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Effects of dietary lipids on immune function in a murine sensitisation model

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We have tested the effect of dietary fatty acids on aspects of innate and specific adaptive T helper (Th) 1- and Th2-driven immune responses in a murine sensitisation model using dinitrochlorobenzene as sensitiser. Six groups of fifteen BALB/c mice were fed diets containing 30 % fat (by energy) for 8 weeks. Diets were rich in saturated fatty acids, *n*-6 polyunsaturated fatty acid (PUFA), or *n*-3 PUFA, each at a sufficient (11, 35 and 68 mg/kg) and a supplemented vitamin E level (1028, 1031 and 1030 mg/kg respectively). Feeding *n*-6 PUFA marginally decreased % phagocytosing cells at the low vitamin E level, but had no other effects on immune function. The *n*-3 PUFA diets decreased production of prostaglandin E_2 while increasing oxidative burst and tumour necrosis factor α production. In addition adaptive Th1-driven responses (immunoglobulin, Ig)G2a, IgG2b, interferon-γ:interleukin 4) were decreased, whereas Th2-driven and mucosal immune responses were increased (IgE) or unaffected (IgG1, IgA). Combination with high levels of α-tocopherol did not affect the reduced prostaglandin E_2 production, augmented the increase of tumour necrosis factor α production and tended to ameliorate the selective suppressive effects of *n*-3 PUFA on certain Th1-driven effects (interferon-γ:interleukin 4 ratio and IgG2a levels). We conclude that the sensitisation model appears useful for application in nutrition research. It allows a broad assessment of the effects of dietary intervention on various aspects of immune responsiveness, and as such provides a valuable model to assess, characterise and rank effects of foods and/or nutrients on a range of immune functions, including Th1–Th2 polarisation.

Immunomodulation: Animal model: Fatty acids: Vitamin E

The immune system has evolved as a defence mechanism against infections and contributes to the maintenance of good health. In recent years, the effect of diet on various aspects of immune function has become increasingly apparent (Chandra, 1997; Bendich, 1999). Systematic research in this area, however, is hampered by the lack of generally accepted, validated animal models that are predictive for effects in man (Bronson *et al.* 1999). Typically, different research groups have studied different nutrients, each using different animal models. Most of these models focus on selected aspects of immune function without assessing other functions. It is therefore difficult to compare the broader immunomodulating effects of diets or individual nutrients. A systematic approach to compare the impact of various nutrients and/or diets using an animal model to assess a range of innate and specific T helper (Th) 1- and Th2-driven adaptive immune responses would therefore be valuable. The aim of the present study is to evaluate the usefulness of a murine sensitisation model for such an approach.

Sensitisation models have previously been used to characterise immunomodulation by contact sensitisers and pharmaceuticals (van't Erve *et al.* 1998; Pieters & Albers, 1999). Here we have used the contact sensitiser dinitrochlorobenzene (DNCB) to elicit specific adaptive responses in BALB/c mice. Although BALB/c mice are prone to develop Th2-driven responses to some infections, both Th1 and Th2 clones can be isolated from them (Cher & Mosmann, 1987) and we have previously demonstrated that modulation of both Th1- and Th2-driven responses can readily be detected in these mice (Albers *et al.* 1998). We therefore set out to determine whether this model can be used to compare the impact of nutrients upon a range of immune functions. Here, we have selected phagocytotic

Abbreviations: ALN, auricular lymph node; DNCB, dinitrochlorobenzene; DTH, delayed-type hypersensitivity; IFN, interferon; Ig, immunoglobulin; IL, interleukin; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; Th, T helper; TNF, tumour necrosis factor.

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activity and oxidative burst of granulocytes as variables relating to innate immune responses. In addition, we measured production of the pro-inflammatory cytokine tumour necrosis factor (TNF) α and of prostaglandin (PG) E_2. Adaptive immunity consists of a balanced mixture of cellular and humoral responses. Cellular, or type 1 immune responses are driven by interferon (IFN)-γ producing Th1 cells and typically comprise delayed-type hypersensitivity (DTH) reactions and, in the mouse, production of immunoglobulin (Ig) G2a- and IgG2b-isotype antibodies (Albers *et al.* 1998). On the other hand, production of IgE and IgG1 antibodies is characteristic for humoral or type 2 immune responses driven by interleukin (IL) 4-producing Th2 cells. In the present study we have measured DTH responses elicited by challenge with DNCB, DNCB-specific antibodies of IgG1, IgG2a, IgG2b and IgE isotype in serum, and production of IFN-γ and IL4. In addition, we assessed production of DNCB-specific IgA and lymphocyte proliferation.

To test the applicability of this model for nutritional intervention studies, we have used diets varying in fatty acid composition and vitamin E contents as these nutrients have been studied extensively for their immunomodulating effects (Calder, 1998; Meydani & Beharka, 1998). Mice received semipurified diets that differed in fatty acid composition: a high-saturated fatty acid (SFA) diet rich in palmitic acid, a high-*n*-6 polyunsaturated fatty acid (PUFA) diet rich in linoleic acid, and a high-*n*-3 PUFA diet with the long-chain *n*-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid. The diets were devised to vary in selected fatty acids while providing sufficient amounts of the essential fatty acids and keeping other fatty acids as constant as possible. In a complete 3×2 design, each dietary fatty acid composition was combined with a sufficient (low) and a high level of vitamin E, taking into account the fatty acid composition of the diet.

Material and methods

Animals and maintenance

The Animal Experimentation Committee of the Faculty of Veterinary Sciences of Utrecht University approved the experimental protocol. Young adult, female BALB/c mice, aged 4–6 weeks at the start of the experiment, were bred and housed at the animal facilities of the Utrecht University. They were kept on wood-chips in filter-topped Macrolon cages under standard conditions (temperature 23 ± 2°C, 50–60 % relative humidity, 12 h dark–light cycle) with free access to food and acidified drinking water. They were randomly divided in six experimental groups (fifteen per group) and were individually identifiable through ink markings on the tail. Persons involved in handling of the animals or sample analysis were blinded for the treatments.

Feeding

The experimental diets were prepared freshly every 2 weeks and were stored under N_2 in single day portions at −20°C to avoid lipid and vitamin E oxidation. The animals were group-fed and fresh diet was given daily. All animals received a run-in diet identical to the SFA, low-vitamin E diet for 2 weeks prior to the intervention. During the 8 weeks intervention, animals received one of six different diets, varying in fatty acid composition and in vitamin E content.

Diets

All animals received pelleted semipurified diets providing 16 % energy as protein, 54 % as carbohydrate and 30 % as fat to mimic the typical human high-fat diets of industrialised societies. Diets were based on AIN-93 recommendations (Reeves *et al.* 1993) and details are given in Table 1. The tocopherol and tocotrienol concentrations in the (stripped) natural oils were analysed and the oils were supplemented with D-α-tocopheryl acetate (Sigma, St Louis, MO, USA) to obtain the desired tocopherol concentrations. The amount of vitamin E was based on the PUFA content, as dietary PUFA impair vitamin E absorption and vitamin E requirement for cell membrane protection increases with increasing numbers of double bonds. The low-vitamin E groups were fed on diets containing sufficient vitamin E to fulfil the biological requirement according to the calculations described by Muggli (1994) and an additional 1000 α-tocopherol equivalent/kg diet was added to the high-vitamin E groups.

Experimental fats

The experimental fat blends were prepared from the following natural oils: palm oil, safflower oil (tocopherol stripped), sunflower oil (tocopherol stripped) all from

Table 1. Analysed composition of diets*†

	Fat blend (g/100 g fatty acids)		
Diet...	SFA	*n*-6 PUFA	*n*-3 PUFA
Fatty acid (g/100 g total fatty acids)			
14 : 0	1·3	0·5	3·3
16 : 0	49·3	20·3	7·0
16 : 1	0·2	0·2	2·9
18 : 0	4·6	4·3	3·2
18 : 1	33·3	32·5	30·6
18 : 2*n*-6	9·7	40·3	11·0
18 : 3*n*-3	0·2	0·3	0·2
20 : 4			0·9
20 : 5*n*-3 (EPA)			14·7
22 : 5*n*-3			2·3
22 : 6*n*-3 (DHA)			10·5
Vitamin E (mgα-TE/kg diet)‡			
Low vitamin E	11	35	68
High vitamin E	1028	1031	1030

SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; TE, tocopherol equivalent; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

* The following ingredients were added at identical levels to all diets (g/kg diet): calcium caseinate 163·3, maize starch 609·7, cellulose 50·0, mineral mixture 35·0, vitamin mixture 10·0, L-cysteine hydrochloride 1·8, choline bitartrate 2·5, fat blend 127·5. Mineral and vitamin mixtures were formulated according to AIN-93 recommendations (Reeves *et al.* 1993), without vitamin E.

† For details of analytical procedures, see p. 293.

‡ 1 mg α-TE = 1 mg natural (*RRR*)-α-tocopherol.

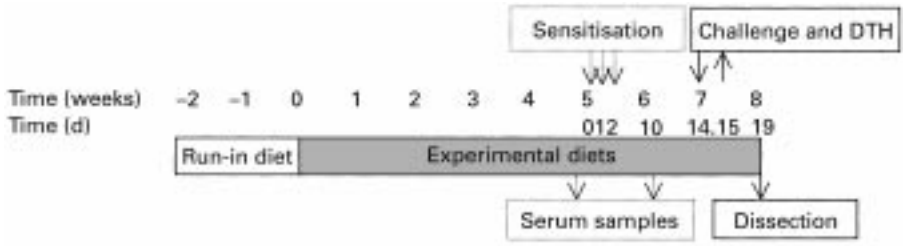

Fig. 1. Design of the study: all animals received a run-in diet identical to the saturated fatty acid-rich, low-vitamin E diet for 2 weeks prior to the intervention. During the 8-weeks intervention, animals received one of six different diets, varying in fatty acid composition and vitamin E content. For details of diets, see Table 1 and p. 292. After 4 weeks, animals were sensitised to dinitrochlorobenzene on three consecutive days. Fourteen days after the first sensitisation, animals were challenged with dinitrochlorobenzene on their ears, delayed-type hypersensitivity (DTH) responses were measured 24 and 48 h later and animals were killed on day 19 after the first sensitisation. For details of procedures, see p. 293.

Chempri BV (Raamsdonksveer, The Netherlands); sunflower oil selected for high-oleic acid content from Contined (Bennekom, The Netherlands); fish oil concentrate (Marinol C45 (Loders Croklaan, Wormerveer, The Netherlands), refined for this trial and directly supplemented with the required level of D-α-tocopheryl acetate) and the solid phase of wet-fractionated palm oil (Loders Croklaan).

Sensitisation

The design of the present study is indicated in Fig. 1. For logistical reasons, the present study was performed in five cohorts each with three mice per experimental group. Animals were sensitised by ear painting with 25 μl DNCB (5·0 g/l) in acetone–olive oil (4:1, v/v) on three consecutive days. Five days before first sensitisation, 8 d and 5 h after the final sensitisation, ten drops of blood were taken via orbital puncture under light diethyl ether narcosis. Fourteen days after start of sensitisation, mice were challenged on both ears with 25 μl DNCB (2·5 g/l) to elicit a secondary immune response. Just prior to this challenge and 24 and 48 h later, ear thickness was assessed with an electronic micrometer (Mitoyota, Veenedaal, The Netherlands) to determine DTH responses. Five days after challenge animals were killed.

Dissection

Mice were anaesthetised by diethyl ether inhalation and blood was obtained by orbital puncture. Ten drops of blood heparinised with 20 μl tromboliquine were used in the phagocytosis and oxidative burst tests. The remainder of the blood was used to prepare serum samples. Animals were subsequently killed by cervical dislocation. Samples of the medial liver lobe were isolated, frozen in liquid N_2 and stored at −70°C. Spleens and auricular lymph nodes (ALN) were used to prepare cell suspensions by mincing in RPMI under sterile conditions.

Antibodies and chemicals

Alkaline phosphatase conjugated anti-IgG1, IgG2a and IgG2b from Southern Biotechnology Association (Birmingham, AL, USA), unconjugated anti-IgA, anti-IgE, anti-IFN-γ, anti-IL4, and biotinylated anti-IFN-γ and anti-IL4 from Pharmingen (San Diego, CA, USA). Concanavalin A and [^{3}H]thymidine from ICN Biomedicals (Costa Mesa, CA, USA), diethanolamine, Tween-20 and casein from BDH (Poole, Dorset, UK), and streptavidin-horseradish peroxidase from CLB (Amsterdam, The Netherlands). DNCB, bovine serum albumin, 3,3′5,5′-tetramethylbenzidine, *p*-nitrophenyl phosphate, *N*-hydroxysuccinimide biotin, and dinitrophenyl–human serum albumin were obtained from Sigma. Dinitrophenyl–human serum albumin was biotinylated as described by Bayer & Wilchek (2001). RPMI-1640 was supplemented with bicarbonate, penicillin–streptomycin, β-mercaptoethanol and L-glutamine, all from Gibco BRL (Life Technology, Breda, The Netherlands). All other chemicals were obtained from Merck (Darmstadt, Germany).

Proliferation and cytokine production

Cells from spleen and ALN were resuspended in RPMI and counted on a Coulter counter (Coulter Electronics, Luton, Essex, UK). Cell suspensions were diluted to 10^6 cells/ml for cytokine production and 5×10^6 cells/ml for proliferation. Autologous heat-inactivated serum was added to a final concentration of 7·5 ml/l. For proliferation, spleen cells were stimulated for 48 h with 0 or 3 μg concanavalin A/ml and [^{3}H]-thymidine (18·5 kBq/ml) was added for the last 8 h. DNA was harvested and counted on a scintillation counter (Beta plate; Wallac, Oosterhout, The Netherlands). For cytokine production, ALN and spleen cells were cultured for 24 h with 5 μg concanavalin A/ml (IL4 and IFN-γ) or 2 μg lipopolysaccharide/ml (TNF-α and PGE_2). After this period, plates were centrifuged and supernatant fractions were collected and stored at −70°C.

ELISA

DNCB-specific antibodies of distinct isotypes were measured using in-house developed ELISA as described previously (van Zijverden *et al.* 2000). In brief, DNCB-specific IgG1, IgG2a and IgG2b were captured on a coat of dinitrophenyl–human serum albumin and were detected with alkaline-phosphatase conjugated isotype-specific antibodies. The lower levels of specific IgE and IgA were detected with reversed capture ELISA, using isotype-specific antibodies for capture and biotinylated

dinitrophenyl–human serum albumin followed by streptavidine-peroxidase for detection. IL4 and IFN-γ were detected with in-house-developed sandwich ELISA as described previously (van Zijverden *et al.* 2000).

Phagocytosis and oxidative burst

Phagocytosis and oxidative burst were assessed using commercial kits (Orpegen Pharma, Heidelberg, Germany). In brief, blood samples were incubated (30 min, 37°C) with fluorescently labelled (phagocytosis) or unlabelled *E. coli* bacteria. For oxidative burst, dihydrorhodamine 123 (fluorescent upon oxidation) was added for the last 10 min of the incubation. Extra-cellular fluorescence was quenched and results were analysed flow-cytometrically, determining the number of granulocytes involved in phagocytosis–oxidative burst and the mean level of activity per granulocyte.

Commercial kits

TNF-α was measured according to the manufacturer's instructions with a kit from Biosource (Nivelles, Belgium) and stable derivatives of PGE_2 with the bicyclo Prostaglandin E_2 kit (Cayman, Ann Arbor, MI, USA).

Vitamin E in oils, fats, diets and serum

Tocopherols from oils, fats and diets were extracted, separated and quantified on HPLC as described by Tijburg *et al.* (1997). Serum samples were mixed with antioxidant (ascorbic acid and pyrogallol) and internal standard (α-tocopheryl acetate), deproteinised with ethyl alcohol, and extracted into *n*-heptane. Solvents were removed under N_2, residues were redissolved in chloroform–methanol (2:1, v/v). Peaks were separated on an RP C_{18} (5 μm, 250 × 4 mm) column (Merck, Amsterdam, The Netherlands) by isocratic elution with methanol–2-propanol–water (50:50:8, by vol.).

Statistics

Data were analysed by ANOVA for each vitamin E level separately, using dietary fatty acid composition as factor and cohort as block. ANOVA was also carried out for both vitamin E levels combined, using dietary fatty acid composition and vitamin E level as factors and cohort as block. Differences between SFA, *n*-6 PUFA and *n*-3 PUFA groups were determined using Student-Newman-Keuls *post-hoc* tests. If ANOVA showed significant interaction between fatty acid composition and vitamin E levels, the effects of the fatty acid composition were also determined for both vitamin E levels separately. Where required, data were log transformed to normalise distribution prior to analysis.

Results

Animal growth

The mean weight of the animals at the start of the intervention did not differ significantly between the groups (range 20·9–21·8 g). However, independently of vitamin E level, animals fed *n*-3 PUFA diets (2·7 (SE 0·2) g) gained more weight during the present study than the other animals (1·2 (SE 0·3) and (1·2 (SE 0·2) g SFA and *n*-6 PUFA groups, $P < 0·001$).

Phospholipids

Phospholipid composition was measured in the liver to verify the dietary intervention. As expected, the fatty acid composition of the phospholipids was markedly affected by the dietary fatty acid composition, but not by the amount of vitamin E given (results not shown). In the *n*-6 PUFA groups the proportion of linoleic acid in phospholipids was increased by about 29 % at the expense of palmitic (16:0) and oleic acid (18:1 *n*-9). Eicosapentaenoic and docosahexaenoic acids were about 5-fold increased in both *n*-3 PUFA groups. Compared with the SFA groups, arachidonic acid (20:4 *n*-6) was somewhat increased in the *n*-6 PUFA groups (+12 %) and was dramatically decreased in the *n*-3 PUFA groups (−60 %).

Serum vitamin E

As anticipated, the vitamin E content of serum was markedly affected by the amount of vitamin E provided in the diet and, to a lesser extent, by the dietary fatty acid composition given. Serum vitamin E was significantly higher in groups fed high levels of vitamin E compared with those fed low levels of vitamin E ($P < 0·001$). In low-vitamin E groups, serum vitamin E was somewhat higher in the *n*-6 PUFA group (14·8 (SE 0·9) μmol/l) compared with the *n*-3 PUFA (11·2 (SE 0·4) μmol/l) and SFA group (10·8 (SE 0·4), μmol/l $P < 0·001$). In the high-vitamin E groups, serum vitamin E was about half the level in the *n*-3 PUFA (20·5 (SE 0·7) μmol/l) than in the SFA (44·0 (SE 1·3) μmol/l) and *n*-6 PUFA groups (39·0 (SE 3·0) μmol/l), $P < 0·001$).

Variables of innate immune responsiveness

Phagocytosis and oxidative burst. Phagocytosis and oxidative burst were only marginally influenced by the diet, and only at the low vitamin E level. The % phagocytosing granulocytes was lower in the *n*-6 PUFA group (53 (SE 5) %) compared with the SFA group (64 (SE 4) %, $P < 0·05$). The mean level of phagocytotic activity per granulocyte (range 270–299) and the % granulocytes responding with an oxidative burst (range 53–57 %) were not affected. However, the mean level of oxidative burst per involved granulocyte was higher in the *n*-3 PUFA group (349 (SE 14)) compared with the SFA group (323 (SE 15), $P < 0·05$).

Concentration of tumour necrosis factor α and prostaglandin E_2. The production of TNF-α was assessed in serum collected 6 h after the first sensitisation and in supernatant fractions of lipopolysaccharide-stimulated ALN and spleen cells. TNF-α was only detectable in supernatant fractions of the spleen cell cultures and levels were similar in SFA and *n*-6 PUFA groups, irrespective of vitamin E intake (Fig. 2). In *n*-3 PUFA groups splenic TNF-α production was almost (low vitamin E) or more than (high

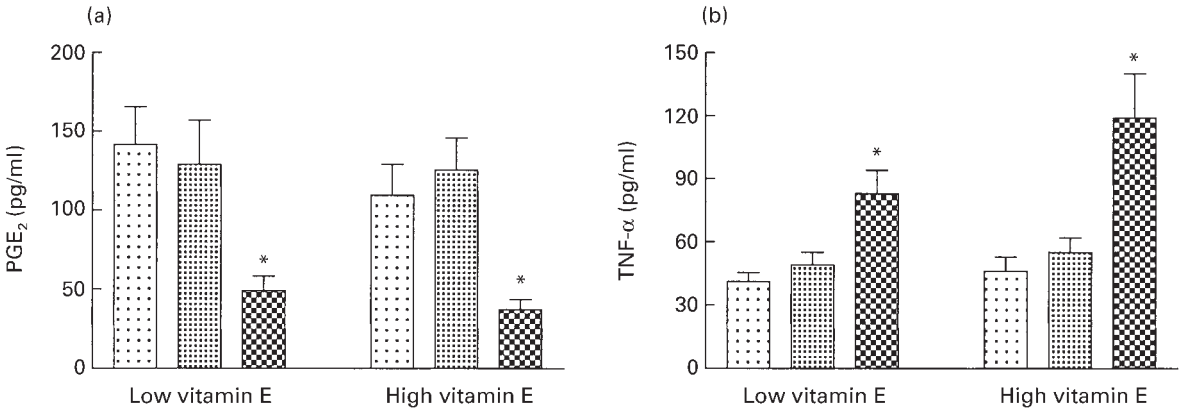

Fig. 2. Production of (a) prostaglandin (PG) E_2 and (b) tumour necrosis factor (TNF) α by lipopolysaccharide-stimulated spleen cells: spleen cells were stimulated with lipopolysaccharide and the level of TNF-α and PGE_2 in the supernatant fraction was determined. (▫), saturated fatty acids (SFA); (▤), *n*-6 polyunsaturated fatty acids (PUFA); (▦), *n*-3 PUFA. For details of diets and procedures, see Table 1 and p. 292. Values are means with standard errors shown by vertical bars (*n* 10–12 for PGE_2 and 15 for TNF-α). Mean values were significantly different from those of the other diets at given vitamin E level: $P<0{\cdot}05$. When analysed for both vitamin E levels combined, values for *n*-3 PUFA were significantly different from those of *n*-6 PUFA and SFA for both PGE_2 and TNF-α ($P<0{\cdot}001$).

vitamin E) twice as high as in the other groups ($P<0{\cdot}001$). Interestingly, effects on production of PGE_2 by lipopolysaccharide-stimulated spleen cells were the reverse of the effects on TNF-α, as production was reduced in both *n*-3 PUFA groups to about a third of the levels in the SFA and *n*-6 PUFA groups ($P<0{\cdot}001$). Vitamin E levels did not affect PGE_2 production.

Variables of adaptive immune responsiveness

Delayed-type hypersensitivity. In all experimental groups, a clear DTH response was observed at 24 h after challenge (Fig. 3). No significant differences in DTH responsiveness were observed between the experimental groups, either at this time point, or at 48 h after challenge when responses had started to subside (results not shown).

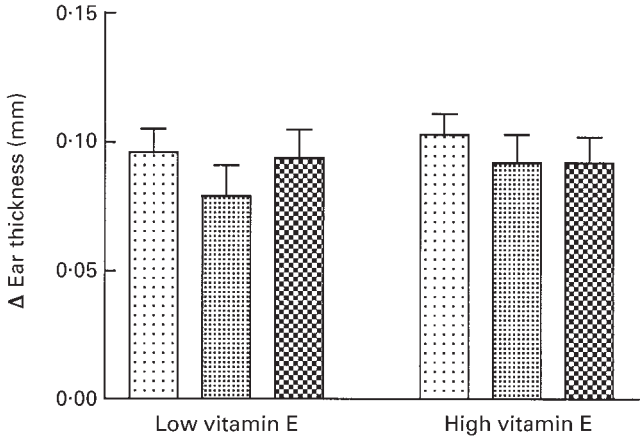

Fig. 3. Delayed-type hypersensitivity response (DTH): ear thicknesses of the sensitised mice were measured before and 24 h after challenge with dinitrochlorobenzene. (▫), saturated fatty acids (SFA); (▤), *n*-6 polyunsaturated fatty acids (PUFA); (▦), *n*-3 PUFA. For details of diets and procedures, see Table 1 and p. 292. The ear swelling is indicated as the Δ ear thickness averaged over both ears of fifteen mice per group. No significant differences were found between mean values for each group.

Levels of dinitrochlorobenzene-specific antibodies in serum. Following sensitisation, a clear time-dependent increase of serum titres of DNCB-specific Ig was observed in all groups, indicating initiation of a specific response to DNCB (results not shown). Table 3 shows the titres of DNCB-specific IgG1, IgG2a, IgG2b, IgE and IgA in sera collected at the end of the present study, 5 d after challenge. Analysed for both vitamin E levels combined, titres of the Th1-dependent isotypes were significantly decreased in the groups receiving the *n*-3 PUFA compared with SFA and *n*-6 PUFA (IgG2a, $P<0{\cdot}01$) or to SFA (IgG2b, $P<0{\cdot}05$). In contrast, the Th2 dependent isotype IgE was significantly increased in the *n*-3 PUFA group compared with SFA ($P<0{\cdot}05$). The other Th2-dependent isotype IgG1 and the mucosa-associated IgA isotype were not different between groups.

Proliferation in auricular lymph nodes and spleen. The number of cells in the ALN draining the site of DNCB challenge reflects mainly *in vivo* activation of lymphocytes. The number of cells in both ALN combined ranged from 20×10^6 (SE $1{\cdot}6\times10^6$) to 22×10^6 (SE $1{\cdot}7\times10^6$), which is clearly increased compared with non-sensitised BALB/c mice (typically 5×10^6–8×10^6 cells/two ALN; M Bol, unpublished results), but was not significantly different between groups. The *ex vivo* polyclonal proliferation of splenocytes (given as stimulation indices) following stimulation by concanavalin A, showed substantial variation but did not differ significantly between diets (41 (SE 9)) and 29 (SE 6) for SFA, low and high vitamin E respectively; 26 (SE 5), 43 (SE 10), for *n*-6 PUFA, low and high vitamin E respectively; 35 (SE 7), 40 (SE 8), for *n*-3 PUFA low and high vitamin E respectively).

Production of interferon-γ and interleukin 4. IFN-γ and IL4 were measured in supernatant fractions of concanavalin A-stimulated cultures of ALN and spleen cells (Table 2). IFN-γ was produced to a greater extent in ALN cultures (ranging from 143–447 pg/ml) compared with spleen cultures (ranging from 60–85 pg/ml), but IL4 levels were similar in spleen and ALN cultures, ranging from 5·3–10·0 pg/ml. No significant differences between

Table 2. Cytokine production by cells from auricular lymph node and spleen*†
(Mean values with their standard errors for twelve mice per group)

	Dietary treatment groups											
	SFA				*n*-6 PUFA				*n*-3 PUFA			
	Low vitamin E		High vitamin E		Low vitamin E		High vitamin E		Low vitamin E		High vitamin E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
ALN												
IFN-γ (pg/ml)	240	64	316	111	143	39	403	110	230	78	447	202
IL4 (pg/ml)	6·0	1·1	7·5	0·8	5·9	0·9	8·0	1·5	8·0	1·4	10·0	2·7
IFN-γ:IL4	40·0	6·3	38·0	11·0	23·0	4·3	42·0	9·0	24·0	6·3	32·0	9·4
Spleen												
IFN-γ (pg/ml)	85	20	68	20	77	30	78	24	60	13	73	24
IL4 (pg/ml)	6·0	0·6	5·3	0·6	7·0	1·5	5·9	0·9	9·0	1·3	7·0	1·4
IFN-γ:IL4	15·0	3·4	11·0	2·3	9·0	1·4	11·0	2·4	5·9	0·9	9·0	1·4

SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; ALN, auricular lymph node; IFN, interferon; IL, interleukin.
* For details of diets and procedures, see Table 1 and p. 292.
† Production of IFN-γ and IL4 after stimulation of ALN and spleen cells with concanavalin A.

experimental groups were observed at the level of absolute production of either IFN-γ or IL4, although high vitamin E levels tended to increase the production of IFN-γ and IL4 by ALN cells ($P<0·1$). The IFN-γ:IL4 ratio was calculated as this can indicate immunomodulation with regard to Th1 *v.* Th2 activation dominance. The IFN-γ:IL4 ratio tended to decrease in mice that received unsaturated fatty acids, but only if they received a diet with low levels of vitamin E. For ALN cells the ratio decreased from about 40·0 in the SFA group to 23·0 in the *n*-6 PUFA group and to 24·0 in the *n*-3 PUFA group. For the spleen cells the ratio decreased from 15·0 in the SFA group to 9·0 in the *n*-6 PUFA group and 5·9 in the *n*-3 PUFA group ($P<0·1$). When analysed at both vitamin E levels separately, the ratio in *n*-3 PUFA low-vitamin E group differed significantly from the SFA, low-vitamin E group ($P<0·05$).

Discussion

In the present study we have investigated the applicability of a murine epidermal sensitisation model for nutrition studies, using prototype diets with different fatty acid profiles and vitamin E content.

Feeding mice the *n*-3 PUFA-rich diets caused significantly higher body-weight gain, suggesting higher food intake in these groups. These diets also greatly increased % long-chain *n*-3 fatty acids eicosapentaenoic and docosahexaenoic acids in the phospholipid fraction, while substantially reducing the level of arachidonic acid (20 : 4*n*-6). Production of PGE_2 by spleen cells was also dramatically decreased in these groups. This is in accordance with results from literature indicating that both eicosapentaenoic and docosahexaenoic acids can reduce PGE_2 production through displacement of arachidonic acid and inhibition of the cyclo-oxygenation of arachidonic acid to PGE_2 (Calder, 1997). The high linoleic acid (18 : 2 *n*-6) content of the *n*-6 PUFA diets raised the levels of this fatty acid in phospholipids and of its elongation product arachidonic acid (20 : 4*n*-6). This elevated arachidonic acid did not result in increased production of

Table 3. Serum titres of dinitrochlorobenzene-specific antibodies of different isotypes*†
(Mean values with their standard errors for fifteen mice per group)

	Dietary treatment groups											
	SFA				*n*-6 PUFA				*n*-3 PUFA			
	Low vitamin E		High vitamin E		Low vitamin E		High vitamin E		Low vitamin E		High vitamin E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
IgG2a‡	12·8	0·5	13·8	0·5	12·8	0·3	12·6	0·5	11·4	0·4	12·4	0·4
IgG2b§	11·5	0·3	11·8	0·4	11·4	0·4	11·6	0·4	10·8	0·4	11·0	0·3
IgG1	17·0	0·3	17·2	0·2	17·0	0·2	17·2	0·4	16·7	0·2	17·3	0·4
IgE‖	8·3	0·2	8·3	0·1	8·7	0·3	8·4	0·2	8·8	0·2	8·8	0·2
IgA	10·3	0·1	10·3	0·2	10·2	0·2	10·2	0·1	10·2	0·1	10·1	0·1

SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; Ig, immunoglobulin.
* For details of diets and procedures, see Table 1 and p. 292.
† Dinitrochlorobenzene-specific antibodies of different isotypes were measured in the sera obtained 5 d after the challenge.
‡ For IgG2a *n*-3 PUFA< *n*-6 PUFA and SFA ($P<0·01$).
§ For IgG2b *n*-3 PUFA< *n*-6 PUFA and SFA ($P<0·05$).
‖ For IgE *n*-3 PUFA> SFA ($P<0·05$).

PGE_2, indicating that production is not regulated solely by substrate availability.

Apart from a slight elevation in the *n*-6 PUFA group, serum vitamin E levels in the low-vitamin E groups were comparable, which demonstrates that the distinct vitamin E requirements of the different dietary fatty acid compositions were successfully compensated. In the groups fed high-vitamin E diets, serum vitamin E was markedly increased. Surprisingly, however, the level in the *n*-3 PUFA high-vitamin E group was only half that in the other high-vitamin E groups. This was not caused by accelerated vitamin E losses during storage of this diet (−12 *v.* −4 and −15 % in SFA and *n*-6 PUFA diets respectively). Possibly there was reduced uptake, increased oxidation in the gastrointestinal tract or increased consumption of vitamin E in the membranes, due to the increased unsaturation of the fatty acids in this diet.

The key difference between the SFA and *n*-6 PUFA diet is an exchange of palmitic for linoleic acids. The only difference in immune function was a marginal decrease in % phagocytosing cells with the *n*-6 PUFA, low-vitamin E diet. This suggests that there is little immunological impact (e.g. detriment) of replacing saturated fat with linoleic acid. Clearly these results do not support the postulate that increased linoleic acid consumption would result in increased production of PGE_2 and subsequent skewing to a Th2 phenotype so favouring allergic inflammation and asthma (Black & Sharpe, 1997; Haby *et al.* 2001).

The main immununological effects were due to feeding the *n*-3 PUFA diets. Of the innate variables studied, oxidative burst was marginally increased by *n*-3 PUFA, but only at the low-vitamin E level and only the mean level of activity per cell, not the % granulocytes involved. Production of TNF-α was clearly increased by *n*-3 PUFA, whereas production of PGE_2 was decreased. The enhancing effects of *n*-3 PUFA on TNF-α production are in accordance with most other studies looking at TNF-α production by *ex vivo* stimulated residential macrophages (Lokesh *et al.* 1990; Blok *et al.* 1996). In contrast, studies with elicited macrophages, i.e. *in vivo* stimulated with lipopolysaccharide, thioglycollate or Freund's adjuvant, tend to report decreased serum on plasma levels of TNF-α due to feeding *n*-3 PUFA (Blok *et al.* 1996; Calder, 1997; Wallace *et al.* 2000). As the much milder inflammatory response induced by topical application of DNCB did not yield detectable serum TNF-α levels we were unable to verify this apparent inconsistency.

The effect of the *n*-3 PUFA diets on adaptive immune responses to DNCB comprise decreased serum levels of the Th1-dependent isotypes (IgG2a and IgG2b), whereas levels of the Th2-dependent and mucosal isotypes increased (IgE) or were unaffected (IgG1 and IgA). As we have noted previously (Albers *et al.* 1998), detection of DNCB-specific antibodies of distinct isotypes is an easier and more sensitive marker of Th1–Th2 modulation than detection of cytokines following mitogen stimulation of T lymphocytes. However, it is important to note that the selective reduction of Th1-driven isotypes is supported by a tendency to decreased ratios of the main immunomodulating cytokines IFN-γ (supporting IgG2a and IgG2b):IL4 (supporting IgG1 and IgE) in the *n*-3 PUFA groups. Stimulation of specific IgE production by consumption of *n*-3 PUFA has been reported in the past (Prickett *et al.* 1982). More recently, others have reported reduced production of the Th1 cytokines IL2, IFN-γ and IL12 (Fritsche *et al.* 1999; Jolly & Fernandes, 1999; Wallace *et al.* 2001), but not of the Th2 cytokines IL4, IL5, IL10 (Jolly & Fernandes, 1999; Wallace *et al.* 2001), after feeding mice diets rich in *n*-3 PUFA.

We did not observe inhibitory effects of *n*-3 PUFA on *in vivo* (ALN cell number) or *ex vivo* (mitogen-induced) lymphocyte proliferation. This contrasts with other studies that have reported suppressed lymphocyte proliferation when feeding *n*-3 containing oils (e.g. linseed or fish oil). Most likely, these effects are only observed when more extreme diets are used. Likewise, DTH responses were not affected by *n*-3 PUFA at current dose levels (about 19 g *n*-3 PUFA/kg), whereas DTH is suppressed when more extreme diets are used (50–200 g/kg)(Dewille *et al.* 1981; Hinds & Sanders, 1993; Sanderson *et al.* 1995; Jeffery *et al.* 1996). Using purified eicosapentaenoic *v.* docosahexaenoic acids at 48 g/kg diet, Tomobe *et al.* (2000) found that this inhibition is elicited by docosahexaenoic, but not by eicosapentaenoic acids. Although scientifically interesting, the relevance of effects observed at such unrealistically high *n*-3 PUFA levels is limited. Nonetheless, these findings underline that regulation of different Th1-driven effector mechanisms like DTH responses and production of IgG2a and IgG2b is partially independently regulated, as we observed previously (Albers *et al.* 1998). It is therefore important to assess various aspects of the different components of the immune response in order to obtain a broad overview of the immunomodulatory activity.

The level of vitamin E intake seemed to modulate some of the effects of *n*-3 PUFA. Increase of oxidative burst as well as suppression of Th1-driven immune responses by feeding *n*-3 PUFA was observed only (oxidative burst) or most clearly (Th1–Th2) in the low-vitamin E group. On the other hand, increased production of TNF-α appeared more pronounced in the high-vitamin E group. In addition, but independent of fatty acid intake, high vitamin E intake tended to increase production of IFN-γ and to a lesser extent also of IL4 by ALN but not by spleen cells. Previously, vitamin E was shown to stimulate various immune variables, including macrophage (Moriguchi *et al.* 1990; Fritsche *et al.* 1999; Wallace *et al.* 2001) and Th cell functions (Tanaka *et al.* 1979; Moriguchi *et al.* 1998; Fritsche *et al.* 1999) in particular of the Th1 type (Han *et al.* 2000*b*). Our current findings are in line with stimulatory effects of vitamin E on Th1 function. Using older mice might increase sensitivity of the model to detect effects of vitamin E, as previous studies have shown that effects of vitamin E are more pronounced in aged mice (Hayek *et al.* 1997; Han *et al.* 2000*a*). Overall, vitamin E and *n*-3 PUFA seem to act via independent mechanisms, as they additively increase TNF-α production, whereas vitamin E tends to ameliorate the selective suppressive effects of *n*-3 PUFA on certain Th1 driven effects (IFN-γ: IL4 ratio and IgG2a levels).

In summary, we have demonstrated the usefulness of a murine sensitisation model to assess diet-induced changes of innate and adaptive immune responsiveness. Whereas

feeding *n*-6 PUFA had little immunomodulatory effects, *n*-3 PUFA decreased production of PGE_2, and increased oxidative burst and production of TNF-α. It selectively reduced (some) adaptive type 1 responses (IgG2a, IgG2b, IFN-γ:IL4) while stimulating (IgE) or not affecting type 2 immune responses. Combination with high levels of vitamin E did not affect reduced PGE_2 production, augmented the increase of TNF-α production, and tended to ameliorate the selective suppressive effects of *n*-3 PUFA on certain Th1 driven effects (IFN-γ:IL4 ratio and IgG2a levels). Based on these results, we conclude that the model used here provides a broad overview of effects of dietary intervention on various aspects of immune responsiveness. As such, it provides a valuable model to assess, characterise, and potentially rank effects of foods and/or nutrients on a range of immune functions including Th1–Th2 polarisation.

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