remain to be clarified. This could be accomplished through a study of similar design to the current study, in which increasing doses of FO and a placebo oil were consumed by volunteers along with increasing doses of vitamin E within each group and the *ex vivo* production of cytokines and lipid peroxidation products were measured.

7.5.9 Effects of FO supplementation on plasma TAGs

There have been a great number of studies which have shown that feeding fish or fish oil to either healthy subjects or to those with cardiovascular disease cause changes in

plasma lipids similar to those reported by Greenland Eskimo (see Harris, 1996 for a review). Marine oils rich in n-3 fatty acids are more effective in lowering the concentration of plasma TAGs than vegetable oils, generally rich in n-6 fatty acids. As with the case of studies investigating the effects of dietary fatty acids on immunity, the studies which have examined the effects of fish oil on plasma TAG levels have tested the dietary effects of fatty fish, fish body or liver oil supplements, and various forms of purified EPA and DHA. This makes studies difficult to compare because of different experimental designs, different sources and amounts of n-3 fatty acids, concomitant changes in saturated fat and cholesterol levels, and differences in the subjects participating in the experiments. The current study clearly shows an inverse dose-response relationship between FO consumption and plasma TAG concentrations after three months of supplementation, when the data were analysed as percentage change from baseline. The mechanisms responsible for the hypotriglyceridemic effects of fish oils are not currently entirely understood, but among the mechanisms that may be responsible for the effect of fish oils on plasma TAG levels are inhibition of the hepatic synthesis and secretion of TAG-containing lipoproteins, or the inhibition of hepatic fatty acid synthesis (Coniglio, 1992). The enhancement of hepatic fatty acid oxidation, decreased activity of enzymes responsible for esterification of fatty acids, and changes in the ratios of the fatty acid esters formed have also been suggested as possible mechanisms (Coniglio, 1992). None of these mechanisms were investigated in the current study. However, work by Halminski *et al* (1991) has attempted to address the relative contributions of some mechanisms to the hypotriglyceridemia produced by fish oils by comparing the effects of three different dietary oils (fish, safflower and palm) on fatty acid oxidation and glycerolipid synthesis in the rat liver. In the Halminski *et al.* (1991) study, the hepatic TAG level was not affected by dietary treatment. This group suggested that the decrease in plasma TAGs due to dietary fish oil is somehow related to a decrease in the capacity of liver to hydrolyze phosphatidate, which would then affect microsomal TAG synthesis from diacylglycerol. However, the precise mechanisms by which fish oils mediate these effects are still unclear, and it appears that both the degree of unsaturation and the precise position of the double bonds may have role. What does emerge from the current study however, is that at least in healthy humans, a clear dose-response effect on plasma TAG lowering occurs. Ingestion of high doses of FO therefore, may be of great benefit in lowering the TAG levels of

patients with hypertriglyceridaemia. The results of the current study however indicate that a dose of 6 g FO/day seems to be required however, and in agreement with a recent study by Pang *et al.* (1998) the lowering effect cannot be obtained by ingesting a similar amount of linseed oil.

7.5.10 Effects of linseed oil

Although α-linolenic acid is a precursor of the long chain n-3 fatty acids, its metabolic behavior in animals and human beings differs substantially from that of EPA and DHA. (Nettleton, 1995). Studies have shown that α-linolenic acid appears to be less effective than fish oil in affecting blood lipid levels and tissue fatty acid composition (Sanders & Roshanai, 1983; Kelley *et al.*, 1993). In the current study, supplementation of the diet with linseed oil caused a significant increase in EPA and DPA and had no effect on DHA in plasma phospholipids. These effects of linseed oil supplementation were not observed in the PBMC lipids however. There were no detectable levels of 18-3 n-3 in either plasma phospholipids or PBMCs. PGE_2 production by LPS-stimulated was decreased compared to baseline values following linseed oil supplementation the diet for the duration of the study. This agrees with previous studies of the effects of supplementation of the human diet with α-linolenic acid rich oil (Caughey *et al.*, 1996; Mantzioris *et al.* 2000). There were no other significant effects of linseed oil supplementation on any of the other cytokines, although IL-6 production by cells from subjects consuming this supplement were decreased compared to baseline measurements after two months supplementation and during the washout period. This has not previously been reported and is an exciting result.

7.5.11 Implications

As mentioned previously in chapters 1 to 5, FO supplementation of the diet may be of use in the treatment of rheumatoid arthritis, and indeed a number of studies have shown that FO supplementation of the diet will cause clinical improvement in this disease (Kremer, 1996; see Belch & Muir, 1998 for a review). The main long term problem in rheumatoid arthritis is progressive erosion of the cartilage in the joints leading to irreversible destruction. It has often been suggested that FO supplementation, by decreasing the production of some pro-inflammatory mediators involved in this disease

might also decrease the need for the anti-inflammatory drugs often prescribed in the treatment of this disease. Some studies (e.g. Lau *et al.*, 1993; Geusens *et al.*, 1994) have shown a significant reduction in use of non-steroidal anti-inflammatory drugs by rheumatoid arthritis patients treated for one year with FO preparation providing 2.6 or 2.9 EPA + DHA g/day. More research in this area is needed however because there are no epidemiological data for fish consumption and arthritis, and only one study exists concerning α-linolenic acid and arthritis, which showed the fatty acid to be ineffective (de Dekere *et al.*, 1998). However, it is possible that beneficial additive effects of drug-diet combinations could be of use in the treatment of rheumatoid arthritis, with anti-inflammatory drugs being decreased in some patients in combination with increased n-3 consumption. This might also be applicable to new drugs or new treatments that aim to suppress cytokine concentrations, i.e. there may be an opportunity for beneficial additive effects with increased n-3 consumption. This requires further investigation.

7.6 Conclusions

This study is unique in design as it is a placebo-controlled, dose-response study of the effects of FO on the fatty acid composition and production of inflammatory mediators by cells of the immune system. It was found that supplementation of the human diet with n-3 fatty acids leads to dramatic changes in the proportions of fatty acids in plasma phospholipids and moderate changes in the fatty acid composition of PBMC total lipids. Dietary supplementation with moderate to high (6-9 g /day) FO for three months was associated with decreased production of some cytokines such as IL-6 and IL-2 by stimulated PBMC compared to supplementation with a placebo oil or linseed oil. No effects were seen on the production of TNF-α, IL-4 or IL-10. There was a significant increase in IL-1β production as a result of supplementation with FO by stimulated cells after one month of supplementation, and a significant decrease during washout. These results clearly show that FO supplementation of the diet can affect the production of some inflammatory mediators, but that a moderately high intake for a moderately long period of time (1-3 months) is required to see results in most cases. The results of this study help to clarify the some of the conflicts in the literature, and provides important information to underpin the use of n-3 fatty acids as a nutritional means of modulating the immune response.

Chapter 8: Overall Discussion

8.1 Overall findings

This thesis has shown that supplementation of the diet of both rodents and humans with FO, rich in long chain n-3 fatty acids, leads to significant changes in the proportions of EPA and DHA present in both immune cells and plasma phospholipids. In parallel with these changes in fatty acid composition, changes are also observed in the ability of cells to produce eicosanoids such as PGE_2, and some pro-inflammatory cytokines. Cellular responses such as the ability of mitogen-stimulated lymphocytes to proliferate were also affected. The effects seen were more pronounced in rodents than in humans. This is probably due to the fact that mice were fed a substantially higher dose of FO than the human volunteers were given. The mice were fed a diet with EPA + DHA containing 20.7 g/100g total fatty acids. In the human study, those subjects in the group taking 9 g FO per day were consuming < 3g EPA + DHA/100g total fatty acids. There is another key difference between mice and humans. The C57BL6 mice used were an inbred strain of mice that are functionally genetically identical. Human cells however exhibit a range of abilities to produce cytokines, in part due to genetic differences between different humans. Nevertheless, the modulatory effects of FO feeding on immune cell responsiveness were observed in the human study and it is hypothesized that the increased incorporation of long chain unsaturated fatty acids into cells may be in part responsible for the changes observed in the abilities of cells to produce cytokines and proliferate. Some of the possible mechanisms as to how this might occur are discussed in the next section.

8.2 How do fatty acids modulate immune cell responses including cytokine production ?

As discussed in the proceeding chapters of this thesis, cytokines have been implicated in the pathology of many diseases (asthma, rheumatoid arthritis, atherosclerois and psoriasis) and modulation of cytokine production by dietary fatty acids has been proposed as a possible strategy for the management of these diseases and has been

shown to be of some clinical benefit (see chapter 1). The mechanisms of how long chain n-3 fatty acids affect cytokine production remain unclear, but there are a number of possibilities. These include causing alterations of membrane structure and fluidity (Rotondo, 1995; Tappia *et al.*, 1997), modulation of intracellular regulatory mechanisms such as enzymes, receptors, lipid mediators or transcription factors (Virella *et al.*, 1989; Murphy, 1990; Simpoulous, 1997) and interference with the flow of arachidonic acid for eicosanoid biosynthesis (Kinsella *et al.*, 1990).

One of the ways in which n-3 fatty acids might be affecting cytokine production is through modulating the amounts and types of eicosanoids produced by activated cells (PGE_2 seems to be particularly important in this regard; see section 1.2.11), which in turn will suppress or enhance the types of cytokines produced. This has been illustrated by Knudsen *et al.* (1986) and Renz *et al.* (1988) for monocyte-derived cytokines and by Gold *et al.* (1994) for lymphocyte derived cytokines. In agreement with the results presented in this thesis, numerous studies have shown that diets rich in the long chain n-3 fatty acids EPA and DHA cause an increase in the amount of those fatty acids in the membranes of cells from animals and humans at the expense of AA (Stubbs & Smith, 1984; Calder *et al.*, 1990; Kinsella, 1990; Gibney & Hunter, 1993; Yaqoob & Calder, 1995). This in turn could result in changes in the amounts and types of eicosanoids produced. However, it is not completely understood how EPA and DHA interfere with eicosanoid production. While they displace AA in cell membrane phospholipids, a significant amount of AA remains available for eicosanoid production. Nevertheless, they reduce AA availability. EPA and DHA are less readily released from membranes upon cell stimulation so that the level of substrate is decreased (Fischer *et al.*, 1984). EPA and DHA are oxygenated by cyclooxygenase (COX) but not as readily as AA (Lands *et al.*, 1973; Lands & Byrnes, 1982). Thus, n-3 fatty acids are not as effective substrates as AA for eicosanoid synthesis. EPA and DHA competitively inhibit COX from acting on AA by binding to the COX enzyme, thereby occupying sites that would be available to AA (Corey *et al.*, 1983). The concentration of EPA and DHA available for competition with AA is also important in determining the extent to which AA metabolism is inhibited. Abundant EPA and DHA, especially in intracellular NEFA pools, will inhibit more enzyme. N-3 fatty acids accumulate in intracellular NEFA pools at higher levels than n-6 fatty acids, making

them more available to the COX enzyme (Lands, 1989). This would lead to greater competitive inhibition in cells which have more n-3 fatty acids present, and the increase in n-3 fatty acid-derived eicosanoids such as PGE_3 as compared to the n-6 derived PGE_2. As these eicosanoids influence the production of certain cytokines (see figure 1.2.11), it is highly possible that the fatty acids eaten in the diet might be influencing the activity of cells of the immune system by modulating the types and amounts of eicosanoids produced by these cells.

Increasing the proportions of n-3 fatty acids present in cell membranes also increases the chain length and degree of unsaturation in cell membranes. This is known to affect membrane fluidity, and it is possible that positional shifts in membrane-associated receptors and enzymes might occur, which might effect their functional capacity (Murphy, 1990). Membrane fluidity was not assessed in the current study. Tappia *et al* (1997) have examined the effects of feeding diets including different types of fatty acids on macrophage membrane fluidity. Fish oil feeding increased membrane fluidity after 4 weeks, but had no effect on membrane fluidity after 8 weeks. However, a study by Yaqoob *et al.* (1995) on rat lymphocyte membranes found no effects on membrane fluidity after 10 weeks of feeding fish oil. It may be that cells within the immune system can compensate for the increased chain length and unsaturation associated with changes in membrane fatty acid composition. The effects of dietary n-3 fatty acids on membrane fluidity in different immune cell membranes have not been directly compared within a single study. This needs to be investigated. It may also be that the duration of supplementation might explain the results of Tappia *et al.* (1997). Yaqoob (1993) has shown that the cholesterol content of lymphocytes increases following FO supplementation of the diet compared to supplementation with saturated fatty acids. It is possible that cells have a mechanism in place to detect when their membranes are getting too 'fluid' and compensate for this by increasing the cholesterol content of the membrane. This might account for the lack of an effect of dietary fatty acids on membrane fluidity, despite clear changes in the proportions of long chain unsaturated fatty acids present in cellular membranes. The cholesterol content of the cells was not assessed in the current study.

Plasma membrane receptors involved in immune cell responses have been shown to be affected by fatty acids. Opmeer *et al.* (1984) showed that PGE_2 receptors can be modulated by dietary fatty acids. This study fed rats for four weeks on either 12.5 % corn oil or linseed oil and found that a corn oil diet caused a two-fold increase in the number of high and low affinity binding sites on rat peritoneal macrophages which coincided with increased binding capacity for PGE_2. Virella *et al.* (1989) showed that the addition of EPA to human PBMC inhibited B cell responses to mitogenic stimulation and depressed the expression of IL-2 receptors. Jolly *et al.* (1998) have recently shown that feeding EPA and DHA affect IL-2 receptor alpha subunit mRNA levels, without affecting IL-2 mRNA levels in murine spleenocytes. This would imply that long chain n-3 fatty acids are exerting their effects on lymphocyte proliferation primarily through modulating transcription of the IL-2 receptor. Dietary n-3 fatty acids have also recently been shown to decrease the level of expression of another Th-1 cytokine receptor, IFN-γR1 on macrophages (Feng *et al.*, 1999). Modulation of cytokine and eicosanoid receptor expression and transcription represent an additional way in which fatty acids may be exerting their effects on the immune system. In contrast to these reports, Jenski *et al.* (1998) have recently shown that DHA does not affect IL-2 receptor expression on rodent splenocytes. An earlier study by Calder & Newsholme (1992) has also shown that addition of a range of specific unsaturated fatty acids to cultured rat lymphocytes did not affect IL-2 receptor expression. The effects of long chain n-3 PUFA on the expression of cytokine receptors needs further study before any conclusions can be made.

A number of intracellular signaling pathways have been shown to be modulated by dietary n-3 fatty acids. Many of these involve the modulation of the production of lipid mediators. Immune cells are activated by cytokines, eicosanoids, antigens, mitogens or antibodies directed against cell-surface structures. This activation is a complex process which involves a number of plasma membrane associated events including the activation of phospholipase C (PLC) with generation of the second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) which lead to calcium mobilisation, protein kinase C (PKC) and tyrosine kinase activation, substrate and ion transport into the cell, appearance of cell-surface receptors and secretion of cytokines.

These processes ultimately lead to the proliferation of the activated cell (Berry & Nishizuka, 1990; Calder & Miles, 1998). In the current study, proliferation of both rodent and human lymphocytes was found to be affected by dietary fatty acids. This indicates that n-3 fatty acids, or indeed other fatty acids such as the saturated fatty acids found in the coconut oil fed in the murine study, might be affecting the generation or activity of mediators involved in the activation and proliferation of lymphocytes.

Most mitogen and cytokine actions depend upon new gene transcription and are thought to be mediated by activation of specific DNA-binding proteins (transcription factors). Lymphocytes, macrophages and other immune and inflammatory cells contain many transcription factors including NFκB, nuclear factor (NF) of activated T cells (NFAT), AP-1, various oncogene products (e.g. myc, fos, jun), steroid hormone receptors and specific nuclear factors such as NF-IL-2, NF-IL-6, NF-ICAM-1 (Abbas *et al.*, 1994) and PPAR-γ (Ricote *et al.*, 1998). NFkB is one of the most important nuclear transcription factors in signal transduction in inflammatory cells. It is a DNA-binding protein that can activate a great variety of genes involved in early defense reactions of animals. It is composed of two proteins of 50 and 65 kDa. NFκB pre-exists in the cytoplasm of most cells in an inactive form complexed to IκB, its inhibitory subunit. Stimulation of the cell by a number of agents such as phorbol ester, LPS and TNF-α results in the disassociation of the IkB-NFkB complex and subsequently the NFkB heterodimer migrates to the nucleus where it binds to its binding site and activates transcription (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Nolan *et al.*, 1991). Binding sites for NFkB are present in the regulatory regions of certain cytokine genes including TNF-α, IL-1β, IL-6, IL-8 and some IFN genes, the IL-2 receptor gene, class I and II hisotocompatability genes, and of enzymes involved in mediator generation such as inducible nitric oxide synthase and a range of acute phase proteins (for a review, see Kopp & Ghosh, 1995). It is interesting that each of the above listed cytokines, enzymes and molecules crucial for immune cell interactions and immune responses been shown to be modulated by long chain n-3 fatty acids (see chapter 1 for references). This suggests that NFκB might be modulated directly by n-3 fatty acids, or that some step in the pathway leading to its activation might be modulated by these fatty acids. The intracellular pathways by which cytokines activate

transcription factors are diverse and not yet completely understood, and a comprehensive review is beyond the scope of this thesis.

The change in the fatty acid composition of cell membrane lipids could affect the types of fatty acids released from membrane phospholipids as a result of cell activation. Cell responses often begin with receptor-mediated activation of a phosphatidylcholine-specific phospholipase–C (PLC). The activity of the main isoform of PLC (PLC-γ1) in lymphocytes has been shown to be decreased by feeding long chain n-3 fatty acids (Sanderson & Calder, 1998), and this might represent one way that these fatty acids affect intracellular signalling processes. The activities of the other isoforms of this enzyme, and of other phospholipases, in response to dietary fatty acids remain to be established. PC-PLC releases diacylglycerol (DAG) from membrane-bound phosphatidylcholine. The lipid composition of DAG species has also been shown to be affected by n-3 fatty acids (Huang & Fritsche, 1992). Certain n-3 fatty acid enriched-DAG species have been shown to be less potent than those species enriched in 18:0 and 20:4n-6 in activating PKC from rat spleen (Bell & Sargent, 1987) (see chapter 1 for PKC involvement in cellular signaling in macrophages and lymphocytes). In agreement with this, diets rich in EPA and DHA have been shown to inhibit PKC activity in rat lymphocytes and macrophages (May *et al.*, 1993; Rossetti *et al.*, 1995; Tappia *et al.*, 1997). DAG released from membrane phospholipids also activates a sphingomyelinase enzyme that liberates ceramide from membrane bound sphingomyelin. The overall concentration of DAG has been shown to decrease in murine splenic cells following n-3 fatty acid feeding (Hosack-Fowler *et al.*, 1993; Jolly *et al.*, 1997). This would lead to decreased DAG for PKC and sphingomyelinase activation, which in turn might lead to a less responsive cell. This represents another way in which n-3 fatty acids could modulate signal transduction in cells. The ceramide released as a result of sphingomyelinase activation binds to and activates a specific ceramide–activated protein kinase, which in turn, is thought to release NFkB from its interaction with IkB. Ceramide generation in mitogen-stimulated spleenocytes has been shown to be decreased by dietary n-3 fatty acids in mice (Jolly *et al.*, 1997) and this study found that this lead to a decrease in the production of IL-2. Modulation of ceramide generation therefore represents yet another way in which n-3 fatty acids could modulate NFkB activation and the production of certain cytokines. PKC is also known

to important in 'on' signal for lymphocyte proliferation (Berry & Nishizuka, 1990) and in the chapter 5, murine lymphocyte proliferation was found to be decreased following a diet rich in unsaturated fatty acids, but was not affected by a diet rich in saturated fatty acids. It is possible that modulation of PKC activity in the lymphocytes might explain these results.

Activation of NFkB is also regulated by peroxisome proliferator activator receptors (PPARs). PPARs regulate the intracellular metabolic fate of fatty acids, and have been shown to repress gene transcription induced by the nuclear factors NFkB, signal transduction and activator of transcription (STAT) and AP-1 (Pineda *et al.*, 1994). N-3 fatty acids themselves, as well as certain eicosanoids such as PGJ_2, act as ligands for PPARs (Jump *et al*, 1997) and it may be that increasing the prevalence of these fatty acids (or their derivatives) in cells through dietary supplementation might influence the activity of these receptors, subsequently affecting cytokine production. At the present time however the effects of n-3 fatty acids on signalling pathways involved in the activation of NFkB and other transcription factors need further investigation, and studies investigating the interactive effects n-3 fatty acids on PPARs, other nuclear factors and cytokine production in immune cells such as macrophages need to be performed.

Other important cellular enzymes have been shown to be modulated by n-3 PUFAs. For example, the activities of adenylate cyclase (fundamental in agonist-induced formation of the second messenger cAMP) and 5'-nucleotidase (fundamental in generating the extracellular agonist adenosine) are markedly influenced by the levels of n-6 and n-3 PUFA in membrane lipids (Spector & Yorek, 1985). In animals fed diets rich EPA and DHA, which leads to increased amounts of these fatty acids in membranes, while the activities of adenylate cyclase and 5'-nucleotidase are increased (Kinsella, 1990). Modulation of the activities of these and other enzymes could explain a number of the results obtained in this thesis. However, the wide involvement of the products of the reactions in which these enzymes are involved in numerous signaling pathways will make the relationships between fatty acid modulation of enzyme activity and the resulting effects on cell signaling difficult to elucidate. For example, an increase in adenlyate cyclase activity by n-3 fatty acids should lead to increased

production of cAMP, leading to increased activity of cAMP-dependent protein kinases. However, cAMP dependent protein kinase activity in the brain has been shown to be inhibited by both n-3 fatty acids and n-6 fatty acids, but is not affected by saturated fatty acids (Speizer *et al.*, 1991). Thus there are a number of possible mechanisms by which n-3 fatty acids could influence immune cell activation and subsequent function. It is clear that more needs to be done to elucidate the mechanisms of action if we are to fully understand how fatty acids interact with the immune system.

8.3 Do n-3 fatty acids have therapeutic uses?

The types of diseases present in cultures which consume large amounts of long chain n-3 fatty acids, such as Greenland Eskimos and the Japanese are very different from the diseases common in Western societies (see chapter 1). The prevalence of cardiovascular diseases and of certain autoimmune diseases are much reduced. This has prompted an extraordinary amount of research into the therapeutic uses of long chain n-3 fatty acids (usually in the form of fish oil) in the treatment of a number of diseases. For example, a great number of studies have investigated the effects of n-3 fatty acids on cardiovascular risk factors, such as high blood pressure and hyperlipidemias (British Nutrition Foundation, 1992). Consumption of n-3 fatty acids have been shown in many studies to decrease plasma TAG concentration (see Harris, 1996) and blood pressure, which may account for some of the protective effects in heart disease. The effects were confirmed in this thesis. The possible uses of long chain n-3 fatty acids in autoimmune diseases such as arthritis, asthma, psoriasis, and Crohn's disease and following organ transplantation and the studies which have examined the use of n-3 fatty acids in these patient groups have been mentioned and discussed in the in the proceeding chapters of this thesis. A number of studies have shown clinical benefits following n-3 fatty acid supplementation in these patient groups. Interpretation of these studies however are hindered by the fact that many are not placebo-controlled studies, and that studies have provided different amounts of the fatty acids for differing lengths of time, and often the numbers of patients in each group were very small (see chapter 1). Overall however, when the great number of studies which have been performed in humans are considered, the majority have shown some clinical benefit of n-3 fatty acids in the amelioration of symptoms often associated with the overproduction of inflammatory

mediators. This would support the hypothesis that supplementation of the diet of patients with n-3 fatty acids will be of benefit in some autoimmune diseases. Patients with autoimmune-type diseases however, are often taking a variety of immunosuppressive and/or anti-inflammatory medications. As long chain n-3 fatty acids have been shown to decrease the production of some proinflammatory cytokines (such as IL-6 as shown in the current human study), supplementation of the diet with n-3 fatty acids might cause further immunosuppression in these already immunosuppressed patients. No studies to date have monitored the frequency of infection following supplementation of the human diet with long chain n-3 fatty acids. The interactions of long chain n-3 fatty acids with specific drugs need also to be established, to identify any risk which might be involved in the administration of these fatty acids to some severely immuno- compromised patients.

8.4 Ways to increase dietary consumption of n-3 fatty acids.

The studies on the Greenland Eskimo were pivotal in drawing attention to dietary n-3 fatty acids and their protective effects on heart disease and inflammation (British Nutrition Foundation, 1992; 1999). They focused medical attention on the influence of dietary fat, as perhaps no other observations had, and they threw the comfortable paradigms about fats into disarray. Current ideas about dietary n-3 fatty acids are partly due to the research stimulated by these Greenland studies. Trying to discern the interrelationships among different fatty acids has made understanding the effects of dietary fat on the body more difficult and complicated. Nevertheless, research on the effects of n-3 fatty acids has prompted organisations responsible for advising on nutritional status in various countries to advise that most individuals increase consumption of oily fish which are rich in n-3 fatty acids. Such fish are listed in chapter 1 (table 1.3). In the UK such organisations are COMA and the British Nutrition Foundation. To meet the COMA recommendations (Department of Health, 1994) for n-3 intake, one small serving of oily fish per person per week would be needed to be consumed. To meet the recommendation set by the British Nutrition Foundation (British Nutrition Foundation, 1992), however, two to three medium servings of oily fish per week would be needed. At present the average household intake of fish in the UK is 144 g per person per week (British Nutrition Foundation, 1999). Only a

proportion of this is specifically classified in the National Food Survey Report as oil-rich fish, and only a third of adults eat oil rich fish (MAFF, 1998). In order to meet the recommendations for consumption of n-3 fatty acids therefore, a substantial increase in consumption would be needed by the majority of the population.

One of the ways in which this increase might be accomplished is through the use of fish oil supplements. Although most nutritionists prefer to recommend increasing the consumption of foods rather than supplements as a first course of action, in the case of oil-rich fish, this is unlikely to be acceptable to certain individuals who do not like or cannot afford fish. Fish oil supplements such as the ones used in the study reported in chapter 7, represent one way in which the intake of n-3 fatty acids can be increased and the weekly dose controlled for, although again they might prove to be too expensive for the average consumer. The use of deodorised capsules also eliminates the fishy aftertaste objected to by a number of people.

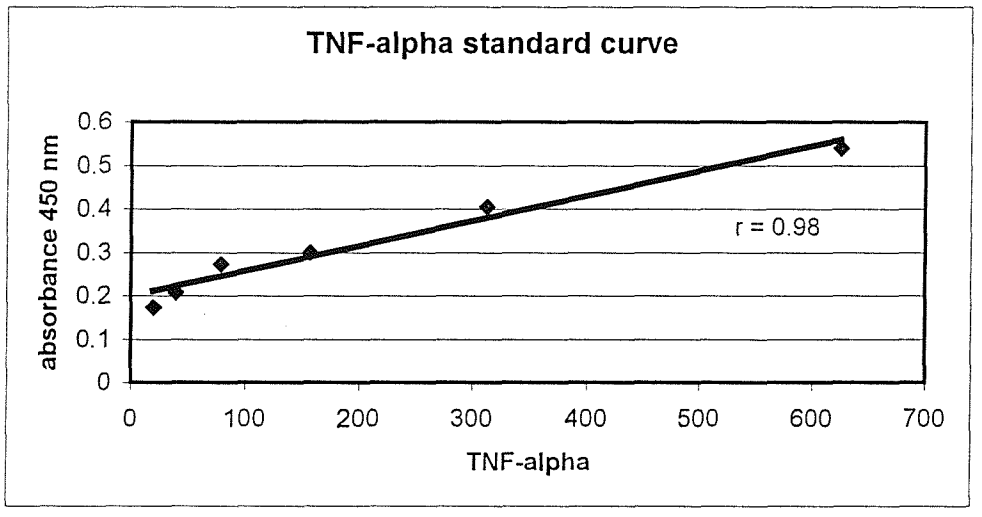
It is also possible that the consumption of n-3 fatty acids could be increased through food fortification, or the use of 'functional foods'. Foods fortified with fish oil such as margarines and spreads could become important sources of EPA and DHA. Advances in food technology have allowed the successful incorporation of EPA and DHA into a range of manufactured foods (British Nutrition Foundation, 1999), while still maintaining palatability, acceptability and shelf life. In Japan and Korea a great range of products are fortified with DHA, ranging from dairy products and pork to teas, sweets and crackers. Eggs enriched in EPA, DHA and α-linolenic acid, as a result of feeding hens linseed oil or fish oil, are also commonly sold in number of countries (e.g. Canada and the USA). There are few reasons, save marketplace appeal, as to why such products could not be marketed in the UK. Micro-encapsulated powders (a technology which reduces the presence of unpleasant fishy odours and reduces the risk of rancidity in highly oxidizable oil), are readily available for use in food fortification, and can potentially be incorporated into almost any food product without affecting the shelf-life, taste or odour. During the micro-encapsulation process, the oil is emulsified in an aqueous solution of coating agent and antioxidants, which is finally dried to a powder.

A limited number of studies have examined whether health benefits occur from the consumption of foods to which n-3 fatty acids have been added, but these have almost all examined the effects of the fatty acids on blood lipids only. One study has examined indicies of immune function, and this trial was not placebo controlled (Mantzioris *et al.*, 2000). Gustafssun *et al* (1996) in a double blind crossover study investigated the effect of seafood products enriched with n-3 fatty acids on blood lipids. The diets included n-3 enriched products providing 3 g of long chain n-3 fatty acids per day, compared to 0.3 g/day in the control diet. The authors reported a significant reduction (25%) in fasting TAG levels, a significant decrease in systolic blood pressure, and positive effects on insulin secretion (19% reduction on the n-3 fatty acid fortified diet). Lovegrove *et al.* (1997) in a study with a cross over design studied the effect of foods enriched in fish oil on both fasting and postprandial lipaemia in 9 middle aged men with normal blood lipid levels. The foods used included n-3 fatty acid enriched bread, biscuits, pasta, cake, and ice cream. Using these foods in place of their habitual brands the subjects were able to increase their intake of EPA and DHA to 1.4 g/day compared to 0.37 g/day during the control diet period. In this study there was no decrease in fasting or postprandial blood TAG concentrations, although there was an increase in HDL-cholesterol. Both of these studies were of relatively short duration (3 weeks) and included small numbers of subjects and so larger and longer studies need to be performed before any firm conclusions about the effectiveness of n-3 fatty acid enriched foods can be made. A recently reported study by Mantzioris *et al.* (2000) has shown that feeding foods enriched in either α-linonenic acid or EPA + DHA to healthy male volunteers caused a significant decrease in the production of TXB_2, PGE_2, and IL-1β by LPS-stimulated PBMCs after four weeks supplementation compared to baseline measurments. It is unfortunate that this study did not include a placebo group for comparison. Nevertheless, by including these n-3 fortified foods both groups managed to substantially increase their consumption of n-3 fatty acids that lead to increases in the proportions of EPA in their plasma phospholipids, similar to the current study. This trial does suggest that common foods fortified with α-linolenic acid, or EPA + DHA are well tolerated and could lead to moderate reductions in the ability of immune cells to produce cytokines. A trial including a greater range of subjects and studying the effects of n-3 fortified foods over a longer period of time is

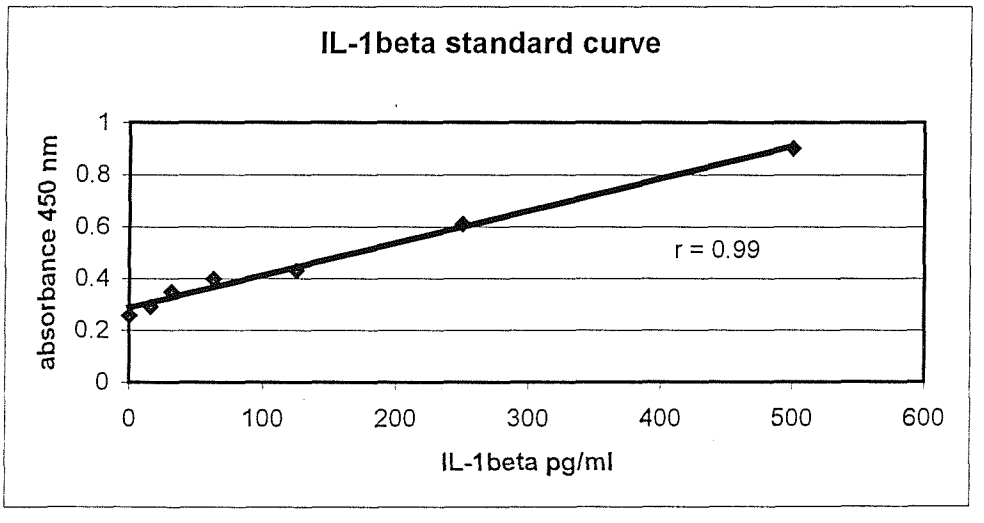
required. Research into the stability of n-3 fortified products also needs to be determined. The precise dose needed to be given to healthy individuals in order to elicit the desired physiological effects also needs to be determined and can perhaps partially be answered as a result of this thesis. This thesis has clearly shown that long chain n-3 fatty acids can affect inflammatory mediator production in both mice and humans, and confirmed that long chain n-3 fatty acids can influence plasma TAG concentrations and blood pressure in humans. The dose required for these changes is, not unsurprisingly, between 6-9 g FO/day, providing 1.3-2.3 g EPA + DHA/day. The average intake of FO reported in the Greenland Eskimo studies three decades ago which began a plethora of research was approximately 7 g EPA + DHA/day (Bang & Dyerberg, 1973). The results of this thesis indicate that a dose of 6-9 g FO/day appears to be necessary in healthy individuals in order to observe the physiological changes associated with FO consumption. At lower doses (3 g/day), for the most part, the immunomodulatory effects of FO are not seen and the effects on plasma TAGs are not observed (see chapter 7). Whether or not this range is appropriate in various patient groups such as patients with autoimmune diseases and patients suffering from hyperlipidemia and/or cardiovascular disease remains to be established, and is an area ripe for future research.

Appendix 1: Murine ELISA standard curves

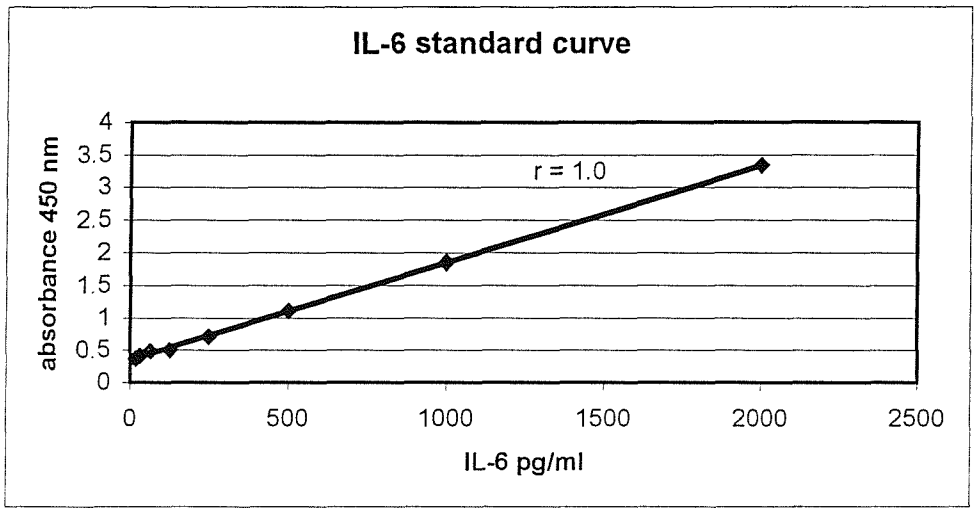
1.1 TNF-α standard curve



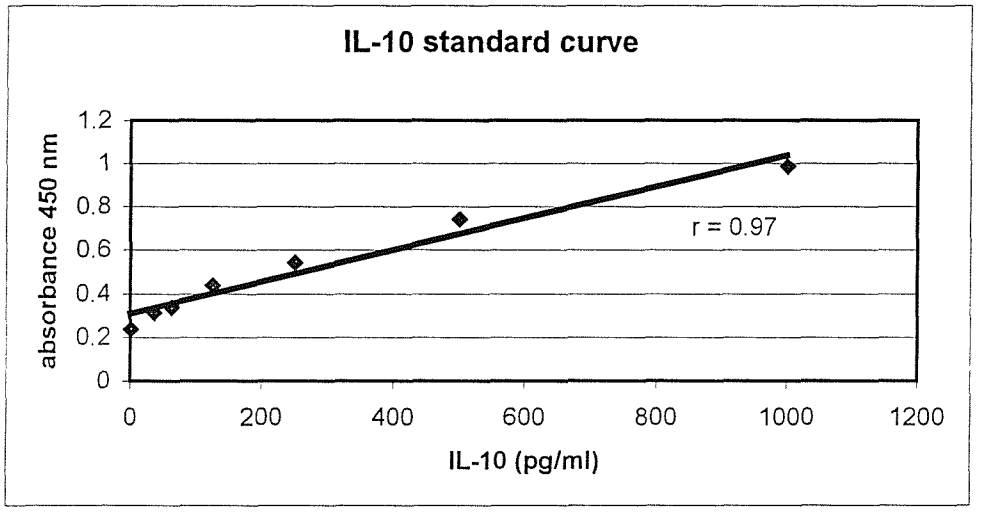
1.2 IL-1β standard curve



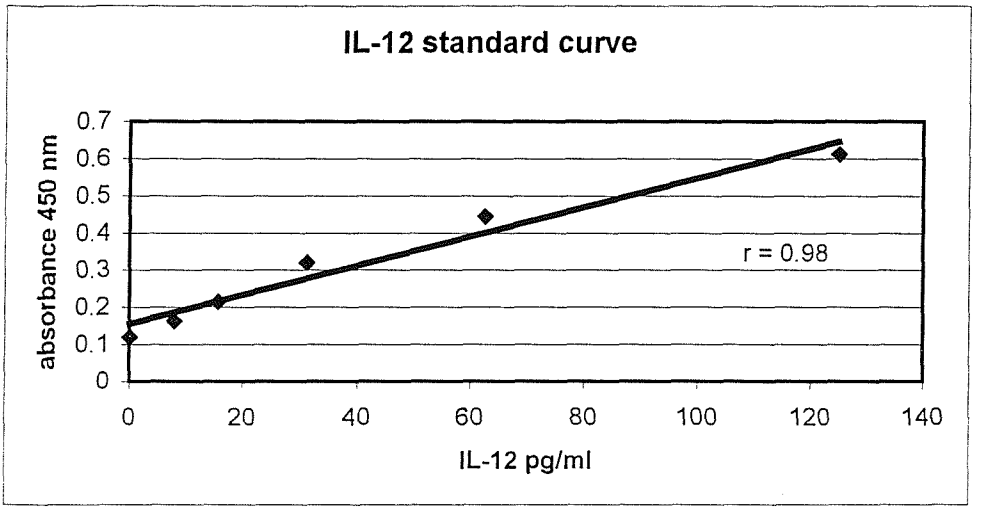
1.3 IL-6 standard curve



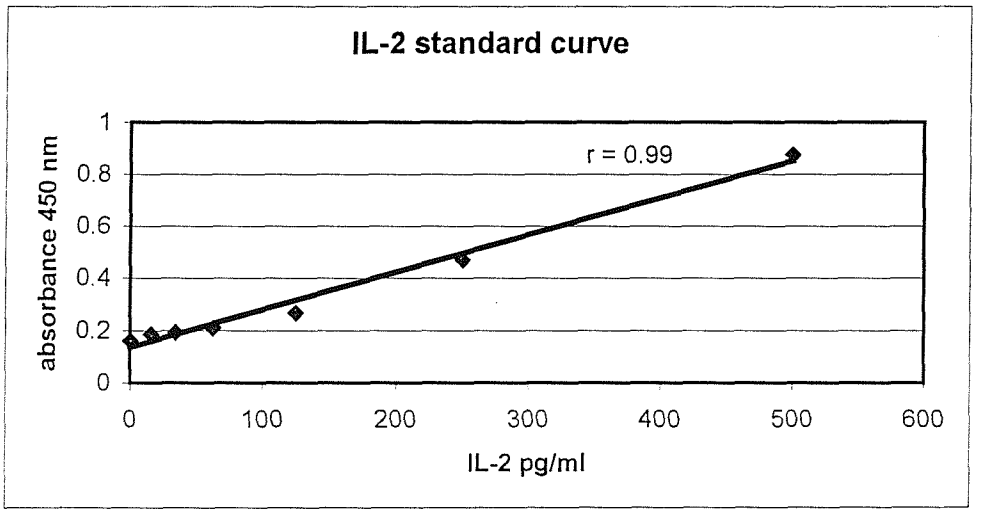
1.4 IL-10 standard curve



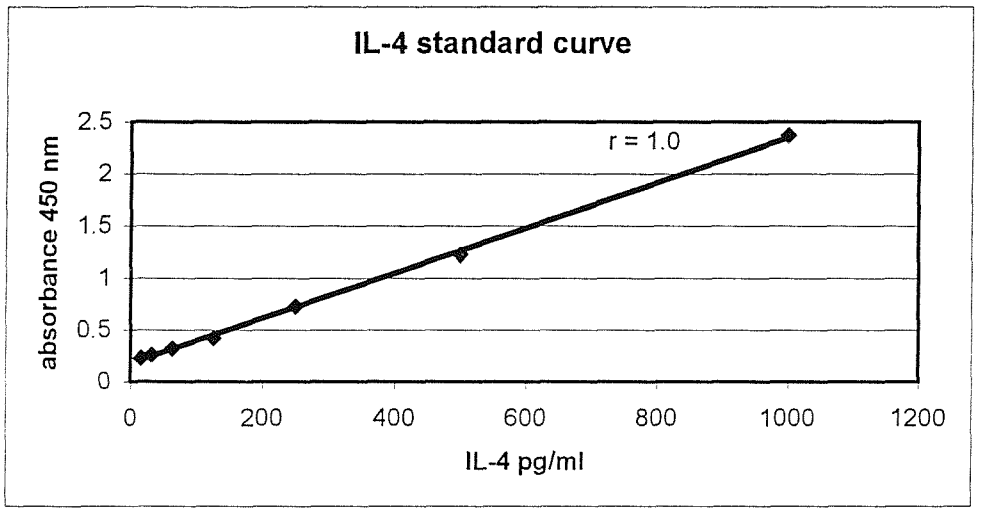
1.5 1L-12 standard curve



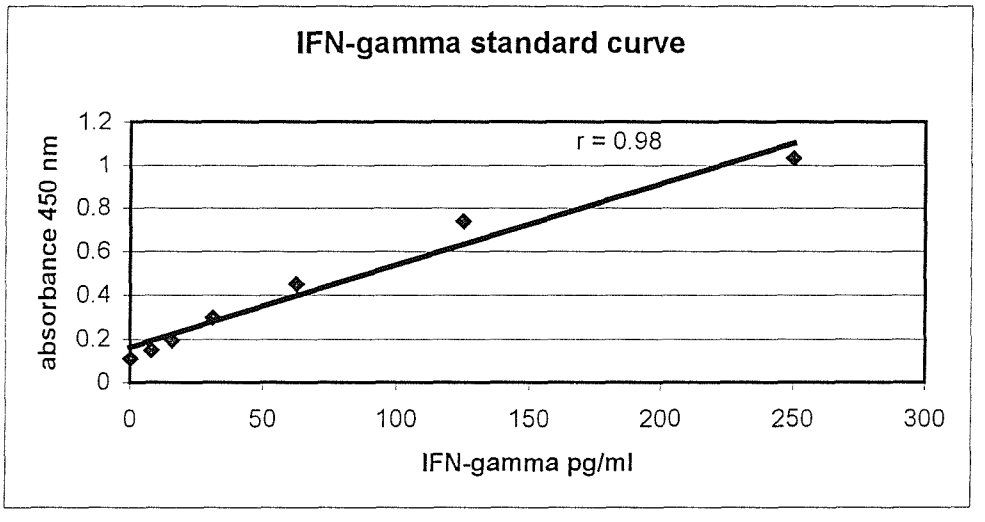
1.6 IL-2 standard curve



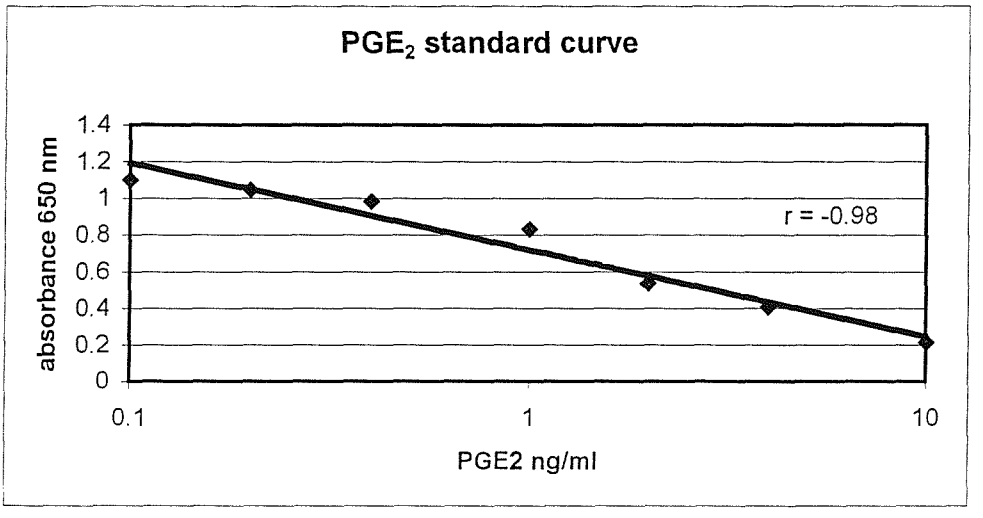
1.7 IL-4 standard curve



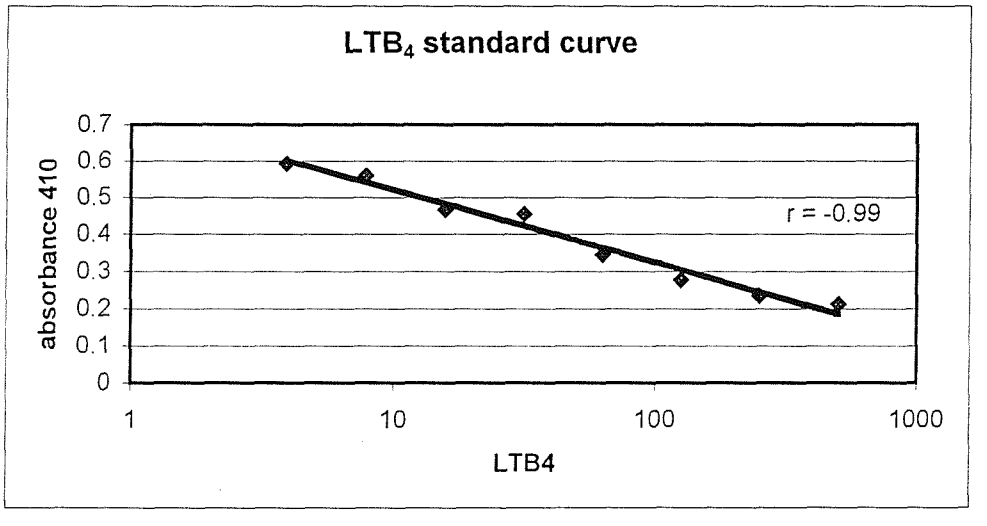
1.8 IFN-γ standard curve



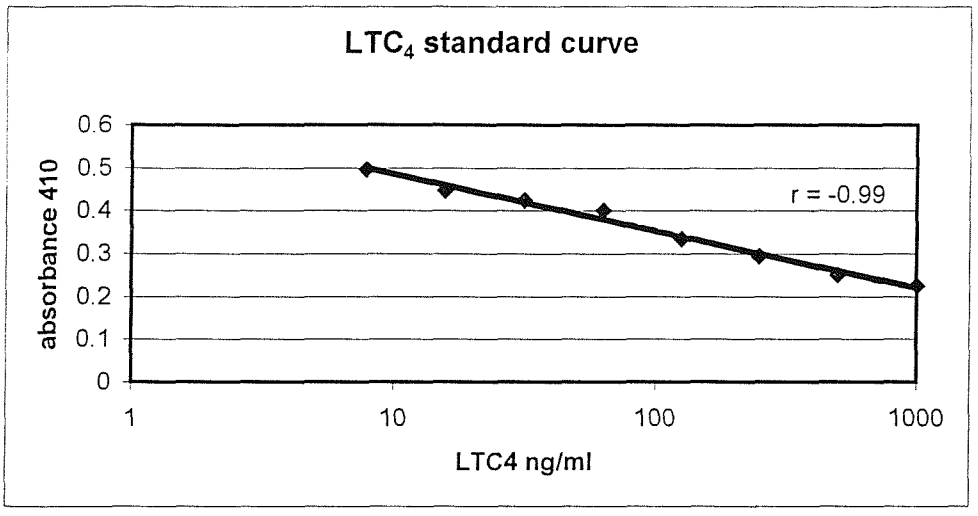
1.9 PGE_2 standard curve



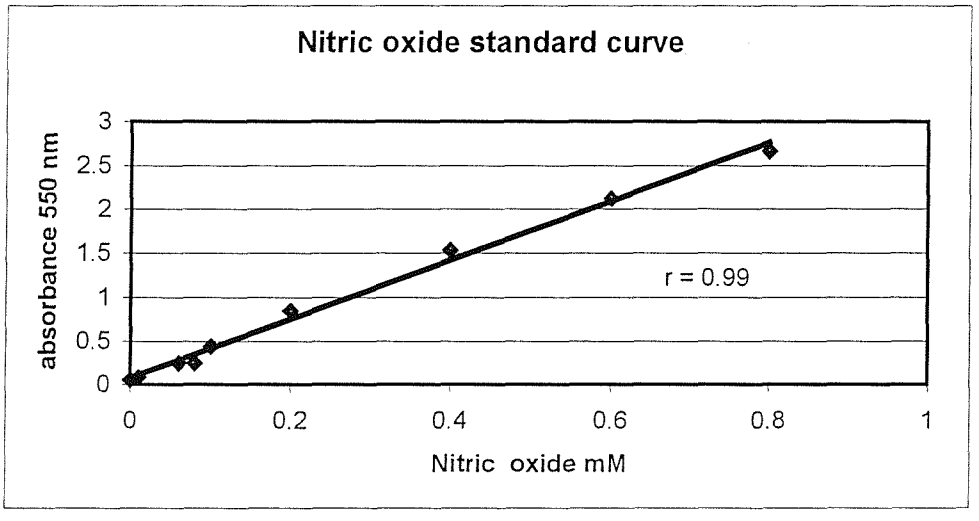
1.10 LTB_4 standard curve



1.11 LTC4 standard curve



1.12 Nitric oxide standard curve



Appendix 2: Example of a MAFF Food Diary

An Investigation of the Effects of Oil Supplements on Human Blood Cells

Dr Liz Miles, Fiona Wallace & Dr Philip Calder
Department of Human Nutrition
University of Southampton
Tel 01703 594689

FOOD DIARY

Surname:

Forename(s):

OXFD/FD/1

We would like you to keep this diary of everything you eat and drink over the next seven days. This is a very important part of the study and will add greatly to the experiments that we will be carrying out on your blood cells. It is very important that you do not change what you eat or drink just because you are keeping a record. Please continue to eat whatever you wish.
When the last day has been filled in, keep the diary in a safe place and bring it in with you on your next visit.
Many thanks for your cooperation.

As you will see, each day is marked in sections, beginning with the first thing in the morning and ending with bedtime. For each part of the day write down all food and drink consumed, the amounts, and a description if necessary. If nothing is eaten or drunk during a part of the day, draw a line through that section. Record everything at the time of eating, not from memory at the end of the day.

On the next four pages is a list of popular foods and drinks. Next to each item is the sort of thing we need to know so that we can tell what it is made of and how much you had. This list cannot cover all foods and drinks, so try to relate to a similar item if any that you have eaten are missing. Please give as much detail as you can. There is an example on page 11

For some foods you may find it easier to describe how much you had by comparing it to one of the photographs on pages 7 to 11.

Many packet foods have weights printed on them, so please use these to show how much you ate.

At the end of each day there is a list of snacks and drinks that can easily be forgotten. Please write any extra items in here if you have not already recorded them in some other part of the day.

[redacted]

WHERE POSSIBLE, ALWAYS STATE WHAT SORT OF OIL OR FAT WAS USED FOR BAKING, FRYING ETC.

Food/Drink	Description & Preparation	Amount
Bacon	lean or streaky; fried or grilled rashers	number
Baked beans	standard or reduced sugar	tablespoons, tin size or picture 12
Beefburger (hamburger)	homemade, from a packet or take away; fried, microwaved or grilled; well-done or rare; large or small; with or without bread roll	number
Beer	stout, bitter, lager; keg, draught, bottled, canned; low alcohol, strong, homemade	number of pints and half pints
Biscuits	plain; savoury; cheese, crispbread, sweet, chocolate, wafer; home-made; include biscuits like Kit-Kat and Penguin; write in the name and brand if you can	number
Bread (also see sandwiches)	wholemeal, white or brown; currant, fruit, malt; large or small loaf; thick, medium or thin slices; sliced or unsliced; give brand if possible	number of slices
Bread rolls	wholemeal, white or brown; alone or with filling (see sandwiches); crusty or soft; give brand if possible	number of rolls
Breakfast cereal, bran, wheatgerm	what sort: cornflakes, Weetabix, muesli etc.; give brand name if possible	number of biscuits, table-spoons or picture 1
Bun	what sort: iced, currant; sweet or plain; large or small	number
Butter for bread	ordinary or low fat dairy spread, write in the name and brand if you can	thick, average, thin spread
Cake - small	what sort & brand: cream, iced; sort of filling	number
Cake - large	what sort: cream, fruit, iced; sort of filling	slices or pictures 15 or 16
Cheese	what sort: cream, cottage, hard, soft; low fat; write in the name if you can	tablespoons or picture 2
Chips	fresh, frozen, oven, microwave or crinkle cut; type of fat for cooking	picture 7
Chocolate	what sort; plain, milk, white, diabetic; give brand name	number or bar weight
Chops	what sort; lean or fatty; large or small; fried, grilled or baked; well done or rare	number
Cider	sweet, dry, vintage, low alcohol	pints and half pints

WHERE POSSIBLE, ALWAYS STATE WHAT SORT OF OIL OR FAT WAS USED FOR BAKING, FRYING ETC.

Food/Drink	Description & Preparation	Amount
Coffee	with milk; half milk/half water; all milk, ground, instant, decaffeinated	cups or mugs
Condiments	pepper, salt or substitute	$^1/_2$ or $^1/_4$ teaspoon, pinch etc.
Cooking oil	type; brand name	teaspoons
Cream	half, single, sour, whipping, double, clotted; low fat; fresh or substitute; sweetened or unsweetened	tablespoons
Crisps	brand name; low fat; low salt	packet weight
Egg	how was it cooked: boiled, fried, scrambled, poached, omelette, etc.	number
Fish	what sort; fried, boiled, grilled, poached, microwaved; with batter or breadcrumbs; tinned with oil or tomato sauce	helping or picture 6
Fish cakes or fingers	what sort; large, medium or small size; fried or grilled	number
Fruit - fresh	what sort and variety eg. Cox apple; with or without skin	number
Fruit - stewed or canned	what sort and variety eg. Bramley apple; in fruit juice or syrup	tablespoons
Fruit - juice	what sort; sweetened or unsweetened	glasses or cups
Gravy	thick or thin, instant or packet, made with or without dripping or meat juices	tablespoons
Herbs	type, fresh or dried	$^1/_2$ or $^1/_4$ teaspoons
Honey, jam	type, specify if low sugar	teaspoons
Ice-cream	dairy or non-dairy; flavour or variety	tablespoons
Liver, kidney	pig, lamb, ox; fried or stewed	picture 4 or 5
Margarine	hard, soft, polyunsaturated, low fat, very low fat; give brand name	thick, average or thin spread
Marmalade	type and brand; specify if low sugar	teaspoons
Mayonnaise	give name and brand; state if low fat	teaspoons
Meat pie, pastie, pastry	what sort; individual or helping, fat used for pastry	number or picture 3

WHERE POSSIBLE, ALWAYS STATE WHAT SORT OF OIL OR FAT WAS USED FOR BAKING, FRYING ETC.

Food/Drink	Description & Preparation	Amount
Meats	what sort; lean or fatty; fried,microwaved, grilled, roast, barbequed; well-done or rare; with or without gravy, cut used	slices, helping or pictures 4 or 5
Milk - for drinking on its own or for cereals	full cream, silver top, semi-skimmed, skimmed, sterilized, UHT, flavoured, powdered, soya	pints, glasses or cups
Minced beef	on its own, with vegetables, fatty or lean	tablespoons or picture 5
Peanuts	dry roasted or ordinary salted	packet weight
Pickled,smoked or salted foods	what sort and brand; how eaten; eg. pastrami, haddock, turkey breast, paté	tablespoons or one of the pictures
Porridge	with sugar or honey; with milk or cream	bowls
Potatoes	baked, boiled, mashed, creamed, fried/chips, instant, roast; with butter	tablespoons, or pictures 10 or 11
Pudding	what sort and brand: e.g. steamed sponge; with fruit; pie (what sort); jelly; blancmange; mousse; instant desserts; milk puddings, give recipe	tablespoons, slices or pictures 3, 15 or 17
Rice	brown or white; boiled or fried; rice pudding	tablespoons or picture 8
Salad	describe ingredients, with dressing; what sort of dressing (e.g. oil and vinegar, salad cream, mayonnaise)	tablespoons or picture 14
Sandwiches and rolls	wholemeal, white or brown bread; type of filling; butter or margarine: large or small loaf; thick, medium or thin slices	number of rolls or slices of bread
Sauce - hot	(for vegetables, meat or fish; puddings) what sort; savoury or sweet; thick or thin, give recipe if possible	tablespoons or picture 12
Sauce - cold	what sort: e.g. tomato ketchup, brown sauce, soy sauce; salad cream; sweet or savoury	tablespoons or picture 12
Sausages	what sort: e.g. pork, beef, pork and beef; low fat; large or small; how cooked	number
Sausage rolls	large or small, type of pastry	number
Scones	what sort: with currants, sweet or plain; cheese	number
Snacks - in packet	what sort: e.g. cheese straws, Twiglets, pretzels (give brand name)	packet weight

WHERE POSSIBLE, ALWAYS STATE WHAT SORT OF OIL OR FAT WAS USED FOR BAKING, FRYING ETC.

Food/Drink	Description & Preparation	Amount
Soft drinks	squash, undiluted or diluted; fizzy drinks; low calorie; give brand name	glasses or cans
Soup	what sort; canned, packet, instant or vending machine, homemade; give brand name	tablespoons, bowl or mug
Soya, Quorn	TVP, mince, burgers or tofu	number or pictures 4 or 5
Spaghetti, other pasta	canned, boiled; white, wholemeal; in sauce	tablespoons or picture 9
Spices	type	½ or ¼ teaspoons
Spreads	on bread, what sort and brand	½ or ¼ teaspoons
Spirits	what sort: e.g. whisky, gin, vodka, rum; at home or in pub	single measures as in pub
Sugar	added to cereals, tea, coffee, fruit etc.	heaped or level teaspoons
Sweets	what sort: e.g. toffees or boiled sweets; diabetic; give brand name	number
Tea	with or without milk; herb, decaffeinated	cups
Vegetables	what sort and variety; with butter, other fat or sauce; how cooked eg. fried, boiled, microwaved, or raw	tablespoons or pictures 12, 13 or 14
Water	state whether tap, filtered, or bottled	glasses
Wine, sherry, port	white, red; sweet, medium, dry; low alcohol	glasses
Yogurt, fromage frais	what sort: e.g. with fruit, natural, plain; flavour; low fat, Greek, creamy, soya	cartons, tablespoons
Homemade dishes	Please say what the dish is called and give recipe or ingredients if possible	tablespoons, or one of the pictures
Ready-made meals	what sort: e.g. pizzas, microwave dishes, slimmers' meals etc. Please give main ingredients on packet, and enclose label and bar code if possible	weight from packet
Meals eaten away from home	what sort: e.g. pizzas, Chinese, Indian dishes, fish and chips, hamburgers, hot dogs etc. Please say what the dish is called and give ingredients where possible. Give the name of the restaurant if it is a well known chain	tablespoons or one of the pictures

Use the pictures to help you to indicate the size of the portion you have eaten. Write down the picture number and size nearest to your own helping eg. 2a, 3b, 1c etc.

The pictures could also be used for foods not shown, e.g. fruit crumble might be a similar portion to shepherd's pie, fruit cake similar to veal and ham pie, and baked beans similar to peas.

Remember that the picture sizes are much smaller than life size. The shaded circle in the background shows the size of the dinner plates used. Please note that items such as cake are photographed on a tea plate which is placed on top of a dinner plate.

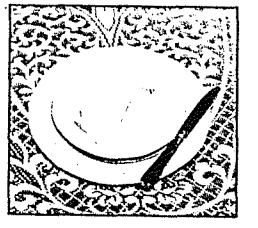
1 a

1 b

1 c

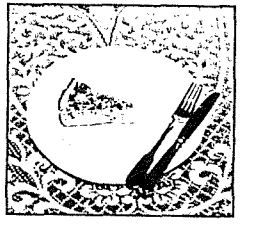
2 a

2 b

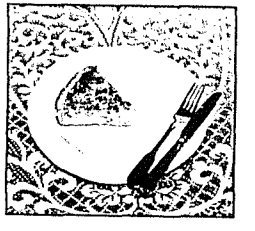


2 c

3 a

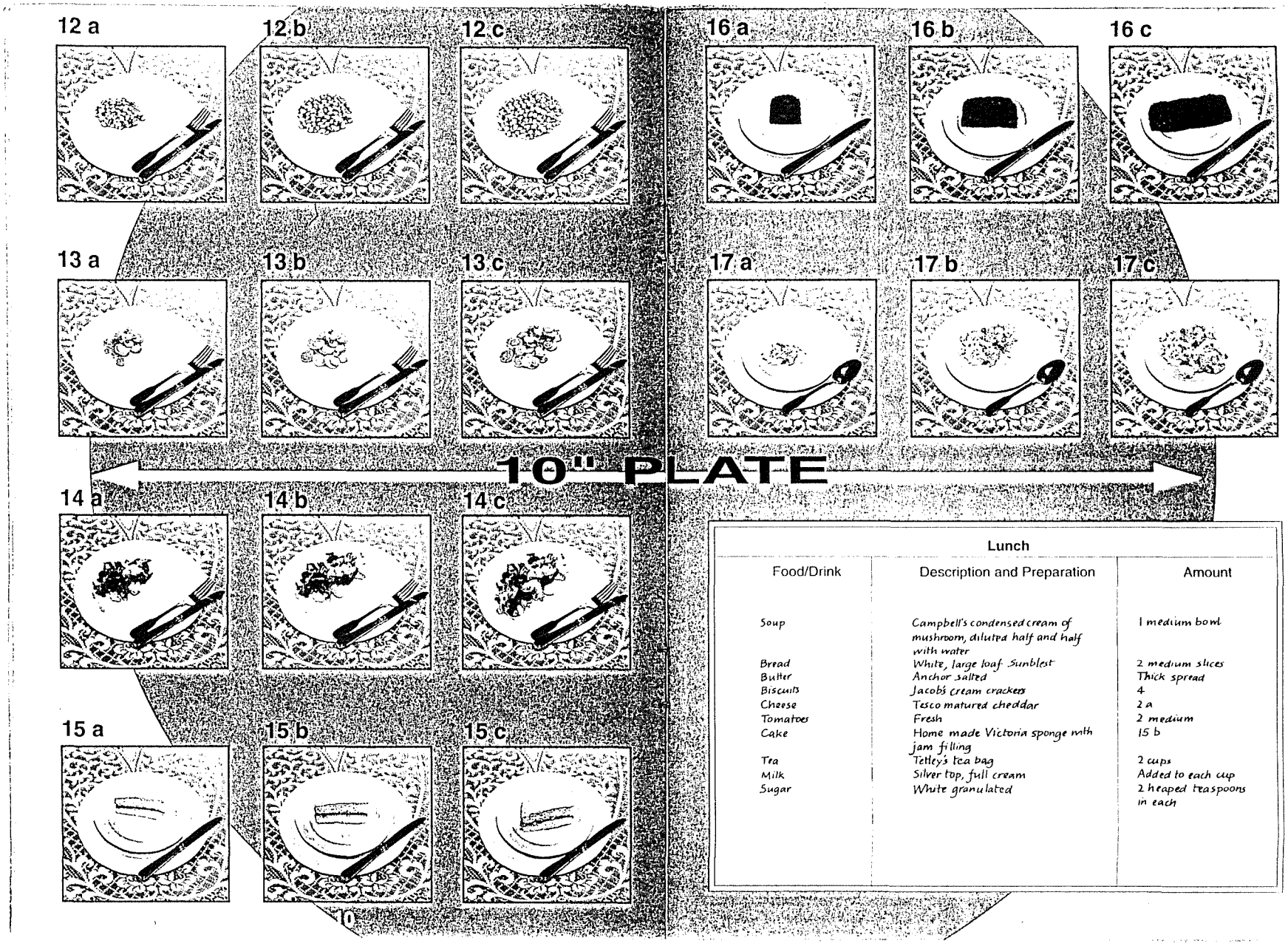


3 b



3 c

10" PLATE
4 a
4 b
4 c
5 a
5 b
5 c
6 a
6 b
6 c
7 a
7 b
7 c
8 a
8 b
8 c
9 a
9 b
9 c
10 a
10 b
10 c
11 a
11 b
11 c



Lunch		
Food/Drink	Description and Preparation	Amount
Soup	Campbell's condensed cream of mushroom, diluted half and half with water	1 medium bowl
Bread	White, large loaf Sunblest	2 medium slices
Butter	Anchor salted	Thick spread
Biscuits	Jacobs cream crackers	4
Cheese	Tesco matured cheddar	2 a
Tomatoes	Fresh	2 medium
Cake	Home made Victoria sponge with jam filling	15 b
Tea	Tetley's tea bag	2 cups
Milk	Silver top, full cream	Added to each cup
Sugar	White granulated	2 heaped teaspoons in each

DATE					1	9			DAY OF WEEK	

BEFORE BREAKFAST

Food/Drink	Description and Preparation	Amount

BREAKFAST

Food/Drink	Description and Preparation	Amount

MID MORNING - between breakfast time and lunch time

Food/Drink	Description and Preparation	Amount

LUNCH		
Food/Drink	Description and Preparation	Amount
TEA - between lunch time and the evening meal		
Food/Drink	Description and Preparation	Amount

EVENING MEAL		
Food/Drink	Description and Preparation	Amount

LATER EVENING - up to last thing at night		
Food/Drink	Description and Preparation	Amount

BETWEEN MEALS, SNACKS AND DRiNKS if not already written in before		
Food/Drink	Description and Preparation	Amount
Chocolate		
Toffees, sweets		
Crisps		
Peanuts		
Other snacks		
Beer		
Wine		
Sherry		
Spirits		
Other cold drinks		
Tea		
Coffee		
Other hot drinks		
Ice cream		
Anything else?		

Space to write in the recipe or ingredients of any homemade dishes, take away meals etc. that you have mentioned but not described previously

END OF DAY No. 6

GENERAL QUESTIONS ON YOUR DIET LAST WEEK

1. Which type of milk did you most often use last week? **Select one only**

Full cream, silver	☐	Semi-skimmed, red/white	☐
Skimmed /fat free	☐	Channel Islands, gold	☐
Sterilized	☐	Dried milk	☐
Soya	☐	Homogenized	☐
None	☐		
Other	☐	State type	

2. How much milk did you usually have in tea?

A lot	☐	Average	☐	Hardly any	☐
None	☐	I did not drink tea	☐		

3. How much milk did you usually have in coffee?

A lot	☐	Average	☐	Hardly any	☐
None	☐	I did not drink coffee	☐		

4. Did you drink decaffeinated tea?

Always	☐	Sometimes	☐	Never	☐

5. Did you drink decaffeinated coffee?

Always	☐	Sometimes	☐	Never	☐

6. Which types of fat did you most often use for baking, frying, spreading and salads?

	Brand name & type used	What did you use it for? Baking	Frying	Spreading	Salads
Butter		☐	☐	☐	☐
Low fat spread		☐	☐	☐	☐
Very low fat spread		☐	☐	☐	☐
Polyunsaturated margarine		☐	☐	☐	☐
Other soft margarine		☐	☐	☐	☐
Hard margarine		☐	☐	☐	☐
Vegetable oils		☐	☐	☐	☐
		☐	☐	☐	☐
		☐	☐	☐	☐
White vegetable fat		☐	☐	☐	☐
Lard		☐	☐	☐	☐
Dripping		☐	☐	☐	☐
Other		☐	☐	☐	☐

7. How thickly did you spread butter, margarine etc. on bread or biscuits?

Thickly spread	☐	Medium	☐	Thinly spread	☐

8. What did you do with the visible fat on meat?

Ate most of the fat	☐	Ate some of the fat	☐
Ate as little as possible	☐	Did not eat meat	☐

9. Which type of bread did you usually eat? **Select one only**

White	☐	Granary	☐
Brown, wheatgerm, Hovis	☐	Wholemeal	☐
Soft grain	☐		
Other	☐	Name	

10. Did you eat the skin on apples, pears, peaches etc?

Yes	☐	No	☐	Did not eat these fruits	☐

11. Was salt usually added to your food during cooking this week?

Yes	☐	No	☐	Don't know	☐

Did you usually add salt to your food at the table this week?

Yes	☐	No	☐	Don't know	☐

Did you regularly use a salt substitute (eg. LoSalt) this week?

Yes	☐	No	☐	Don't know	☐

If YES, which brand

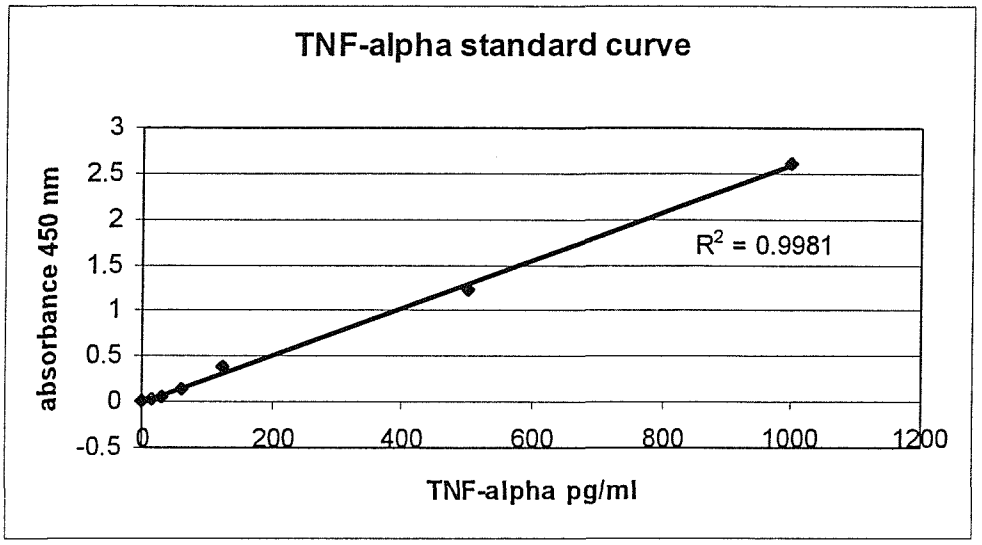
12. Please name any vitamins, minerals or other food supplements taken on each day of last week.

	Brand and name	Amount taken
Day 1		
Day 2		
Day 3		
Day 4		
Day 5		
Day 6		
Day 7		

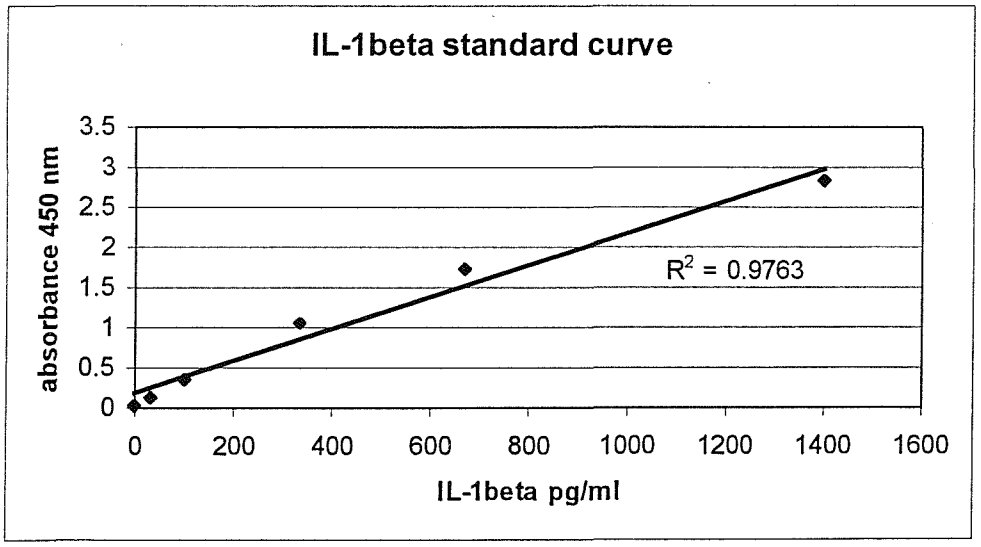
If you have any queries, please contact:
Dr Liz Miles, Fiona Wallace or Dr Philip Calder
Department of Human Nutrition
University of Southampton
Tel 01703 594689/594320

Appendix 3: Chapter 7 (Human study) ELISA standard curves

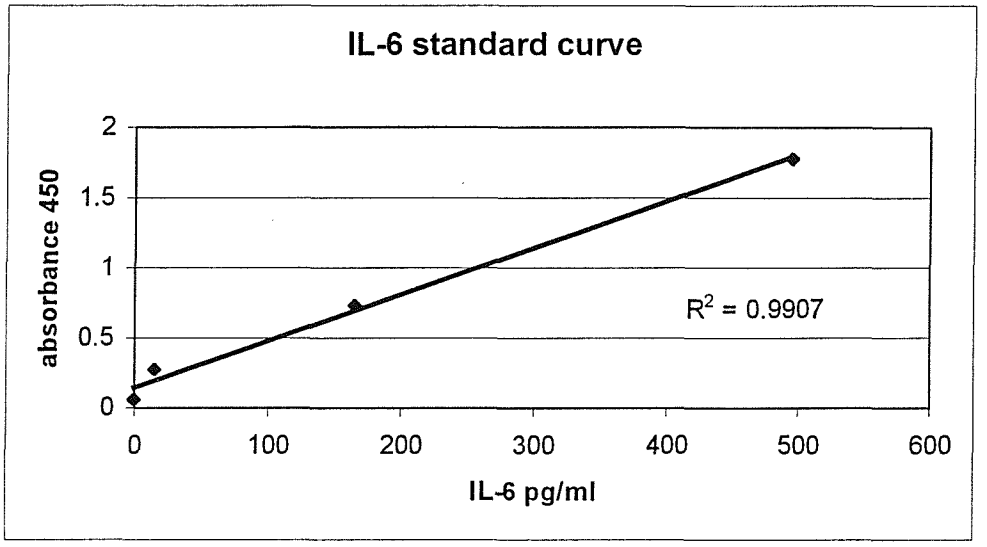
3.1 TNF-α standard curve



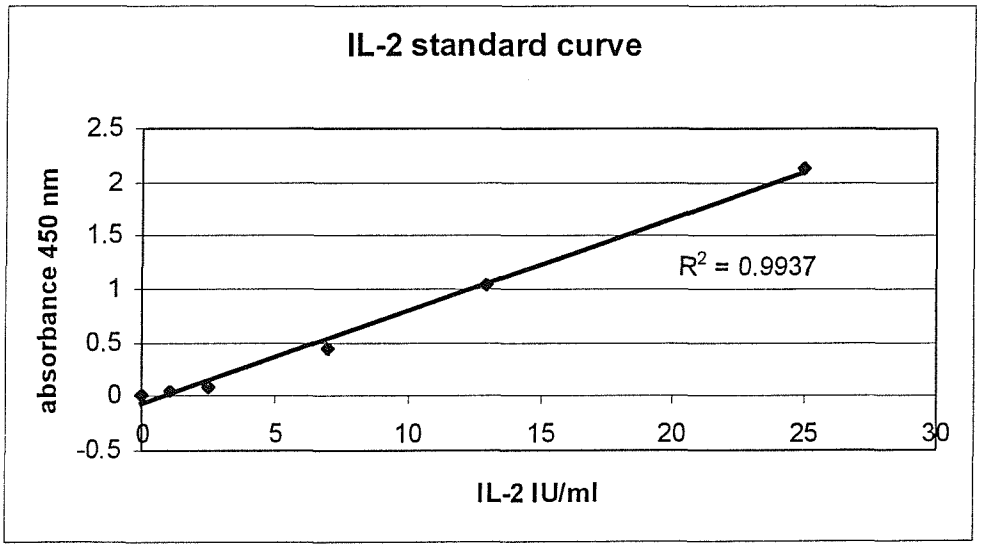
3.2 IL-1β standard curve



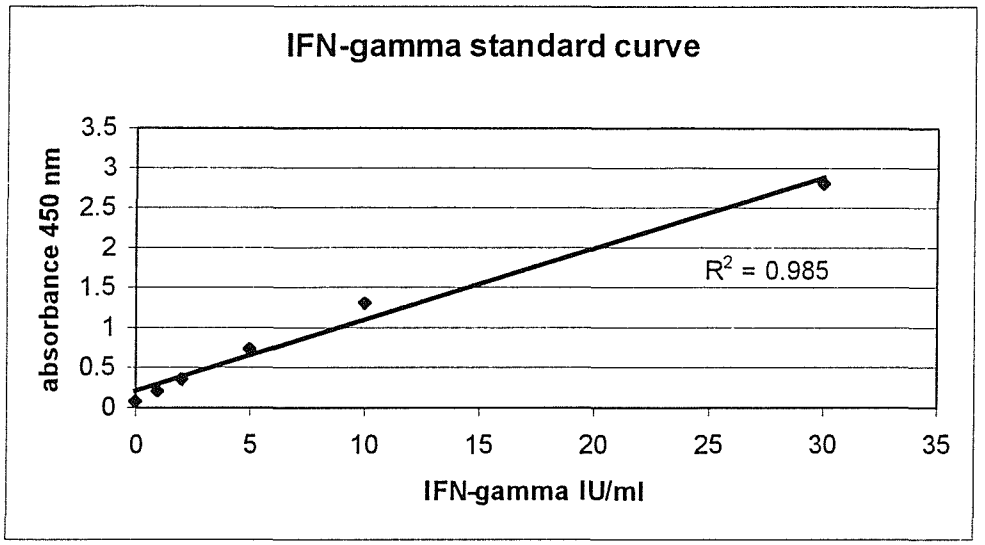
3.3 IL-6 standard curve



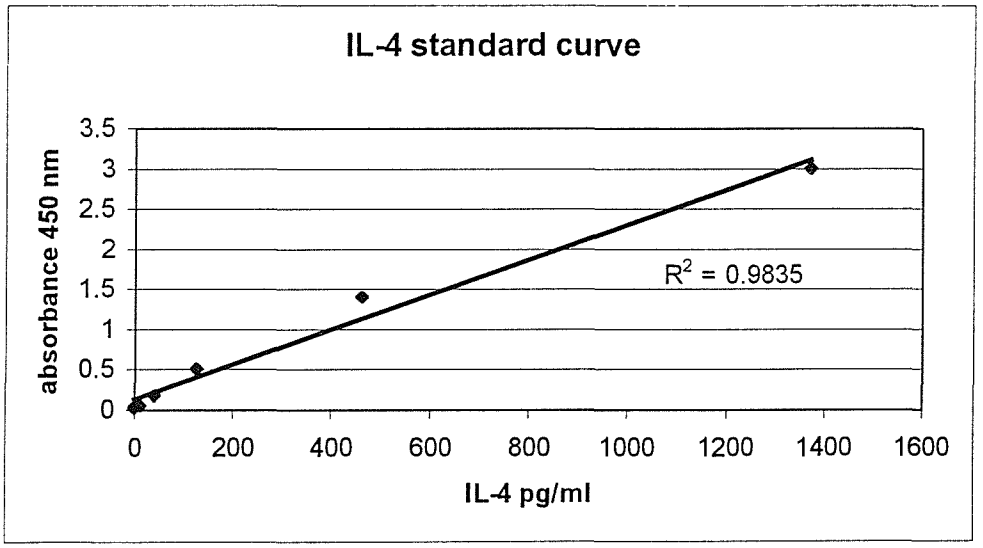
3.4 IL-2 standard curve



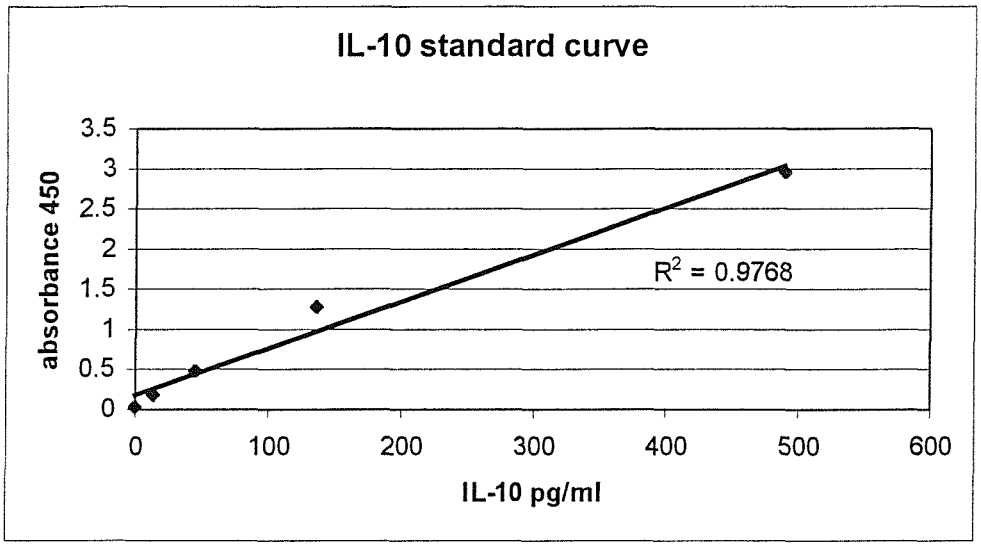
3.5 IFN-γ standard curve



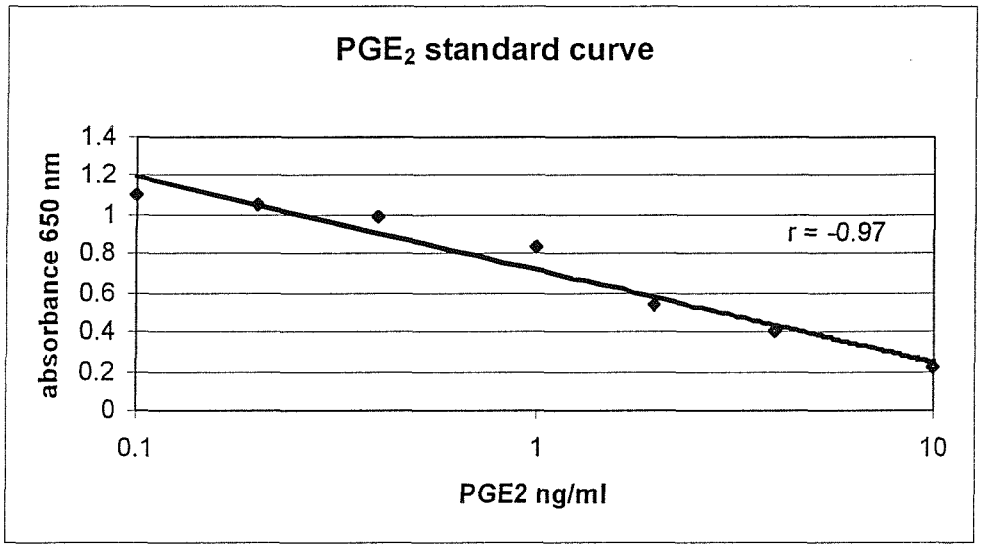
3.6 IL-4 standard curve



3.7 IL-10 standard curve



3.8 PGE_2 standard curve



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