British Journal of Nutrition (2001), **85**, 251–269
DOI: 10.1079/BJN2000239

Review article

Antioxidants and fatty acids in the amelioration of rheumatoid arthritis and related disorders

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(Received 5 August 1999 – Revised 16 August 2000 – Accepted 21 September 2000)

The generation of reactive oxygen species (free radicals) is an important factor in the development and maintenance of rheumatoid arthritis in humans and animal models. One source of free radicals is nitric oxide produced within the synoviocytes and chondrocytes and giving rise to the highly toxic radical peroxynitrite. Several cytokines, including tumour necrosis factor-α (TNFα) are involved in the formation of free radicals, partly by increasing the activity of nitric oxide synthase. Indeed, nitric oxide may mediate some of the deleterious effects of cytokines on bone resorption. Aspirin, tetracyclines, steroids and methotrexate can suppress nitric oxide synthase. Dietary antioxidants include ascorbate and the tocopherols and beneficial effects of high doses have been reported especially in osteoarthritis. There is also evidence for beneficial effects of β-carotene and selenium, the latter being a component of the antioxidant enzyme glutathione peroxidase. The polyunsaturated fatty acids (PUFA) include the *n*-3 compounds, some of which are precursors of eicosanoid synthesis, and the *n*-6 group which can increase formation of the pro-inflammatory cytokines TNFα and interleukin-6, and of reactive oxygen species. Some prostaglandins, however, suppress cytokine formation, so that *n*-3 PUFA often oppose the inflammatory effects of some *n*-6-PUFA. γ-linolenic acid (GLA) is a precursor of prostaglandin E_1, a fact which may account for its reported ability to ameliorate arthritic symptoms. Fish oil supplements, rich in *n*-3 PUFA such as eicosapentaenoic acid have been claimed as beneficial in rheumatoid arthritis, possibly by suppression of the immune system and its cytokine repertoire. Some other oils of marine origin (e.g. from the green-lipped mussel) and a range of vegetable oils (e.g. olive oil and evening primrose oil) have indirect anti-inflammatory actions, probably mediated via prostaglandin E_1. Overall, there is a growing scientific rationale for the use of dietary supplements as adjuncts in the treatment of inflammatory disorders such as rheumatoid arthritis and osteoarthritis.

Arthritis: Oils: Antioxidants: Fatty acids: Free radicals

Rheumatoid arthritis as an inflammatory disorder

Rheumatoid arthritis (RA) is a chronic, systemic disorder with symmetrical, inflammatory polyarthritis which may produce progressive joint damage, and extra-articular involvement of many organs. Inflammation of the joint tissues is associated with the release of toxic substances in the synovium which lead to cartilage destruction. There is

Abbreviations: COX, cyclo-oxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPO, evening primrose oil; GLA, γ-linolenic acid; GSHpx, glutathione peroxidase; IL-1, interleukin-1; iNOS, inducible nitric oxide synthetase; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; NFκB, nuclear factor κB; NOS, nitric oxide synthetase; NSAID, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; PARS, poly(ADP-ribose) synthetase; PGE_2, prostaglandin E_2; PUFA, polyunsaturated fatty acids; RA, rheumatoid arthritis; ROS, reactive oxygen species; SAARD, slowly-acting anti-rheumatic drugs; SNAP, *S*-nitroso-*N*-acetyl penicillamine; SOD, superoxide dismutase; Th1, T helper 1; TNFα, tumour necrosis factor-α.

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joint swelling with morning stiffness, fatigue and malaise. RA has a prevalence of about 2 % worldwide, but is three times more common in women than men and can begin at any age.

The cause of RA remains unclear but it is now generally accepted that it is an autoimmune disease with auto-antibodies (including rheumatoid factor – a circulating IgM immunoglobulin present in about 80 % of patients), immune complexes, locally synthesised immunoglobulins and lymphokines in the synovial fluid, defective cell-mediated immunity and an association with other auto-immune diseases.

Treatment is normally with analgesics, anti-inflammatory and anti-rheumatic drugs, corticosteroids and anti-tumour necrosis factor-α (anti-TNFα) agents but increasing evidence for the role played by free radicals suggests that antioxidant therapy may represent an alternative approach. There is also increasing evidence that dietary fatty acids can modify the generation of cytokines and eicosanoids in ways which can influence patient symptoms and the course of the disease, and it is the function of this review to summarise some of the key findings in these two areas.

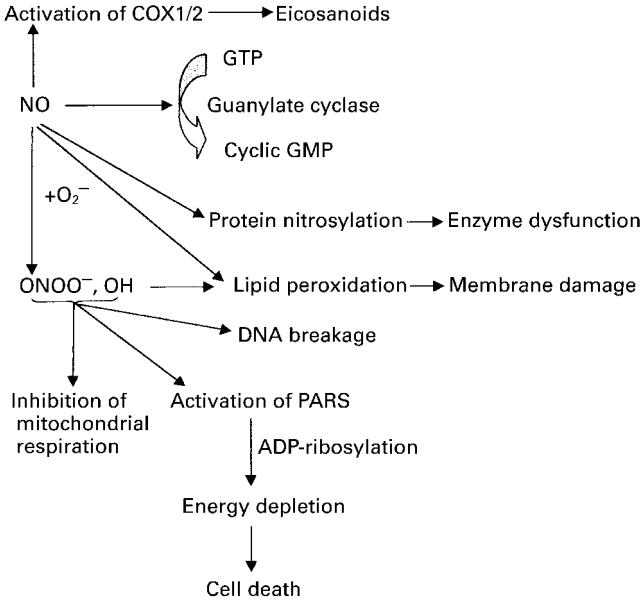

Fig. 1. A summary of the mechanisms by which nitric oxide (NO) can induce tissue damage, especially after conversion to the peroxynitrite radical (ONOO-) and the hydroxyl radical.

Antioxidants

Reactive oxygen species and rheumatoid arthritis

Reactive oxygen species (ROS) are highly reactive atoms and molecules with unpaired electrons. They include superoxide anions ($O_2^{-\bullet}$), formed when molecular oxygen acquires an additional electron, hydrogen peroxide, hydroxyl and peroxynitrite radicals, and nitric oxide. The first three of these are produced by xanthine oxidase and are also generated by activated macrophages and neutrophils as a result of respiratory chain activity known as the 'oxidative burst'. This is primarily due to NADPH oxidase activity leading to the formation of hypochlorous acid (HOCl) as a bactericidal agent. Hydrogen peroxide is formed partly by superoxide dismutase (SOD), by the reaction between superoxide radicals and protons. Hydrogen peroxide is metabolised by catalase and peroxidase enzymes, chiefly glutathione peroxidase.

Oxidative stress All of these ROS can cause oxidative stress – major cellular damage produced as a result of chain reactions leading to a disruption of macromolecular structure. The unsaturated fatty acid components of the cell wall are a major target, easily reacting with ROS to accept an extra electron which induce covalent interactions between neighbouring molecules and causing severe disruption to membrane function. This aspect of ROS activity is readily quantifiable by the measurement of lipid peroxidation products such as 4-hydroxynonenal and malondialdehyde. The latter can then react with lysine residues in proteins to produce immunogenic molecules which can exacerbate inflammation. 4-Hydroxynonenal can also directly suppress mitochondrial respiration (Picklo *et al.* 1999) and monoamine transporter function (Morel & Baroiki, 1998) both of which may further compromise cellular viability.

ROS may also damage nucleic acid structure, compromising cell survival directly and potentially modifying gene expression, leading to disorders of cell proliferation. The oxidation of thiols and the formation of carbonyl groups on proteins can lead to widespread deterioration in cell viability, wih loss of receptor, enzyme and transporter functions (Brown-Galatola & Hall, 1992).

Nitric oxide The reaction of nitric oxide (NO) with superoxide generates peroxynitrite (Beckman *et al.* 1994) which, under the acid conditions often found in regions of inflammation and ischaemia, yields the hydroxyl radical $OH^{\bullet}$, the most highly reactive and toxic of the ROS (Fig. 1). The study of experimental arthritis in animals has confirmed an increased activity of inducible NO synthetase (iNOS) (McCartney-Francis *et al.* 1993; Sakurai *et al.* 1995) with a raised production of NO (Cannon *et al.* 1996; Grabowski *et al.* 1996*a*; Yang *et al.* 1998). The inhibition of NOS can suppress disease activity in parallel with a fall in plasma nitrotyrosine or nitrite (McCartney-Francis *et al.* 1993; Kaur & Halliwell, 1994; Connor *et al.* 1995; Cannon *et al.* 1996; Santos *et al.* 1997; Stichtenoth & Frolich, 1998). There is an increased activity of NOS in MRL-lpr/lpr mice (a strain which shows pronounced lymphoproliferative activity and develops severe autoimmune disorders) and enzyme inhibition reduces the degree of arthritis (Weinberg, 1998).

However, in one study, NOS inhibition only reduced adjuvant-induced arthritis if injected before or close to the time of adjuvant application, not if administered after the establishment of inflammation. This suggests that NO may be involved in the initial stages of RA but not in the maintenance of chronic inflammation and subsequent joint destruction (Fletcher *et al.* 1998).

Poly(ADP-ribose) synthetase (PARS) is one of the regulators of the expression of NOS and collagenase (Szabo, 1998; Szabo *et al.* 1998). It is activated as a result of free-radical-induced DNA breakage, when it transfers

adenosine diphosphate-ribose to nuclear proteins and depletes cells of intracellular NAD^+, leading to acute cell dysfunction and apoptosis (Fig. 1). Inhibitors of the enzyme can protect against cell damage and reduce the inflammation in arthritis models (Miesel *et al.* 1995). Similarly, fibroblasts from PARS (−/−) knockout mice possess reduced iNOS activity and a reduced synthesis of NO by cells when stimulated (Szabo *et al.* 1998).

Nitric oxide and bone A biphasic effect of NO on bone metabolism has often been observed. At high levels, NO antagonises the effects of prostaglandin E_2 (PGE_2) and inhibits bone resorption by depressing the growth and development of osteoblasts (Ralston, 1997). At low levels, NO potentiates the cytokine-promoted resorption of bone and has been considered essential for osteoclast activity.

Cytokines and the generation of reactive oxygen species

A number of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) appear to be associated with joint inflammation and their secretion can be suppressed by anti-inflammatory agents such as steroids (Barnes & Adcock, 1993). The plasma levels of TNFα are correlated directly with the ability of phagocytes to generate superoxide (Miesel *et al.* 1996*a*,*b*) although there is no correlation with C-reactive protein. The removal of TNFα by dialysis diminished superoxide generation to control levels in RA patients, implying an important intermediate role for the cytokine. Both TNFα and interferon-γ increase the secretion of hydrogen peroxide by rabbit chondrocytes (Tiku *et al.* 1990).

Oxidative stress leads to the activation of the nuclear transcription factor (NFκB), which is normally held in an inactive form complexed with protein 1κB. Oxidants and cytokines activate NFκB which then binds to and activates genes regulating the expression of cytokines and acute phase proteins (Morel & Baroiki, 1998; Chen *et al.* 1999). A positive feedback cycle is thus initiated which may cause severe tissue injury unless it is broken. Glucocorticoids inhibit the activation of NFκB (Barnes, 1997).

Nitric oxide and cytokines NOS is induced by several cytokines, including IL-1β and TNFα (Fig. 1), in human chondrocytes (Rediske *et al.* 1994; Sakurai *et al.* 1995; Perkins *et al.* 1998; Stichtenoth & Frolich, 1998), while glucocorticoids prevent this induction and reduce disease activity in parallel (Stichtenoth *et al.* 1995). NOS activity in blood mononuclear cells correlates with disease severity (St. Clair *et al.* 1996), while antibodies to TNFα reduce disease severity and decrease NOS activity (Perkins *et al.* 1998). The greater amount of NO produced in RA is reflected in increased levels of nitrotyrosine and nitrite or nitrate in the serum and synovial fluid of patients but not in normal subjects (Farrell *et al.* 1992; Kaur & Halliwell, 1994). Hydroxy-L-arginine is probably a better measure of NO formation than nitrate since the latter is influenced by diet (Wigand *et al.* 1997).

Interleukin-1β and TNFα increase NO and PGE_2 formation and bone resorption (Ralston & Grabowski, 1996) (Fig. 2). The resorption was prevented by indomethacin or the NOS inhibitor L-monomethyl-L-arginine, indicating that both cyclo-oxygenase (COX) and NOS are

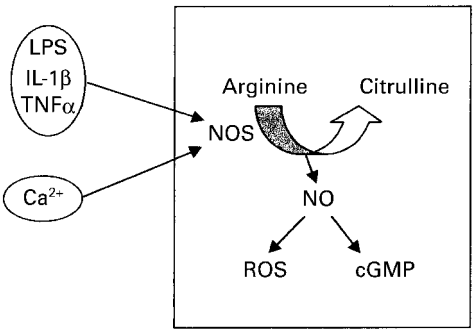

Fig. 2. The activation of nitric oxide synthase (NOS) can be induced by lipopolysaccharide (LPS), and several cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNFα). These and other stimuli raise the intracellular calcium levels which activate the enzymic conversion of arginine to citrulline with the release of NO which subsequently activates the guanylate cyclase system.

able to mediate the cytokine effect (Fig. 1). In bovine cultured chondrocytes and human osteoarthritis (OA) cartilage explants, IL-1β and TNFα induced both NOS and COX. If NOS activity was inhibited, COX was also inhibited, suggesting that NO may be an important modulator of COX. This view was supported by evidence that NO donors increase, whereas NO scavengers inhibit, prostanoid synthesis (Manfield *et al.* 1996). The induction of NOS by IL-1β may involve the formation of IL-18, which is increased by IL-1β and which is itself able to increase NOS, NO production and COX, and to increase the breakdown of human cartilage (Olee *et al.* 1999).

In addition to its activity as a free radical and its ability to lead to the formation of OH^-, NO may modulate the activity of enzymes involved in joint maintenance. TNFα and IL-1β increase the activities of NOS and collagenase (a metalloproteinase) in explants of bovine and human cartilage. NOS inhibition prevented the collagenase activation, while NO donors such as *S*-nitroso-*N*-acetyl-penicillamine (SNAP) increased the activity (Murrell *et al.* 1995).

Bone metabolism In rat osteoblasts, fibroblasts and chondrocytes, as well as explants or cultures of human cartilage from patients with RA or OA, combinations of cytokines including IL-1β TNFα and interferon-γ induce NOS activity (Grabowski *et al.* 1996*b*; Murrell *et al.* 1996; Miyasaka, 1997; Amin & Abramson, 1998). The release of NO and PGE_2 by cartilage explants from human OA patients was reduced by IL-1β-receptor antagonist, suggesting an essential role for IL-1β in their synthesis (Attur *et al.* 1998). Since the soluble TNFα-receptor did not share this action, this cytokine would appear to be less crucial. The levels of serum NO correlate with the amounts of TNFα and IL-6 as well as disease activity, especially joint stiffness (Ueki *et al.* 1996). The increased levels of NO suppressed osteoblast activity, as measured by the amounts of DNA synthesis, cell proliferation and osteocalcin production (Hukkanen *et al.* 1995; Ralston, 1997), depressed chondrocyte function and promoted apoptosis (Amin & Abramson, 1998).

There is a complex interplay between several cytokines in the regulation of lymphocyte T helper cell subsets, and it

is likely that some of those interactions are mediated by NO. The inhibitory effect of IL-10 on T helper type 1 (Th1) cells, for example, is produced by the down-regulation of the expression of the IL-12 gene and this, in turn, is regulated by NO. NO donors such as SNAP induced IL-12 whereas NOS inhibition suppressed its expression (Rothe *et al.* 1996).

Effects of reactive oxygen species

Hydroxyl radicals, in particular, cause the breakdown of hyaluronic acid (Rowley *et al.* 1984; Grootveld *et al.* 1991) but can also disrupt proteoglycans (Cooper *et al.* 1985), collagens (Davies *et al.* 1993) and tissue and fluid proteinase inhibitors such as α-antiproteinase (Wasil *et al.* 1987). They may also induce covalent cross-linking of immune complexes (Uesugi *et al.* 1998). Superoxide anions can affect adversely the structure and integrity of collagen *in vitro* and may *in vivo* cause depolymerisation of hyaluronate in synovial fluid (Grootveld *et al.* 1991; Davies *et al.* 1993).

Direct confirmation that hydrogen peroxide can produce severe tissue damage and arthritis has come from injections of a peroxide-generating system (glucose oxidase) into the joints of mice (Schalkwijk *et al.* 1986; Kasama *et al.* 1988). The induction of experimental arthritis in mice by collagen injections increases xanthine oxidase (XO) activity in the serum and joint tissues.

Reactive oxygen species in humans

Following the initiation of the autoimmune process, activated macrophages and neutrophils accumulate in the synovial fluid. Rheumatoid pannus contains many macrophages that can liberate ROS (McCord 1974; Gutteridge *et al.* 1982; Gutteridge 1987; Nurcombe *et al.* 1991; Farrell *et al.* 1992; Robinson *et al.* 1992), leading to joint damage (Blake *et al.* 1981; Rowley *et al.* 1984; Cooper *et al.* 1985; Schalkwijk *et al.* 1986; Chapman *et al.* 1989; Situnayake *et al.* 1991; Davies *et al.* 1993). There is strong evidence to suggest that hydroxyl radicals are generated in the synovial fluid of arthritic subjects (Kaur *et al.* 1996) and there are raised levels of peroxidation markers, accompanied by low levels of SOD activity, in cases of juvenile arthritis (Sklodowska *et al.* 1996; Araujo *et al.* 1998). In adult RA patients, stimulated phagocytes produced greater levels of superoxide compared with controls and subjects with non-rheumatic internal disorders. The release of superoxide by neutrophils is inhibited by α_1-antitrypsin, the levels of which are elevated in RA serum, so that it may play a compensatory role in limiting the inflammatory process (Miesel *et al.* 1996*a*,*b*).

Superoxide ($O_2^{-\cdot}$) and H_2O_2 become converted into the highly reactive hydroxyl radical ($OH^{\cdot}$) in the presence of free ferrous ions and synovial fluid from RA patients often contains measurable quantities of iron (Gutteridge, 1987) capable of catalysing oxidative damage *in vivo* (Gutteridge *et al.* 1982).

The ability of sera to resist attack by peroxyl radicals is less in RA patients than healthy controls and varies inversely with disease severity. Resistance is mainly due to uric acid, the levels of which are also inversely correlated with disease activity. In control subjects, resistance is largely due to the serum levels of vitamin E (Situnayake *et al.* 1991).

Xanthine oxidase The activity of XO is increased up to fifty-fold in the serum of RA patients (Miesel & Zuber, 1993; Miesel *et al.* 1994; Zuber & Miesel, 1994) compared with healthy controls or patients with other disorders. The combination of xanthine oxidase with acetaldehyde is especially potent in producing ROS, a finding which may suggest that RA patients should be advised to avoid or limit their alcohol intake. The inhibition of xanthine oxidase by allopurinol reduces significantly the oxidative burst in leucocytes. The administration of cortisone rapidly restores xanthine oxidase levels to those of control subjects in parallel with improvement in disease activity.

Nitric oxide The inducible form of the synthetic enzyme nitric oxide synthetase (iNOS) is found primarily in synovial lining cells (especially $CD68^+$ macrophages and fibroblasts and type A synoviocytes), chondrocytes and endothelial cells (Grabowski *et al.* 1997). The synovial cells appear to the primary source in RA, whereas the chondrocytes form the primary source in OA (Melchiorri *et al.* 1998). Cells from more superficial regions of human or bovine cartilage explants produced more NO than deep tissue when stimulated with bacterial lipopolysaccharide, TNFα or IL-1β (Hayashi *et al.* 1997). NOS is undetectable in tissue form normal subjects.

Reactive oxygen species and ischaemia – reperfusion

In view of the presence of inflammation in the joint, movement and rest induce alternating periods of relative ischaemia and reperfusion (Blake *et al.* 1989). Such changes, which in other tissues such as the heart and brain have been clearly associated with the generation of ROS as a direct result of the re-introduction of calcium ions enhancing the activity of NOS and related enzymes. It appears, however, that this situation occurs primarily in severely inflamed synovial tissue of human RA and OA patients. Thus, the generation of free radicals seems to be under the control of xanthine oxidase, since inhibitors of this enzyme prevent ROS generation during reperfusion (Singh *et al.* 1995). Such an increased formation of ROS has been shown directly using spin-trap procedures (Henderson *et al.* 1991) and correlated with the increased reperfusion-induced damage to proteins, lipids and glycosaminoglycans (Dowling *et al.* 1990; Henderson *et al.* 1991; Merry *et al.* 1991) as well as the depolymerisation of hyaluronate (Grootveld *et al.* 1991).

Clinical studies of dietary antioxidants in rheumatoid arthritis and osteoarthritis

Many raw foods contain natural antioxidants, including enzymes such as superoxide dismutase, glutathione peroxidase and catalase, which are usually inactivated during food processing, and non-enzymic antioxidants such as carotenoids (e.g. canthaxanthin and astaxanthin in some farmed fish), β-carotene, lutein, lycopene, tocopherols (in oils) and other phenolic compounds in plants. The latter

include other carotenoids and ascorbate (vitamin C). The plasma concentrations of these are largely determined by dietary intake. Two or more antioxidants can act together synergistically (Chaudiere & Ferrari-Iliou, 1999; Lakatos & Szentmihalyi, 1999; Pryor, 2000).

In view of the high antioxidant content of the French diet, rich in fruit, vegetables and red wine (Renaud & De Lorgeril, 1992), it is intriguing to note the relatively low incidence of RA in France (Guillemin *et al.* 1994).

More work is required on tissue distributions and bioavailability of antioxidant molecules within joints since lipophilic antioxidant molecules, such as vitamin E or β-carotene, may not have the same access to tissues as hydrophilic antioxidants, such as vitamin C. It may be, therefore, that different effects in disease processes may depend on the hydrophilicity of the antioxidant molecules concerned and the resulting pattern of tissue distribution in different tissue areas. One major problem is that there is no test currently available to measure oxidant activity within joints themselves and activity could occur from an alternative mechanism.

Vitamin E and vitamin C

α-Tocopherol is the most biologically important form of vitamin E. It scavenges free radicals before these can initiate a destructive chain reaction. Such 'chain-breaking' antioxidants are consumed in the process, although vitamin E may be regenerated by GSH and by ascorbic acid. Most cells contain enzymes which reduce dehydroascorbate back to ascorbate using either reduced NADH or GSH. Since dehydroascorbate is a very unstable molecule, however, there is an overall loss of ascorbate at sites of oxidative damage (Chaudiere & Ferrari-Iliou, 1999).

Vitamins C and E also have non-antioxidant effects. Ascorbate stimulates procollagen secretion (Henderson *et al.* 1991) and vitamin C deficiency is associated with defective connective tissue. Vitamin C is needed for the vitamin C-dependent enzyme lysyl-hydroxylase for the post-translational hydroxylation of specific prolyl and lysyl residues in procollagen – actions necessary for the stabilisation of the mature collagen fibril (Dowling *et al.* 1990). Vitamin C is also thought to be necessary for glycosaminoglycan synthesis (Merry *et al.* 1991).

Vitamin E blocks arachidonic acid formation from phospholipids and inhibits lipoxygenase activity, resulting in a mild anti-inflammatory effect. Benefit from vitamin E treatment has been claimed from several small studies of human OA (Doumerg, 1969; McAlindon & Felson, 1997). However, combined supplementation with vitamins C and E is more immunopotentiating than supplementation with either vitamin alone in healthy adults (Jeng *et al.* 1996).

Data from the Framingham Knee OA Cohort Study (McAlindon *et al.* 1996) did not support the hypothesis that diets rich in antioxidant micronutrients reduced the risk of incident knee OA but they did suggest that antioxidants might protect people with established disease from disease progression. Using radiographic parameters of progression, there was a three-fold reduction in risk for those in the middle and highest tertiles for vitamin C intake (adjusted odds ratio AOR 0·3, 95 % CI 0·1, 0·6). Those in the highest tertile for vitamin C intake also had reduced risk for developing knee pain (AOR 0·3; 95% CI 0·1, 0·8). Reduction in risk of progression was also seen for β-carotene (AOR 0·4; 95% CI 0·2, 0·9) and vitamin E (AOR = 0·7; 95% CI 0·3, 1·6) but less consistently since the β-carotene association diminished substantially after adjustment for vitamin C, and the vitamin E effect was seen only in men.

Vitamin A

Fairney *et al.* (1988) reported a difference in vitamin A metabolism or intake between patients with RA and controls: serum retinol and retinol binding protein levels were lower in RA than in matched control sera ($P < 0{\cdot}01$) and in OA patients ($P < 0{\cdot}001$).

β-*Carotene* If the diet of healthy volunteers is supplemented with β-carotene, significant increases can be demonstrated in the percentage of monocytes expressing the major histo compatibility class II molecule, HLA-DR, and the adhesion molecules intracellular adhesion molecule-1 and leucocyte function-associated molecule (Hughes *et al.* 1997). β-Carotene may also quench singlet oxygen which may reduce the free radical burden and protect membrane lipids from peroxidation.

Fourteen of over 1400 people studied for antioxidant status in Finland developed RA and their antioxidant status, measured by a combination of β-carotene, vitamin E and selenium, was significantly lower than that of other subjects (Heliovaara *et al.* 1994). Low β-carotene levels seemed to carry the highest risk of RA. The measurement of low antioxidant levels before the onset of disease, suggest that low antioxidant status may contribute to the pathogenesis of RA rather than being a result of the disease process.

Selenium

Selenium concentrations are relatively low in the serum of patients with RA when compared with healthy controls (Aeseth *et al.* 1978). Selenium is an essential component of the enzyme glutathione peroxidase (GSHpx) at the active centre of which selenium catalyses reduction of hydroperoxides produced from oxidised species such as superoxide and lipoperoxides (Comb & Comb, 1986).

Tarp *et al.* (1987), described long-term supplementation of RA patients and controls with selenium. Even after 26 weeks of treatment, patients with RA had granulocyte GSHpx activities still significantly lower than those of controls, regardless of nutritional selenium status. The low enzyme activity may allow the intracellular accumulation of ROS sufficient to maintain inflammation. The unresponsiveness of granulocyte GSHpx to selenium supplements may explain the predominantly negative effects seen with selenium in patients with RA (Tarp *et al.* 1985). Some clinical improvement has been reported for patients with RA (Munthe *et al.* 1986) but other evidence suggests that any role of GSHpx in RA must be indirect since D-penicillamine, a useful drug in the treatment of RA, is a specific inhibitor of GSHpx (Chaudière *et al.* 1984). Nevertheless it is intriguing that an Se-containing organic compound (2-phenyl-1,2-benzisoselenazol-3(2H)one),

frequently called 'PZ51', which has an GSHpx-like effect in catalysing the glutathione-dependent reduction of hydroperoxides, has anti-inflammatory activity (Parnham & Graf, 1987).

Osteoarthritis and antioxidants

OA is an age-associated disease to which patients may be predisposed by weaker cartilage and in which ROS have been implicated. Several small studies in humans have suggested benefit from vitamin E treatment. In a 6 week, double blind, placebo-controlled trial of 400 mg of α-tocopherol in fifty-six patients with OA, those treated with vitamin E experienced greater improvement in every efficacy measure (Blankenhorn, 1986). Intra-articular administration of SOD (orgotein), a superoxide radical inhibitor has long been used to treat equine osteoarthropathy and also, with benefit, in placebo-controlled clinical trials in human OA.

Drug effects and reactive oxygen species

The use of copper and zinc for RA may be justified by the requirement of these metals for the cytoplasmic form of SOD (Cu/Zn-SOD). Intra-articular injections of SOD reduce joint inflammation. It has been proposed that D-penicillamine could exert its therapeutic effects by forming a complex with copper which then acts as a SOD-mimetic (Aeseth *et al.* 1998). D-Penicillamine also scavenges hydrogen peroxide and hypochlorous acid and suppresses the stimulated release of ROS from human neutrophils (Ledson *et al.* 1992).

In mice with chemically induced arthritis, disease severity has been correlated with the generation of ROS by phagocytes. It has been claimed that this can be prevented by a number of commonly used anti-rheumatoid drugs including non-steroidal anti-inflammatory drugs (NSAID), slowly-acting anti-rheumatic drugs (SAARD) and steroids. However, these suggestions are usually based on experiments using drug concentrations far higher than those achieved *in vivo* (Aruoma & Halliwell, 1998). Thus, not only is this an unlikely mode of action for most anti-inflammatory agents but, for several drugs, the reverse may be true since myeloperoxidase, haem proteins and prostaglandin synthetase can oxidise many drugs into reactive metabolites (Uetrecht, 1990).

Drug effects and nitric oxide

Aspirin reduces NOS expression (Kwon *et al.* 1997). Tetracyclines have been reported to ameliorate the symptoms of RA. Not only do they combat the deleterious effects of NO, as reflected in their prevention of tyrosine nitration and α_1-antiproteinase inactivation by peroxynitrite (Whiteman *et al.* 1996), but they also inhibit the expression of NOS protein (Amin *et al.* 1996). In addition, these antibiotics inhibit metalloproteinase activity, thus reducing collagen breakdown (Greenwald *et al.* 1992). Methotrexate inhibits the synthesis of biopterin – a cofactor for NOS. As a result methotrexate suppresses the release of NO. These findings may explain the reports of beneficial actions of tetracyclines and methotrexate in RA.

Corticosteroids diminish NOS activity and NO production in several animal models of RA including that promoted by the direct application of cytokines to joint tissue (Yang *et al.* 1998; Grabowski *et al.* 1996*b*). Dexamethasone reduced NO produced by synovial explants and cultures of synovial macrophages from rats with adjuvant-induced arthritis (Yang *et al.* 1998). This inhibition of iNOS was associated with an increase of intracellular lipocortin levels, indicating parallels with other anti-inflammatory actions of these compounds.

While the mechanism of the anti-rheumatoid activity of gold compounds remains obscure, it is clear that they can depress macrophage function. It has been noted that locally-produced free radicals could oxidise gold to Au(III) or aurocyanide, both of which are highly toxic and could kill or damage activated white cells (Whitehouse & Graham, 1996).

Sex hormones

NO may mediate the effects of oestrogenic hormones on bone cell activity since oestrogens increase NO production by bone. Mechanical strain also induces NOS, leading to the proposal that NO donor compounds might be useful in the maintenance of bone density in the absence of oestrogens or during prolonged immobility. This effect of oestrogens may be relevant to the finding that polymorphonuclear cells from pregnant women show less stimulation of the oxidative respiratory burst than cells from control subjects, and also less activation of NOS (Crouch *et al.* 1995). This phenomenon may be the result of the high progestogen levels in pregnancy exerting a functional opposition to endogenous oestrogens, and could contribute to the amelioration of rheumatoid symptoms often reported during pregnancy.

Fatty acids

Mechanisms of action

Polyunsaturated fatty acids (PUFA) fall into 3 major classes - the *n*-3, *n*-6 and *n*-9 groups, the inter-relationships between which are summarised in Fig. 3. A fundamental hypothesis underlying the use of oils and PUFA is that Western diets are relatively low in *n*-3 PUFA and relatively high in *n*-6 PUFA compared with Eastern diets or with the diet of more primitive humans. The ratio of *n*-6:*n*-3 PUFA is approximately 25:1 in the modern Western diet, whereas it was nearer to 2:1 in pre-industrialised societies. Oxygenases metabolise the n3 and n6-PUFA in competition, so that a high proportion of n6 compounds leads to a relative deficiency of the products of n3 metabolism. This is seen in the generation of thromboxane A_2 (from *n*-6-PUFA) rather than thromboxane A_3 (from *n*-3-PUFA), a difference which partly accounts for the longer bleeding time and lower incidence of heart disease encountered in populations such as Inuits consuming fish-based diets rich in *n*-3-PUFA (Cleland, 1991; Cleland & James, 1997).

Conversely, an increased intake of eicosapentaenoic acid

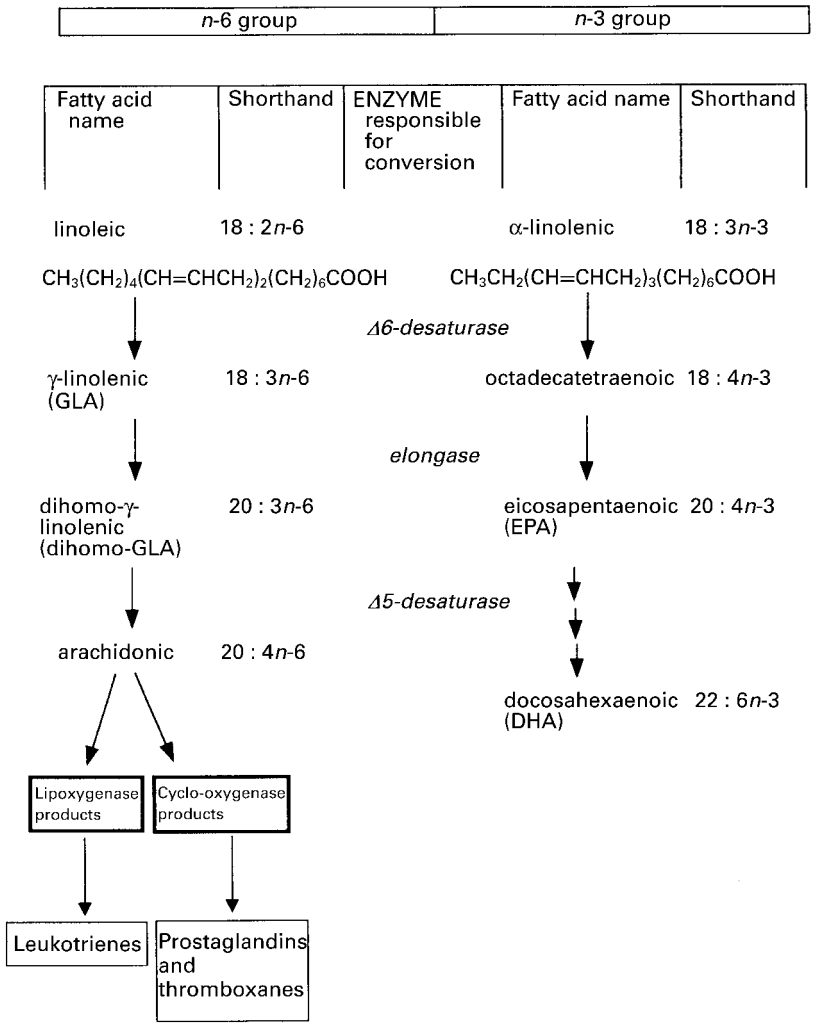

Fig. 3. The nomenclature of, and relationships between, fatty acids. Fatty acids are often referred to by a shorthand form which illustrates their structural relationships. Thus, linoleic acid possesses eighteen carbon atoms and two double bonds, the first of which is on the sixth carbon counting from the methyl group. It is therefore known as 18:2*n*-6. Plants, but not animals, are able to make the *n*-3 and *n*-6 parent fatty acids, α-linolenic acid (18:3*n*-3) and linoleic acid (18:2*n*-6) respectively, from oleic acid (18:1). Animals can, however, elongate and desaturate α-linolenic acid and linoleic acid from the diet for conversion to the *n*-3 PUFA γ-linolenic acid (GLA) (C18:3*n*-6) and octadecatetraenoic acid (18:4*n*-3) respectively. Further chain elongation gives the *n*-6 metabolites such as dihomo-γ-linolenic (20:3*n*-6, dihomo GLA), arachidonic (20:4*n*-6), docosatetraenoic (22:4*n*-6) and docosapentaenoic (22:5*n*-6) acids, and the *n*-3 longer-chain fatty acids eicosapentaenoic (20:5*n*-3, EPA) decosapentaenoic (22:5*n*-3, DPA) and docosahexaenoic (22:6*n*-3, DHA) acids. Some of the PUFA containing three, four and five carbon–carbon double bonds are precursors of biologically-active eicosanoids. The efficiency of these interconversions is illustrated by the finding that the intake of relatively small amounts of GLA increase human blood dihomo GLA and arachidonic levels (Gazso *et al.* 1989). The diagram also indicates the use of arachidonic acid as the starting material for the biosynthesis of the various eicosanoid families.

(EPA) or other *n*-3 PUFA, for example, leads to their incorporation into macrophage and neutrophil membranes in preference to the n6 compound arachidonic acid (Lokesh *et al.* 1986; Gazso *et al.* 1989; Chapkin *et al.* 1992; Hardardottir & Kinsella, 1992; Luostarinen & Saldeen, 1996). The non-essential *n*-9 PUFA include the mono-unsaturated fats found in olive oil. When present in the diet these can replace *n*-6 PUFA in several aspects of cell metabolism and, by reducing the competition between *n*-6 and *n*-3 PUFA, can lead to the increased use and incorporation of *n*-3 PUFA.

As the proportion of different PUFA is changed, there is a corresponding change in the fatty acid composition of cell membranes leading to a variety of biochemical effects. In U397 cells, the catecholamine stimulation of adenylate cyclase was reduced by 30 % after incubation with γ-linolenic acid (GLA; Cantrill *et al.* 1996). An increased dietary intake of EPA and docosahexaenoic acid (DHA)

reduces the activity of phospholipase A_2, and protein kinases A and C. Interestingly, activity of the latter was increased by linoleic and oleic acids.

The clinical work with diets containing different proportions of PUFA has clearly demonstrated an anti-inflammatory effect, although the mechanism remains the subject of debate. At least two, overlapping hypotheses are prominent. One relates to the effects of PUFA on cytokine levels, while the second deals with the effects on oxidative stress.

Polyunsaturated fatty acids and cytokines

Human studies The *n*-6 PUFA increase the amounts of inflammatory cytokines in the serum (Tappia & Grimble, 1994; Grimble, 1998; Hayashi *et al.* 1998). On the other hand, *n*-3 PUFA and monounsaturated fatty acids generally reduce the synthesis of IL-1β and TNFα by mononuclear cells stimulated *in vitro* (Sperling *et al.* 1987; Billiar *et al.* 1988; Endres *et al.* 1989; Tappia & Grimble 1994; Grimble, 1998). Diets low in *n*-6 PUFA may also lead to reduced TNFα formation in stimulated macrophages (Yaqoob & Calder, 1995). The effect can be demonstrated clearly in RA subjects and can be shown to correlate with the plasma levels of EPA, the main *n*-3 competitor of arachidonic acid (*n*-6) metabolism. This result may partly be explained by the stimulatory effect of thromboxane-A_2 on IL-1β and TNFα synthesis, since thromboxane-A_2 would be replaced by A_3. The varying results with IL-1β and IL-6 may be reconciled by the work of Yaqoob & Calder (1995), who found a biphasic effect of a diet with reduced *n*-6 PUFA: after 4 weeks, the formation of IL-1β and IL-6 was reduced, but formation was increased thereafter. Experiments *in vitro*, however, show clearly that the production of IL-6 by stimulated human endothelial cells is suppressed in the presence of EPA or DHA (Khalfoun *et al.* 1997). Human monocytes have been found to generate increased amounts of IL-1β upon incubation with GLA or dihomo-GLA, while EPA had little effect (Rothman *et al.* 1997).

T cells are the primary source of interferon-γ and their number shows a positive correlation with disease activity in patients with RA (Schuerwegh *et al.* 1999). One of the immunological changes observed in patients with RA is an alteration in the balance of T1 and T2 cell activity, producing destruction and protection respectively of articular cartilage and reflected in their production of interferon-γ and IL-14 respectively (Verhoef *et al.* 1999). Production of interferon-γ is particularly sensitive to manipulations of the lipid environment, since the addition of fatty acids, saturated or unsaturated, changed the formation of this cytokine to a greater extent than others in human lymphocytes (Karsten *et al.* 1994). Supplementation of culture media with GLA, EPA or DHA suppressed the production of interferon-γ by human lymphocytes in addition to TNFα, IL-1β and IL-2 (Purasiri *et al.* 1997).

An important study by Li *et al.* (1996) revealed that several fatty acids, including EPA, DHA, linoleic and linolenic acids could potentiate the action of TNFα in promoting generation of ROS by human neutrophils. Such an effect could result in the activation of compensatory antioxidant enzymes as noted above, but the result emphasises the greater potential for fatty acid effects in inflammatory conditions compared with normal individuals.

Animal studies Di-homo-γ-linolenic acid (dihomo-GLA) can modulate the activity of immune cells independently of prostaglandins (Santoli & Zurier, 1989; Santoli *et al.* 1990) and those effects may be mediated by changes in cytokine levels or sensitivity. In contrast to the human studies quoted above, there are reports of an increase in TNFα and IL-6 secretion by rat macrophages *in vitro* (Lokesh *et al.* 1990; Watanabe *et al.* 1991; Hardardottir & Kinsella, 1992; Tappia *et al.* 1995). Macrophages stimulated with bacterial lipopolysaccharide (LPS) generated larger amounts of TNFα when isolated from mice fed fish oil rich in *n*-3 PUFA. Interestingly, the rates of TNFα synthesis were comparable in animals fed *n*-6 PUFA-rich safflower oil, suggesting that the different cytokine levels may have resulted from an enhanced rate of removal or destruction in the animals treated with *n*6 compounds. The concentrations of PGE_2 which facilitate the removal of TNFα are lower than those which suppress its synthesis. Treatment with *n*-3 PUFA selectively reduces TNFα removal.

On the other hand, Yaqoob & Calder (1995) reported that mice fed *n*-3 PUFA in the form of fish oil possessed macrophages which yielded less PGE_2, thromboxane-B_2, IL-6 and TNFα in response to LPS stimulation. Interestingly, there was no change in the production of IL-1β. However, there are several reports which contradict this view, with data showing that *n*-3 PUFA increased production of the pro-inflammatory IL-6 (Tappia & Grimble, 1994) and either increased (Lokesh *et al.* 1990) or decreased (Billiar *et al.* 1988; Tappia & Grimble, 1994) the levels of IL-1β. In man the *n*-3 PUFA decreased the production of TNFα, IL-1β and IL-6 (Meydani *et al.* 1991; Baldie *et al.* 1993; Endres *et al.* 1993).

Following a variety of dietary lipid additions, mice supplemented with olive or safflower oils possessed T lymphocytes with increased secretion of IL-2 and a trend towards a lower production of IL-10 (Yaqoob & Calder, 1995). Diets containing a variety of PUFA additions caused a decline of natural killer lymphocyte cell activity in rats (Yaqoob *et al.* 1994; Hughes & Pinder, 1997), supplementation with *n*-3 PUFA having the greatest effect. T lymphocytes from essential fatty acid-deficient mice produced less interferon-γ than controls (Benhamou *et al.* 1995), while macrophages secreted increased amounts of TNFα and IL-1β. The effects on lymphocyte proliferation are controversial. Calder (1997) has demonstrated that the mitogenic response of lymphocytes is suppressed by *n*-3 PUFA, while Miyasaka *et al.* (1998*b*) has claimed that they increase lymphocyte proliferation. Miyasaka *et al.* (1998*a*,*b*) have found that *n*-3 PUFA had no effect on macrophage phagocytosis capacity.

Polyunsaturated fatty acids and eicosanoids

The relationships between PUFA, eicosanoids and cytokines are complex. Since the *n*-6 PUFA, linoleic acid, and the *n*-3 compound GLA can be converted by mammals into arachidonic acid, they can increase the formation of prostaglandins. PGE_2 can reduce TNFα and IL-6 synthesis (Kunkel *et al.* 1988; Tappia *et al.* 1995), which should

produce an anti-inflammatory action. Conversely, the *n*-3 PUFA EPA and DHA reduce PGE_2 synthesis by macrophages (Leslie *et al.* 1985; German *et al.* 1988; Somers *et al.* 1989), an effect which appears to involve changes of both gene expression and receptor transduction systems (Yaqoob & Calder, 1995). Linoleic acid raises TNFα secretion but increases PGE_2 formation (Tappia *et al.* 1995).

The usual explanation of dietary lipid effects is that the levels of inflammatory arachidonic acid oxidation products are reduced, with the formation of less active prostanoids (Callegari & Zurier, 1991). GLA is converted to dihomo-GLA, the immediate precursor of PGE_1 (Fig. 3). This is a potent anti-inflammatory agent (Zurier, 1980), partly by virtue of its reducing IL-1β production (Baker *et al.* 1989; Callegari & Zurier, 1991). In RA patients, this effect is accompanied by a fall in PGE_2 and leukotriene B_4 (LTB_4) synthesis by stimulated monocytes (Pullman-Mooar *et al.* 1990). The clinical efficacy of GLA may be via this elevation of endogenous PGE_1, a potentially valuable strategy in view of the prostaglandin's short half life. The use of GLA permits endogenous levels to be raised at the sites and times needed physiologically, without the need to use analogues, with potential side effects, or inhibitors of degradation.

Polyunsaturated fatty acids and leukotrienes In addition to their interactions with prostaglandins, *n*-3 PUFA can modify tissue responses to other arachidonate metabolites such as the leukotrienes. They reduce LTB_4-mediated human neutrophil adherence and chemotaxis (Begin *et al.* 1988) and also suppress their responsiveness to interferon-γ (Somers *et al.* 1989; Hubbard *et al.* 1991).

LTB_4 has been considered to be a pivotal agent in the development of inflammatory responses (Devchand *et al.* 1996). Dihomo-GLA cannot be converted to inflammatory leukotrienes by 5-lipoxygenase but, on the contrary, it is converted to 15-OH-dihomo-GLA which suppresses 5- and 12-lipoxygenases leading to the fall in LTB_4 (Kuratko & Constante, 1998). Since LTB_4 is a potent pro-inflammatory compound (Devchand *et al.* 1996), its decrease is an important contributory factor to the anti-inflammatory effect of dihomo-GLA. *n*-3 PUFA diets also decrease the levels of thromboxane-A_2 (Luostarinen *et al.* 1997). In the study by Pullman-Mooar *et al.* (1990), a period of 12 weeks of raised GLA intake increased the formation of dihomo-GLA, leading to a reduction of PGE_2, LTB_4 and leukotriene C4 LTC_4 formation by stimulated monocytes. Dietary *n*-6 PUFA, in the form of corn oil fed to rats, increased the number of PGE_2 binding sites. This could account for the increased sensitivity of cells to PGE_2 (Opmeer *et al.* 1984), which suppresses the production of both TNFα and IL-1β (Knudsen *et al.* 1986; Okusawa *et al.* 1988).

Polyunsaturated fatty acids and oxidative stress

Contradictory results have been obtained with respect to the effect of dietary fish oil (*n*-3 PUFA) on free radical formation (Somers & Erickson, 1994). Yaqoob & Calder (1995) have summarised evidence that after a high *n*-3 PUFA intake, murine macrophages generated more superoxide, hydrogen peroxide and NO than in those from control animals. Crosby *et al.* (1996) have also claimed a three to four-fold increase of lipid peroxidation in preparations of vascular endothelial cells in response to incubation with EPA or DHA. Other studies, however, have reported that fish oil did not modify the production of superoxide by macrophages stimulated by phorbol esters, but did increase hydrogen peroxide release. No change was noted in these studies in the antioxidant enzymes SOD, catalase and GSHpx in the macrophages or lymphoid organs (Miyasaka *et al.* 1998*a*,*b*). The overall increase in cellular oxidation was reflected in an increase in lipid peroxidation products measured as thiobarbiturate-reactive substances in the plasma. In the study by Crosby *et al.* (1996), GSHpx activity was induced by EPA and DHA, leading the authors to speculate that this induction could represent a major element in the cellular protection afforded by fish oil.

The longer chain PUFA are especially potent at increasing lipid peroxidation and causing cell damage by oxidative stress (Zurier, 1993). The cytotoxicity of fatty acids seems to depend especially on their ability to stimulate superoxide production rather than hydroxyl radicals or hydrogen peroxide (Begin *et al.* 1988; Howie *et al.* 1993). GLA and arachidonate easily generate superoxide anions. The antioxidants vitamin E and butylated hydroxyanisole prevent damage caused in this way. Of the major PUFA, DHA (*n*-3) is the least effective at raising superoxide generation (Begin *et al.* 1988; Howie *et al.* 1993).

The deleterious effects of the *n*-6 PUFA are reflected in the decrease in anti-oxidant protection produced by linoleic acid treatment of cultured endothelial cells. This leads to a decrease of intracellular glutathione levels and activation of the oxidative stress-sensitive nuclear transcription factor NFκB. Another of the major antioxidant systems, SOD is also modified by PUFA. Oils with a high linoleic, EPA or GLA content increase mitochondrial Mn-SOD activity (Phylactos *et al.* 1994; Luostarinen *et al.* 1997; Kuratko & Constante, 1998) although the cytoplasmic enzyme, Cu/Zn-SOD is unaffected. Predictably, fat-free diets lead to a decline in tissue Mn-SOD activity.

Horrobin (1991) has pointed out that the oxidative damage in cells may be due to the loss of fatty acids from cell membranes due to their peroxidation, rather than to the accumulation of toxic oxygen and peroxidation products.

Channels and enzymes

Diets high in *n*-6 PUFA cause changes in Na^+K^+-ATPase and acetylcholinesterase activity in brain membranes (Srinivasarao *et al.* 1997*a*,*b*). Neuronal membranes are particularly rich in DHA, and the application of this PUFA depresses potassium currents (Poling *et al.* 1996). In fibroblasts, DHA inhibited the depolarisation-induced potassium current by acting on the outside of cells only, suggesting that it interacted with a specific site on the channel, rather than simply modifying the local lipid environment of the channel.

While these effects on neurones may seem removed from any possible relevance to RA, it is known that IL-2 secretion is regulated partly by intracellular potassium and

calcium levels (Palanki & Manning, 1999). Raised extracellular potassium can promote the release of IL-1β (Mancuso *et al.* 1998), while the production and secretion of IL-1β and TNFα are enhanced by lowering intracellular potassium (Gantner *et al.* 1995; Perregaux & Gabel, 1994, 1998) at least partly because the activation of interleukin converting enzyme is enhanced by the lowering of intracellular potassium (Cheneval *et al.* 1998). The non-selective blockade of potassium channels with quinine (Deakin *et al.* 1994) inhibited both TNFα and IL-1β release from cells, while glipizide (Pfizer) and glibenclamide (Hoechst Marion Roussel), inhibitors of ATP-sensitive K^+ channels, reduced IL-1β release but not TNFα. Apamin, a selective inhibitor of low-conductance Ca^{2+}-sensitive channels, inhibited TNFα release, potentiated IL-1β but had no effect on IL-6 or IL-8 release. These results suggests that K^+ channels may differentially regulate cytokine production by THP-1 cells. The ability of PUFA to modulate ion channels, therefore, provides one indirect mechanism by which cytokine production could also be changed.

Ion channels and polyunsaturated fatty acids The relevance of these studies to PUFA is that the *n*-3 PUFA EPA and DHA activate potassium current in smooth muscle cells (Asano *et al.* 1998). The effect of DHA on one particular channel, Kv1·2, was antagonised by micromolar concentrations of zinc. EPA and DHA inhibited agonist-induced non-selective cation current even when the current was induced by guanosine-5′-*O*-(3-thiotriphosphate) applied to the internal surface of cells. This result strongly suggests that these *n*-3 PUFA are interacting directly with the ion channel and not with any agonist receptor (Asano *et al.* 1998).

PUFA interact with several ion channels, including those carrying sodium and calcium (Vreugdenhil *et al.* 1996). DHA and EPA are the most effective, while the monounsaturates, such as oleic acid, or saturated compounds such as palmitic acid, were ineffective. The effect is normally one of inhibiting ion movement through the channels (Hazama *et al.* 1998), and it may explain the ability of the *n*-3 PUFA to protect against cell damage and death caused by calcium overload induced by depolarising agents such as glutamate in neurones (Okada *et al.* 1996; Calvert *et al.* 1999).

EPA treatment of vascular muscle cells decreases the intracellular resting concentration of calcium and reduces the increase of calcium levels produced by agonists. The consequence is to inhibit the cell migration response to platelet-derived growth factor (Asano *et al.* 1998). Similar effects of *n*-3 PUFA in joint tissues could suppress the infiltration by macrophages and related cells. Cell movements are also likely to be affected by changes in the cytoskeleton. The *n*-6 PUFA, GLA, increases the expression of catenin, one of several proteins linking cadherin to cytoskeletal components (Jiang *et al.* 1995*a*). Possibly as a result of this, GLA decreases the motility and invasiveness of several cell types, including cancer cells. GLA also changes the expression of cell adhesion molecules which would contribute to altered cell movement (Jiang *et al.* 1995*b*).

It is also probable that cellular lipid composition can modify several aspects of intercellular communication. Both GLA and EPA can reduce cellular permeability to large molecules, and both were also found to increase the production by cells of occludin, a protein involved in the formation and maintenance of cellular tight junctions (Jiang *et al.* 1998). Linoleic and arachidonic acids had no effect.

Clinical studies of oils in autoimmune and inflammatory disease

Before presenting the evidence for oil-induced benefits in RA, it is worth emphasising some of the difficulties which plague attempts at scientific study in this area. All studies of dietary therapy in RA should be carefully controlled since disease severity waxes and wanes, giving a false impression of improvement, and patients also tend to show a high placebo response rate. Cross-over trials are exceptionally difficult to design and interpret unequivocally because of the known effect of fatty acids remaining in tissue lipids for up to three months after withdrawal of fatty acid supplements. In general it is advisable to avoid a cross-over trial design.

Problems with clinical trials in inflammatory diseases

Many of the oils used as the placebo arm of clinical trials are unacceptable since they may have intrinsic beneficial effects. For example, in many of the early trials designed to test the efficacy of fish oil, (*n*-3 PUFA), in human autoimmune and inflammatory diseases, the placebo chosen was olive oil (65–85 % oleic acid). However, olive oil significantly reduced by incidence of experimental autoimmune encephalomyelitis in the guinea pig (Meade *et al.* 1978) and increased the survival rate of MLR/lpr mice, which are prone to autoimmune disease (Godfrey *et al.* 1986).

Cleland *et al.* (1988) compared fish oil and olive oil supplements in patients with RA in a double-blind, non-cross-over study and found improvements in painful joint score and grip strength at 12 weeks with fish oil, while morning stiffness and analogue pain score improved in both groups. This result was only significant with olive oil, consistent with an earlier report by Brzeski et al. (1991). A beneficial effect of olive oil was also reported by Darlington *et al.* (unpublished results) who found reduced levels of C-reactive protein (an acute phase protein which correlates with disease activity in RA) with olive oil treatment.

Coconut oil has been suggested as a control for *n*-6, *n*-3 (other than EPA and DHA) and monounsaturated fatty acid biological effects. However, coconut oil contains saturated fatty acids (88 %), linoleic acid (<2 %) and monounsaturated fatty acids (<10 %).

Fish oils and α-tocopherol requirements The addition of antioxidants to encapsulated fish oil, to prevent oxidation in the capsules and *in vivo* after ingestion, should include α-tocopherol (3 mg/g fish oil), alone or with the additional antioxidants dodecyl gallate (100 μg/g) or ascorbyl palmitate (vitamin C). The concentration of α-tocopherol (3 mg/g fish oil) is based on the best estimate for tocopherol adequacy in diets and supplements as 0·6 mg D-α-tocopherol per gram linoleic acid ingested plus the

dependency of vitamin E requirements on the degree of fatty acid unsaturation. To allow for the effects of vitamin E in the fish oil the same concentration of vitamin E should be added to the placebo oil.

A 3-month treatment with EPA 1·68 g/d and DHA 720 mg/d in fifteen young and ten older women produced significant reductions in plasma triacylglycerols and a fall in plasma α-tocopherol levels, with an increase in lipid peroxides (Meydani *et al.* 1991*a*,*b*). A similar result was obtained by Sanders & Hinds (1992). These two studies may indicate that the vitamin E content of fish oil supplements is not sufficient to provide adequate antioxidant protection and that increased fish oil intake may require a graded increment in vitamin E intake – a fact not appreciated by many clinicians and certainly not by many patients taking fish oil without medical supervision. It is also possible that the altered balance of cholesterol levels (raised HDL-cholesterol and lowered VLDL-cholesterol) could have produced secondary changes in vitamin E levels.

Fish oils

In a review of thirty-seven human studies involving supplementation with fish oil or fish diets ranging from EPA supplement 1–6 g/d and from 2 weeks to 8 months duration, Kristensen *et al.* (1989) reported prolonged bleeding time, inhibition of adenosine diphosphate – and collagen-induced platelet aggregation, decreased thromboxane levels and a favourable shift in the prostacyclin–thromboxane balance, with decreased erythrocyte sedimentation rate. Fish oil feeding increased phospholipid EPA composition (Vidgren *et al.* 1997) and lowered systolic blood pressure (Bonaa, 1989).

EPA and DHA supplementation in normolipidaemic and hypertriacylglycerolaemic subjects results in a reduction in plasma triacylglycerol and cholesterol levels, the decrease being observed for VLDL-cholesterol and LDL-cholesterol (Goodnight *et al.* 1981; Phillipson *et al.* 1985; Herzberg, 1989). Saynor & Gillott (1992) found significant reductions in serum triacylglycerol and fibrinogen levels and in total cholesterol (in subjects with pre-treatment levels of >6·5 mmol/l), with a significant increase in HDL-cholesterol on fish oil administration. In a similar study in the same year Sanders & Hinds (1992) described a fall in plasma concentrations of triacylglycerol and VLDL-cholesterol and increased HDL- and HDL_2-cholesterol and apoprotein B. Both systolic and diastolic blood pressures fell during supplementation and increased after discontinuation of the fish oil supplement.

Fish oil in arthritis The ingestion of long-chain *n*-3 PUFA, such as EPA, and DHA, from fish oil is likely to have an anti-inflammatory effect in RA (Linos *et al.* 1991; reviewed in Cleland, 1991 and Darlington, 1994) whereas *n*-6 PUFA, found in polyunsaturated cooking oils and margarine, tend to exacerbate inflammation. Arthritic patients taking cod liver oil showed biochemical and clinical improvement (Brusch & Johnson, 1959) and a significant decrease in joint pain index and in patients' assessment of disease activity after 6 weeks dietary supplementation with EPA 20 g/d (Sperling *et al.* 1987). In another open study, Kremer *et al.* (1985) gave MaxEPA 10 g/d for 12 weeks to patients with RA, in combination with other dietary modifications, and reported modest improvement in morning stiffness and in the number of painful joints – improvements that deteriorated after stopping the oil. The same group subsequently completed a well-controlled, double-blind cross-over study of twenty-one patients with active RA (Kremer *et al.* 1987) showing significantly fewer tender joints and improvement in time to onset of fatigue after MaxEPA 15 g/d for 14 weeks compared with olive oil 15 g as placebo. The improvement in symptoms was correlated with a decrease of neutrophil LTB_4 production.

A number of studies have examined the effects of fish oils or their constituent fatty acids on cytokine levels in parallel with clinical symptoms. In a 24-week double-blind, randomized trial, twenty patients with RA were given dietary supplements of EPA and DHA at a low dose (27 mg/kg and 18 mg/kg respectively) or a high dose (54 mg/kg and 36 mg/kg). Symptomatic improvements were noted in patients with both dose levels, together with a decreased synthesis of LTB_4 by neutrophils and a 40 % decrease in IL-1β production by macrophages (Kremer *et al.* 1990). Similarly, in a shorter, 12-week, double-blind, randomized study, thirty-two patients with RA were given a mixture of *n*-3 fatty acids or placebo (3·6 g/d). Plasma levels of IL-1β were reduced significantly, although there was no change on TNFα levels (Espersen *et al.* 1992). Clinical symptoms improved in parallel in the treated groups but not the placebo patients.

Fish oils in systemic lupus erythematosus Significant clinical benefit has been claimed in systemic lupus erythematosus patients given a low-fat diet with *n*-3 PUFA-rich fish oil supplements at around 20 g/d (Walton *et al.* 1991; Robinson *et al.* 1993), although Clark *et al.* (1993) reported on the use of fish oil in a randomized crossover trial on twenty-one patients with lupus nephritis. Olive oil was employed as the placebo arm. No benefit was obtained from fish oil given at 15 g/d after 1 year of treatment, in terms of either renal function or disease activity. Indeed the only changes noted were in serum lipids, with significant decreases in triacylglycerol and VLDL cholesterol levels. Das (1994) has also concluded that the *n*-3 fatty acids, EPA and DHA, are useful in the management of systemic lupus erythematosus. Mohan & Das (1997) proposed that EPA and DHA can modulate oxidant stress and nitric oxide synthesis and may have a role as regulators in the synthesis of antioxidant enzymes such as SOD and GSHpx.

Animal studies of systemic lupus erythematosus Robinson *et al.* (1993) examined the effects of fish oil components in a mouse model of systemic lupus erythematosus, administering diets containing EPA, DHA or a combination of these over a period of 14 weeks, Renal disease, assessed by histology and proteinuria was reduced by 10 % fish oil, EPA 10 % or DHA 6 % diets. A diet containing EPA and DHA in the approximate ratio 3:1 were more effective than either individual fatty acid.

Immunosuppression associated with long-term fish oil therapy Immunosuppression may be one mechanism by which high-dose fish oil is beneficial. Subjects supplemented with EPA and DHA 1·23 g/d exhibited a reduced

lymphocyte responsiveness to mitogen stimulation (Wu *et al.* 1996) with a reduced delayed-type hypersensitivity response (Meydani *et al.* 1993). After 24 weeks, a fall in the proportion of peripheral blood $CD4^+$ cells and an increase in $CD8^+$ cells was observed (Meydani *et al.* 1993). Supplementation with *n*-3 PUFA depressed immune reactivity in volunteers by suppressing the expression of monocyte surface molecules associated with their antigen-presenting function (Hughes *et al.* 1995).

Fish oil reduces cytokine production (IL-1α, IL-1β, IL-2, IL-6 and TNFα) from human peripheral blood mononuclear cells (Billiar *et al.* 1988; Endres *et al.* 1989, 1993; Yaqoob & Calder, 1995; Caughey *et al.* 1996; Bonner *et al.* 1997) and suppresses neutrophil LTB_4 and LTB_5 production in subjects with inflammatory diseases (Schmidt & Dyerberg, 1989).

In summary, the long-term consequences of alterations in the *n*-3–*n*-6 balance in favour of the *n*-3 PUFA is incompletely understood in humans but it could lead to detrimental immunological and haematological effects. If fish oil is to be taken or used in clinical trials, therefore, the lowest possible effective dose should be used i.e. equivalent to EPA 500–750 mg/d. More studies are clearly required to investigate the safety of long-term supplementation with fish oil in man.

Marine oils

Seatone® Seatone® (Peter Black Health Care, Swadlingcote, UK) is an oily extract of the New Zealand green-lipped mussel, *Perna canaliculus*, which was found to have anti-inflammatory activity if given to rats intraperitoneally, but not orally (Miller & Ormrod, 1980), associated with an inhibition of prostaglandin biosynthesis (Couch *et al.* 1982; Miller & Wu, 1984). A 12-week, double-blind, clinical trial, randomised with placebo but without cross-over (Gibson *et al.* 1980) suggested that Seatone® was effective in RA and OA, with reduction in pain and stiffness and with a low incidence of side effects. Other groups have failed to demonstrate significant benefit using a randomised, cross-over design (Huskisson *et al.* 1981; Larkin *et al.* 1985) but the duration of treatment was limited to only 4 weeks.

Lyprinol® In 1997, an extract of *Perna canaliculus*, rich in biologically-active oils and natural antioxidants was reported to have protective and therapeutic effects against inflammatory arthritis in rats (Whitehouse *et al.* 1997). The extract, known as *Lyprinol*® (Lyprinol UK Ltd., Tunbridge Wells, UK) induces a slow reduction of inflammation and is not analgesic. Whitehouse *et al.* (1997) describe anti-inflammatory effects similar to those of NSAID, but without any deleterious effect on the gastro-intestinal tract in rats and without risk of seafood allergy. More work is obviously required with *Lyprinol*® to establish its efficacy and toxicity but, in the light of the toxicity associated with current NSAID, a new, safer medicine in this therapeutic area would be welcome.

Vegetable oils

A number of vegetable oils have been claimed to provide benefit in RA, Darlington, Sanders and Hinds, (unpublished work), and Cleland *et al.* (1988) found improvement in the symptoms of RA in patients taking olive oil for 14 weeks. Improvement was also seen in RA patients consuming evening primrose oil (EPO), which is rich in GLA, and olive oil (Brzeski *et al.* 1991). Leventhal *et al.* (1993) conducted a randomised, double-blind placebo-controlled trial of GLA in thirty-seven patients with RA. Patients were treated with GLA 1·4 g/d and a range of clinical assessments conducted of the patients' physical status and ability to perform daily tasks. A significant difference was demonstrated ($P < 0{\cdot}05$) between the treated patients, who showed clear improvement, and the placebo (cottonseed oil) group who showed either no improvement or a deterioration during the 24-week period of the trial. The improvements in joint stiffness, swelling and tenderness were improved by 30–40 %, with no change in the placebo subjects. Watson *et al.* (1993) found significant improvement in morning stiffness in patients with RA after taking blackcurrant seed oil, which also contains GLA. Monocytes cultured from the patients exhibited a lower secretion of the inflammatory cytokines IL-1β, IL-6 and TNFα compared with control subjects given sunflower seed oil.

Evening primrose oil

EPO is rich in GLA, a precursor of PGE_1 (Fig. 3) and of 15-hydroxy-dihomo-GLA. As noted earlier, PGE_1 is a known anti-inflammatory agent while dihomo-GLA inhibits both 5- and 12-lipoxygenases, which generate pro-inflammatory eicosanoids. There are synergistic interactions between GLA and EPA; the latter inhibits conversion of dihomo-GLA to arachidonic acid and, as a result, GLA has a greater effect in raising concentrations of dihomo-GLA. Combined administration, therefore, raises the levels of two anti-inflammatory essential fatty acids, dihomo-GLA and EPA, while reducing levels of the pro-inflammatory arachidonic acid. GLA has also been reported to inhibit the formation of leukotrienes from arachidonic acid via a metabolite of dihomo-GLA (Shimizu *et al.* 1984).

Kunkel *et al.* (1981) demonstrated that the responsiveness of rat polymorphonuclear leucocytes to a synthetic chemoattractant was significantly impaired, and chronic proliferative adjuvant arthritis was greatly suppressed in rats treated with EPO. In human patients it has been claimed that EPO, both alone and when combined with fish oil, produced significant subjective improvement and allowed more than 70 % of patients to reduce, or even to terminate NSAID therapy. Belch *et al.* (1988), for example, treated sixteen rheumatoid patients with GLA 540 mg/d in the form of EPO, and a further fifteen patients with GLA 450 mg/d plus EPA 240 mg/d. Eighteen patients were given placebo. After 12 months of treatment, both GLA-treated groups showed improvement in their symptoms and a reduced requirement for NSAID. Within 3 months of ceasing the oil intake, however, the treated patients had returned to their pre-trial NSAID intake levels (Belch *et al.* 1988). In a similar trial using twenty RA patients treated with EPO, no significant benefit was demonstrated (Hansen *et al.* 1983), but treatment was given for only 12 weeks,

compared with the 12-month duration used by Belch *et al.* (1988).

One multicentre, double-blind, randomised, placebo-controlled parallel group trial incorporating 402 patients with RA treated with EPO 2–3 g/d (with sunflower oil placebo) was undertaken to assess the extent to which the dosage of NSAID could be reduced. Each test capsule contained approximately GLA 280 mg, EPA 45 mg and DHA 30 mg. The trial yielded no support for the use of EPO as a NSAID-sparing agent in RA (Scotia Pharmaceuticals, personal communication, 1999).

Jantti *et al.* (1989) studied eighteen patients with RA for 12 weeks, each of whom received either EPO 20 ml containing 9 % GLA, or olive oil. Serum concentrations of oleic acid, EPA and apolipoprotein-B decreased, and those of linoleic acid, GLA, dihomo-GLA and arachidonic acid increased during EPO treatment. The authors felt that the increases in PUFA, which are eicosanoid precursors, might raise the levels of the pro-inflammatory compounds PGE_2 and leukotrienes.

Conclusion

Overall, the relationship between PUFA, eicosanoids and cytokines is clearly emerging as an area of great interest and potential clinical relevance. The results obtained to date are often inconsistent and vary with the nature of the experimental preparation or model, the concentrations of agents used and whether they are tested acutely or chronically. There is no doubt, however, that dietary manipulation of fatty acid levels do produce changes in the generation of eicosanoid hormones and cytokines, and can modify their cellular actions. With the increasing evidence that either or both of these groups of compounds play a pivotal role in the disease process underlying arthritis and other inflammatory disorders, dietary control of fatty acid intake would be expected to modify the disease process and provide a useful adjunctive strategy in the treatment of these disorders.

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