

Use of fish oil to prevent graft rejection

Helmut Grimm^{1*}, Friedrich Grimminger², Stephan Korom¹ and Werner Seeger²

Departments of ¹Surgery and ²Internal Medicine, Justus-Liebig University, D-35385 Giessen, Germany

Immunonutrition combining parenteral nutrition and pharmacological intervention has become of particular interest over the last few years. *n*-3 Fatty acid-rich lipid emulsions have been developed for parenteral application in clinical situations, designed to shift the arachidonic acid (AA): eicosapentaenoic acid (EPA) value toward predominance of the latter lipid-mediator precursor. Many of the EPA-derived eicosanoids, i.e. 5-series cysteinyl leukotrienes (LT), LTB₅ and thromboxane (TX)A₃, possess markedly reduced inflammatory and vasomotor potencies and even exert antagonistic effects to AA-derived lipid mediators (Leaf & Weber, 1988). Dietary supplementation with *n*-3 fatty acids has been shown to cut down on polymorphonuclear leucocyte (PMN)-mediated and thrombocyte-related inflammatory events (Leaf & Weber, 1988) and to reduce proinflammatory cytokine synthesis in mononuclear cells (Endres *et al.* 1989).

Based on the properties mentioned previously, *n*-3 fatty acids might even have advantageous effects on the acute rejection process after allotransplantation, in which inflammatory and specific immunological reactions are involved. Both experimental and clinical studies have recently shown that *n*-3 fatty acid-enriched diets reduce nephrotoxicity and graft rejection in cyclosporine A-treated recipients (Kelley *et al.* 1990; van der Heide *et al.* 1993). Since dietary supplementation with EPA is associated with slow kinetics and limited availability for inflammatory processes, the intravenous route of *n*-3 lipid administration might prove superior in an immunological reaction as grave as acute rejection.

In the present study, a fish oil-based lipid emulsion was continuously infused after heterotopic rat heart allotransplantation to assess the impact on graft rejection, with particular focus on inflammatory and specific immunological processes. Graft survival, mobilization of immunocompetent cells, cytokine release by mononuclear cells, plasma fatty acid profiles and lipid mediator generation were evaluated and compared with data derived from the infusion of a soybean oil-based lipid preparation.

Material, methods and experimental design

Animals

The experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1980) after approval of the local committee of ethics.

Inbred PVG (class 1 major histocompatibility antigen RT 1^c, male, 100–150 g) and Wistar/Kyoto rats (class 1 major histocompatibility antigen RT 1¹, male, 200–250 g; Mollegaard Breeding Center, Skensved, Denmark) served as donors and recipients respectively. The rats were housed in plastic cages with stainless-steel-wire bottoms in a laboratory with controlled temperature (20°), humidity (50 %) and 12 h light–dark cycle. The animals were allowed to adapt to the environment for at least 1 week before transplantation. They were fed on R3-EWOS-ALAB Brood Stock Feed (ALAB, Sollentuna, Sweden).

Intravenous catheter

A 50 mm long, spiral-shaped piece of PE 10 (polyethylene) catheter (Clay Adams, Parsippany, NJ, USA) attached to a silicon tube (Silastic[®], 0.012 in × 0.025 in, no. 602–105 HH 061999; Dow Corning Corp., Midland, MI, USA) was heat fused to a 300 mm piece of a PE 20 catheter. The silicon part of the catheter was placed in the animal's left jugular vein, as described elsewhere (Weeks, 1972), and the PE 20 end diverted to the exterior immediately before transplantation. This catheter was connected to a SAGE pump (Ismatec, Wertheim, Germany) allowing 24 h continuous infusion.

Surgical technique

The recipients were anaesthetized with phentanylcitrate (Hypnorm[®]; Janssen, Beerse, Belgium; 0.315 mg/kg body weight given intramuscularly). The abdomen was opened by a midline incision. The left kidney was removed and the kidney vessels were cuffed, as described elsewhere (Heron, 1973). The donors were anaesthetized with pentobarbital (Mebumal vet.[®]; Nord Vacc, Stockholm, Sweden; 60 mg/kg body weight intraperitoneally). Heparin (300 IU) was injected intravenously before the harvesting of the heart. The grafts were flushed with cold Ringer lactate solution containing 50 IU heparin/ml. Immediately after harvesting the graft was anastomized with the cuffed vessels; the cold ischaemia time was less than 5 min.

Fat emulsions

Emulsions containing 200 ml oil/l were prepared using soyabean oil or fish oil. The fatty acid composition of these oils is given in Table 1. The isotonic water phase containing

Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; LT, leukotriene; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear leucocyte; TNF- α , tumour necrosis factor- α ; TX, thromboxane.

*Corresponding author: Dr H. Grimm, fax +49 641 9944709

Table 1. Fatty acid composition (g/100 g total fatty acids) of fish oil and soyabean oil

Fatty acid	Fish oil	Soyabean oil
12 : 0	0.2	
14 : 0	5.5	0.1
16 : 0	14.2	11.0
16 : 1	6.5	0.1
17 : 0		0.1
18 : 0	3.2	4.4
18 : 1 n -9	10.1	23.3
18 : 1 n -7	3.1	1.55
18 : 2 n -6	2.6	51.6
18 : 3 n -6	0.2	
18 : 3 n -3	0.7	6.6
18 : 4 n -3	2.5	
20 : 0	0.2	0.3
20 : 1 n -9	1.5	0.3
20 : 1 n -7	1.8	
20 : 1 n -5	0.2	
20 : 2 n -6		0.5
20 : 3 n -6		0.04
20 : 4 n -6	1.6	0.1
20 : 5 n -3	18.1	
22 : 0		0.4
22 : 1 n -11	2.0	
22 : 1 n -9	0.5	
22 : 1 n -7	0.1	
22 : 5 n -3	2.7	
22 : 6 n -3	12.9	
22 : 6 n -6		0.3

distilled water, purified egg phospholipids and glycerol was heated to 60–70° and the lipid phase was added in a mixer (KABI Pharmacia, Stockholm, Sweden) at high speed. The emulsions contained the antioxidant vitamin E at a concentration of 1 mg/ml. A fine emulsion was created in a valve homogenizer (KABI Pharmacia) at high pressure. The emulsion was dispensed into glass vials and heat sterilized.

Experimental design and treatment groups

Animals were randomized into groups of ten and given one of the two fat emulsions. Infusion was started immediately after transplantation and terminated after rejection was completed. Fat (9 g/kg body weight per d) was administered by continuous intravenous infusion over 24 h. Controls were infused with a corresponding volume of saline (9 g NaCl/l).

Assessment of graft rejection

The transplanted hearts were palpated twice daily. Rejection was considered to be complete when no pulsations were palpable and electrocardiography showed no activity of the transplant. Following removal of the grafts, rejection diagnosis was verified histologically.

Assessments at a defined time point of the rejection process

In a second set of experiments graft recipients (n 10 per group) were exsanguinated on day 4 after transplantation and their transplants were harvested. Blood collected by puncture of the recipient's abdominal aorta through a laparotomy

incision was used for the analyses described later and the grafts were subjected to an immunohistological screening.

Immunohistological studies

Grafts were snap-frozen in liquid N₂ immediately after harvesting. Cryostat sections (7 µm) were cut and air-dried on gelatinized slides, fixed in acetone, and stored at –20° until used. To stain the sections, the slides were thawed and the sections were incubated with the appropriate mouse anti-rat monoclonal antibody (W 3/25, MRC OX-8 and MRC OX-1; Camon Co., Wiesbaden, Germany) at room temperature for 30 min (dilution 1 : 100; v/v) and then washed twice in Tris-buffered saline. The second incubation with rabbit anti-mouse immunoglobulin bridging antibodies (diluted 1 : 20; v/v) was done at room temperature for 30 min. The slides were washed and then incubated with alkaline phosphatase–anti-alkaline phosphatase complex (diluted 1 : 50; v/v) for 30 min at room temperature. The colour was developed with alkaline phosphatase (EC 3.1.3.1) substrate (incubation for 30 min at room temperature). Sections were counterstained with haemalaun. Labelled cells within twenty high-power fields per section per rat were counted with the aid of an ocular grid micrometer (Leitz Co., Wetzlar, Germany).

Analysis of peripheral blood lymphocyte subpopulations by fluorescein-activated cell sorter

Fluorescein-conjugated monoclonal antibodies (Camon, Wiesbaden, Germany; 10 µl) against CD (cluster of differentiation)45R (B-cells), CD 4 (T4-cells), CD 8 (T8-cells), CD 5 (T-cells), CD 25 (interleukin-2-receptor-positive T-cells) and immunoglobulins G1 or G2a (negative controls) respectively were mixed with 100 µl EDTA-treated blood and incubated for 15 min in the dark. Lysing solution (1 µl) was added and, after incubation for another 5 min, cells were centrifuged at 300 g. The pellet was resuspended, washed with fluorescein-activated cell sorter flow solution (Becton-Dickinson, Erembodegem-Aalst, Belgium) and again centrifuged for 5 min. After resuspending the pellet with 0.5 ml fluorescein-activated cell sorter flow solution, lymphocyte subpopulations were measured in the fluorescein-activated cell sorter.

Stimulation of peripheral blood mononuclear cells in vitro

Heparinized fresh whole blood (10 IU heparin/ml) was diluted 1 : 2 (v/v) with PBS. The peripheral blood mononuclear cell (PBMC) fraction was obtained by Ficoll-Hypaque centrifugation (Böyum, 1968). The cells were washed in PBS before culturing. The PBMC were cultured for 24 h at 37° at a density of 1×10^6 cells per well in RPMI 1640 (Biowhittaker, Ingelheim, Germany) supplemented with fetal calf serum (50 ml/l), either unstimulated or after addition of 25, 50 or 100 g concanavalin A. The supernatant fraction was collected after centrifugation at 2000 rev./min for 10 min. The tumour necrosis factor- α (TNF- α) concentration in the PBMC culture media was assayed using a commercial murine TNF- α ELISA kit (Endogen, Boston MA, USA), as described previously (Meager *et al.* 1989).

Plasma fatty acids

Non-esterified plasma fatty acids were quantified by one-step rapid extractive methylation for GC analysis (Pace-Asciak, 1989). Briefly, citrate-treated plasma was spiked with heptadecanoic acid as internal standard; free fatty acids were converted to methyl esters by mixing with ethereal diazomethane; the ether layer was dried, redissolved in chloroform, and transferred to the GC. GC analysis was performed using a Chrompack gas chromatograph CP 9000 with a CP-88 fused silica capillary column (50 m × 0.25 mm; Chrompack AG, Frankfurt, Germany). The programme used an initial oven temperature of 160°, lasting 2 min. The temperature then was raised at a rate of 4°/min to 220°, where it was held for 3 min. Injector and detector were maintained at 250 and 300° respectively. The fatty acid methyl esters were detected using a flame-ionization detector, and peak area integration was performed. For quantification of total plasma fatty acids, 30 µl plasma was subjected to hydrolysis and methylation in methanol–2 M-HCl for 15 h at 100°. After evaporation, the residue was redissolved in methanol–water, extracted with hexane, evaporated to dryness, redissolved in chloroform and subjected to GC analysis.

Neutrophil leukotriene profile

Rat PMN were isolated by an adaptation of the technique described by Hjorth *et al.* (1981) for human PMN. Blood was immediately mixed with heparin (50 U/ml) and centrifuged in a discontinuous Percoll gradient to yield a fraction of approximately 97 % purity. Cell viability, as assessed by Trypan Blue exclusion, ranged above 96 % under all experimental conditions, and lactate dehydrogenase (*EC* 1.1.1.28) release was consistently below 3 %. PMN were incubated in RPMI 1640–fetal calf serum (100 ml/l), washed twice with Hank's balanced salt solution–4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid (HEPES) (25 mmol/l) without Ca²⁺ and Mg²⁺, resuspended in Hank's balanced salt solution–HEPES (25 mmol/l), and stimulated for 10 min with 1 µmol A23187/l (Calbiochem, Bad Soden, Germany). After terminating the reaction on ice, cells were removed by centrifugation (3000 g for 5 min at 4°), and LT of the 4- and 5-series were extracted from the supernatant fraction using octadecylsilyl solid-phase extraction columns as described by Grimminger *et al.* (1988). Conversion into methyl esters was performed by addition of freshly prepared diazomethane in ice-cold diethyl ether. Reverse-phase HPLC of non-methylated compounds was carried out on octadecylsilyl columns (Hypersil; Shandon, Astmoor, UK; 5 µm particles) with a mobile phase of methanol–water–acetic acid (72 : 28 : 0.16, by vol., pH 4.9). In addition to the conventional u.v. detection at 270 nm (LT) and 237 nm (5-hydroxyicosatetraenoic acid and HEPES), a photodiode array detector (Waters model 990; Waters/Millipore, Eschborn, Germany) was used, which provided full u.v. spectra (190–600 nm) of eluting compounds and allowed checking for peak purity and subtraction of possible co-eluting material. For additional verification, samples were collected in 15 s fractions in selected experiments, and subjected to post-HPLC radioimmunoassay with anti-LTB₄, as previously described (Grimminger *et al.* 1988). Reverse-

phase HPLC of methylated compounds was performed isocratically (methanol–water–acetic acid; 66 : 34 : 0.16, by vol., pH 4.9) for 5 min, followed by a linear gradient to methanol–water–acetic acid (90 : 10 : 0.16, by vol.) over 10 min (Gynkotheek gradient former, model 250; Gynkotheek, Munich, Germany). Straight-phase HPLC of methylated compounds was carried out using a modification of the method of Nadeau *et al.* (1984). The mobile phase consisted of hexane–propan-2-ol–acetate (86 : 14 : 0.1, by vol.) and the column was eluted isocratically at a flow-rate of 1.0 ml/min. All values obtained by the different analytical techniques were corrected for the respective recoveries of the overall analytical procedure and are given in pmol/10⁶ PMN throughout the experiments. Recovery was determined by separate recovery experiments using different quantities of the individual compounds in the appropriate concentration range. For quantification of the different LT, it was required that values calculated from u.v. absorbance in two different chromatographic procedures should agree (deviation < 10 %). For LTB, quantification was confirmed additionally by the use of post-HPLC radioimmunoassay.

Platelet thromboxane generation

Blood was collected in 3 ml plastic tubes containing 700 µl EDTA solution (75 g/l). After centrifugation at 200 g for 10 min, the platelet-rich plasma was decanted and spun again (1500 g for 10 min). Pelleted platelets were washed with isotonic PBS (pH 7.4), recentrifuged, and resuspended in Tris buffer (20 mmol/l, pH 7.4, containing (mmol/l): 132.8 NaCl, 4.3 KCl, 1.1 KH₂PO₄, 2.4 CaCl₂, 1.3 MgPO₄, 2400 mg glucose/l). Platelet count was adjusted to 10⁸/ml, and stimulation was performed with 2 µmol A23187/l (vehicle dimethyl sulfoxide, final concentration 5 ml dimethyl sulfoxide/l). Incubation was terminated after 15 min by adding 500 µl Tris buffer with bovine albumin (1 ml/l, pH 7.0) and 4 ml ice-cold Tris buffer. After centrifugation at 1500 g for 10 min, TXA₂ and TXA₃ in the supernatant fraction were measured as their stable hydrolysis products TXB₂ and TXB₃. The analytes were extracted from the buffer solution by solid-phase extraction, subjected to reverse-phase HPLC separation, and quantified by post-HPLC ELISA as detailed recently (Krämer *et al.* 1993). To avoid chemical decomposition of the analytes, ionic pair reagent, antioxidant and high buffer strength were used during critical steps of the analytical procedure. Briefly, buffer samples, including zero controls as well as controls with known amounts of TX, were supplied with the ionic pair reagent Tris buffer with bovine albumin and submitted to solid-phase extraction with pre-conditioned C₁₈ columns. Elution was performed by addition of acetone–acetonitrile (50 : 50, v/v) into microreaction vessels containing 10-fold concentrated PBS (pH 7.4) and α-tocopherol for protection of prostanoids in the following freeze-drying procedure under vacuum. Dried eluates were extracted with acetonitrile and submitted to reverse-phase HPLC separation (C₁₈, column length 20 × 150 mm, 3 µm particles; mobile phase: acetonitrile–water (28 : 72, v/v), 0.5 mmol Tris buffer with bovine albumin, pH 7.3; flow-rate 1 ml/min) to separate 2- and 3-series TX. Eluate fractions of 0.3 ml corresponding to the known retention times of TXB₂

and TXB₃ were collected, freeze-dried, redissolved in water, and subjected to ELISA. A monoclonal mouse antibody against TXB₂ with established cross-reactivity to TXB₃ (90 %) was used.

Statistics

Differences between the groups in graft survival, PBMC cytokine release and peripheral blood lymphocyte subpopulations were analysed by Student's *t* test, after a normal distribution had been confirmed by the Kolmogorov–Smirnov test. Significance was assumed to be $P < 0.05$. Values are presented as means with their standard errors.

The Mann–Whitney U test was used to compare immunohistological differences in the number of graft-infiltrating cells between the different groups. The Bonferroni procedure was applied to adjust for repeated comparisons.

For all other determinations one-way ANOVA with Tukey's honestly significant difference *post hoc* test was used to test for differences between the various groups after a normal distribution had been confirmed by the Kolmogorov–Smirnov test. $P < 0.05$ was considered to indicate statistical significance.

Results

Graft survival

In saline-treated controls acute rejection was complete after an average of 7.8 (SEM 0.3) d. There was a moderate prolongation of graft survival with continuous infusion of soyabean oil (10.4 (SEM 0.7) d, *v.* controls $P < 0.01$) and a considerable delay in acute rejection following fish oil infusion (12.3 (SEM 0.4) d, *v.* controls $P < 0.01$, *v.* soybean oil $P < 0.05$) (Table 2).

Immunohistological studies

The number of infiltrating cells was highest in the grafts of the control animals. The overall numbers of both leucocytes and all classified subtypes were markedly decreased in the fish oil group (Table 3). T4- and T8-cells were reduced to less than 50 % as compared with controls. T4 : T8 was markedly increased in the fish oil group as compared with both the control and the soyabean oil groups, which showed identical T4 : T8 values.

Table 3. Immunohistological distribution of graft-infiltrating cells (cells per high-power field) in Wistar/Kyoto rats after PVG rat heart transplantation and infusion with saline (9 g NaCl/l; control), soyabean oil or fish oil*

(Mean values and standard deviations)									
Antibodies	Marked cells	Isografts		Saline		Soyabean oil		Fish oil	
		Mean	sd	Mean	sd	Mean	sd	Mean	sd
MRC OX-1	Leucocytes (CD45+)	3.2	0.5	13.9 ^a	0.7	8.9 ^b	0.6	8.3 ^b	0.4
W 3/25	T4-cells	4.9	0.5	8.6 ^a	0.5	7.3 ^b	0.5	5.0 ^{bc}	0.2
MRC OX-8	T8-cells	1.3	0.5	8.2 ^a	0.5	7.5	0.6	3.3 ^b	0.2
W 3/25, MRC OX-8	T4 : T8	4.0		1.0		1.0		1.5	

^{a,b,c} Mean values in the same row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of animals and procedures, see pp. 577–579.

Lymphocyte subpopulations in the peripheral blood

In the fish oil group the percentage of T lymphocytes (CD 5⁺) in the peripheral blood was significantly reduced (59.8 (SE 2.6) %, $P < 0.05$) compared with that of the controls (69.0 (SE 2.8) %). However, the proportional distribution of the T-cell subgroups (CD4⁺, CD8⁺ and interleukin-2 receptor positive (CD25⁺) cells) was not significantly changed. The percentage of B-cells (CD45R⁺) was approximately the same in both groups. The proportion of CD45R⁺, CD5⁺, CD4⁺, CD8⁺ and CD25⁺ lymphocytes in the soyabean oil and the control groups varied only marginally (Table 4).

Peripheral blood mononuclear cell tumour necrosis factor- α secretion

In all groups PBMC TNF- α secretion was maximal on stimulation with 50 μ g concanavalin A (Fig. 1). The TNF- α secretory potential was significantly decreased in the fish oil group (51.9 (SE 13) pg/10⁶ cells), but not in the soyabean oil group (67.1 (SE 9) pg/10⁶ cells) as compared with the controls (70.8 (SE 10.9) pg/10⁶ cells; $P < 0.002$).

Plasma fatty acids

In saline-infused rats linoleic acid (18 : 2) and AA (20 : 4) were the predominant compounds within the total plasma fatty acid pool (Fig. 2). No substantial change occurred within the fatty acid profile of rats as a result of infusion with soyabean oil. In rats undergoing fish oil infusion the total plasma fatty acid concentration increased by 50 % and the fatty acid profile shifted toward EPA and docosahexaenoic acid, the sum of which even surpassed that of 18 : 2 and

Table 2. Survival of PVG rat heart transplanted into Wistar/Kyoto rats receiving infusions of saline (9 g NaCl/l; control), soyabean oil or fish oil \ddagger

Infusion	Graft survival (d)		
	Individual rats	Mean	SEM
Saline	7,7,7,7,7,8,8,9,9,9	7.8	0.3
Soyabean oil	8,8,8,9,10,11,12,12,13,13	10.4**	0.7
Fish oil	10,11,12,12,12,12,12,13,14,15	12.3*** \ddagger	0.4

Mean values were significantly different from those for saline (control) group: ** $P < 0.01$.

Mean values were significantly different from those for soyabean oil group: \ddagger $P < 0.05$.

\ddagger For details of animals and procedures, see pp. 577–579.

Table 4. Percentage distribution of circulating peripheral blood mononuclear cells in Wistar/Kyoto rats after PVG rat heart transplantation and infusion with saline (9 g NaCl/l; control), soyabean oil or fish oil*

		(Mean values and standard deviations)					
		Saline		Soyabean oil		Fish oil	
Antibodies	Marked cells	Mean	SD	Mean	SD	Mean	SD
anti-CD45R	B-cells	25.7 ^a	1.7	24.6 ^b	2.8	34.0 ^b	1.3
anti-CD5	T-cells	69.0 ^a	2.8	67.2 ^b	3.4	59.8 ^b	2.6
anti-CD4	T4-cells	48.3	2.1	49.2	2.1	42.5	1.9
anti-CD8	T8-cells	29.7	2.7	30.1	2.2	35.8	5.3
anti-CD25	IL2-R ⁺ T-cells	3.4	0.2	3.3	0.3	5.3	1.7

IL2-R⁺, interleukin-2 receptor positive.

^{a,b} Mean values in the same row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of animals and procedures, see pp. 577–579.

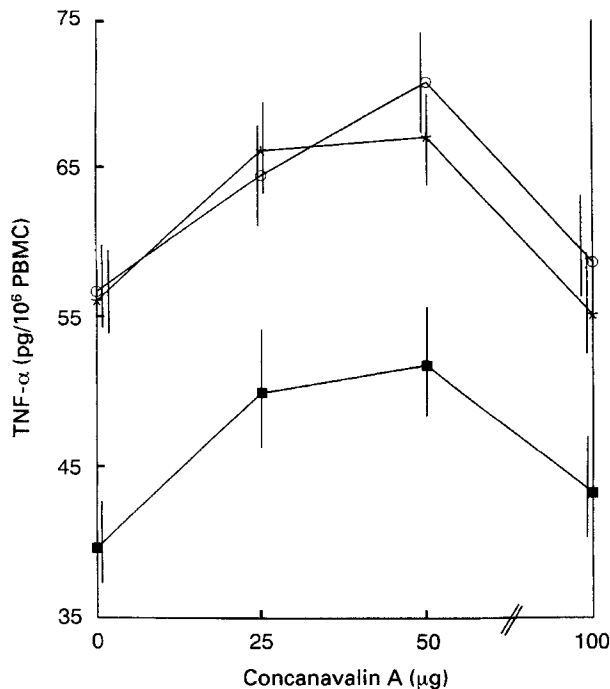


Fig. 1. Concanavalin A-stimulated tumour necrosis factor (TNF)- α secretory potential of peripheral blood mononuclear cells (PBMC) from fish oil (■), soyabean oil (*) and saline (○)-infused Wistar/Kyoto rats after PVG rat heart transplantation. Values are means and standard deviations represented by vertical bars. For details of animals and procedures, see pp. 577–579.

20 : 4. Even more dramatic were the changes in the free fatty acid profile (Fig. 3). Fish oil infusion induced an approximately 2.5-fold increase in the total sum of free fatty acids, and the sum of EPA and docosahexaenoic acid markedly exceeded that of 18 : 2 and 20 : 4.

Polymorphonuclear leucocyte leukotriene synthesis

Only 4-series LT, including LTB₄, its oxidation products 20-COOH- and 20-OH-LTB₄, LTA₄, and its hydrolysis products 6-*trans*- and 6-*trans*-epi-LTB₄ were released on *in vitro* stimulation of PMN isolated from saline-treated

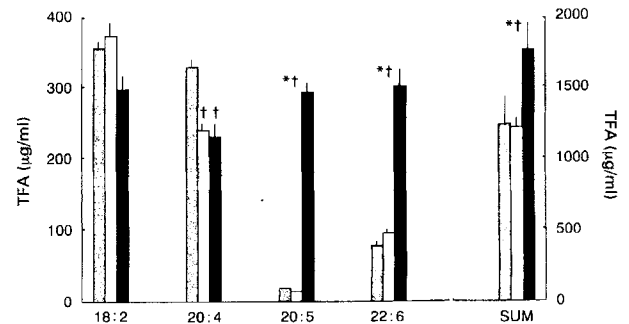


Fig. 2. Total plasma fatty acids (TFA) in saline (9 g NaCl/l; control; ■), soyabean oil (□) and fish oil (▨)-infused Wistar/Kyoto rats after PVG rat heart transplantation. A selection of fatty acids (linoleic acid, 18 : 2; arachidonic acid, 20 : 4; eicosapentaenoic acid, 20 : 5; docosahexaenoic acid, 22 : 6) and the total sum of plasma fatty acids are presented. Values are means with their standard errors represented by vertical bars. Mean values were significantly different from those for soyabean oil group: * $P < 0.05$. Mean values were significantly different from those for saline group: † $P < 0.05$. For details of animals and procedures, see pp. 577–579.

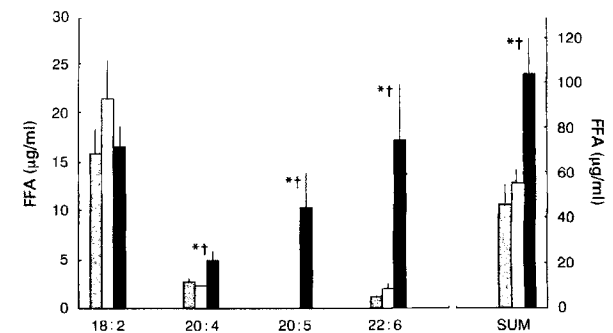


Fig. 3. Free plasma fatty acids (FFA) in saline (9 g NaCl/l; control; ■), soyabean oil (□) and fish oil (▨)-infused Wistar/Kyoto rats after PVG rat heart transplantation. A selection of fatty acids (linoleic acid, 18 : 2; arachidonic acid, 20 : 4; eicosapentaenoic acid, 20 : 5; docosahexaenoic acid, 22 : 6) and the total sum of free plasma fatty acids are presented. Values are means with their standard errors represented by vertical bars. Mean values were significantly different from those for soyabean oil group: * $P < 0.05$. Mean values were significantly different from those for saline group: † $P < 0.05$. For details of animals and procedures, see pp. 577–579.

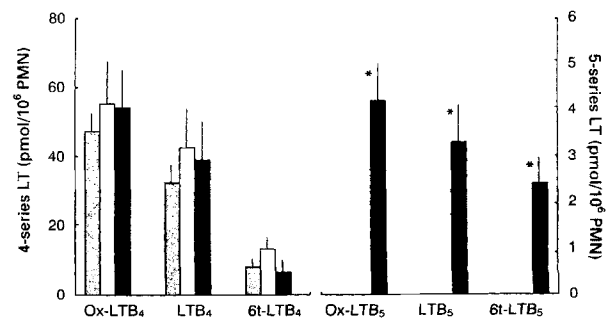


Fig. 4. A23187-induced polymorphonuclear leucocyte (PMN) generation of 4-series (*n*-6 derivatives) and 5-series leukotrienes (LT; *n*-3 derivatives) in saline (9 g NaCl/l; control; ■), soyabean oil (□) and fish oil (▨)-infused Wistar/Kyoto rats after PVG rat heart transplantation. Ox-LTB₄₍₅₎, sum of 20-COOH- and 20-OH-LTB₄₍₅₎; 6t-LTB₄₍₅₎, the sum of 6-*trans*- and 6-*trans*-epi-LTB₄₍₅₎. Values are means with their standard errors represented by vertical bars. Mean values for fish oil-infused rats were significantly different from those for the other groups: * $P < 0.05$. For details of animals and procedures, see pp. 577–579.

controls (Fig. 4). Neither the profile nor the total amount of PMN LT synthesis were substantially changed in rats undergoing soyabean oil infusion. Intravenous administration of the fish oil-based lipid emulsion, however, resulted in marked formation of 5-series LT (LTB₅, its oxidation products, and LTA₄ hydrolysis products) without completely suppressing 4-series LT generation.

Platelet thromboxane synthesis

Platelets isolated from saline-infused controls and from rats undergoing soyabean oil infusion liberated only TXA₂ on *ex vivo* stimulation with A23187. TXA₂ synthesis was only slightly increased following *n*-6 fatty acid supplementation (Fig. 5). In response to fish oil infusion platelet TXA₂ generation was reduced by over 50 % in favour of marked TXA₃ synthesis, resulting in a TXA₃:TXA₂ value of approximately 1 : 1.

Discussion

In our experimental rat heart allotransplant model a fish oil-derived lipid emulsion proved to be immunosuppressive, indicated by significant prolongation of graft survival. In transplantation research the prolongation of graft survival is accepted as a means of establishing the efficacy of new immunosuppressive drugs. Due to the well-defined histoincompatibility of the different rat strains chosen the entire spectrum of immune defence, i.e. specific cellular and humoral or non-specific mechanisms, is activated in a standardized and reproducible reaction allowing correlation between graft survival and biochemical, cell physiological and morphological variables.

Compared with oral application, intravenous infusion gives the advantage of continuous body-weight-related lipid administration. The high level of lipids provided intravenously to rats (approximately 9 g/kg body weight per d) reflects the manyfold increased energy turnover per kg body weight in comparison with that for human subjects (Nakagawa *et al.* 1991). Approximately 30 % of the total energy expenditure of rats was provided as intravenously-

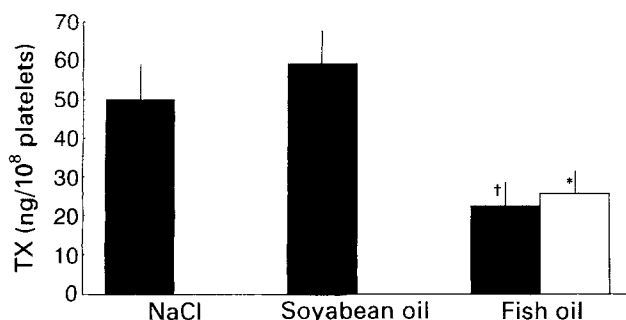


Fig. 5. A23187-induced platelet thromboxane (TX) generation in saline (9 g NaCl/l; control), soyabean oil and fish oil infused Wistar/Kyoto rats after PVG rat heart transplantation. Values for TXB₂ (■) and TXB₃ (□; stable hydrolysis products of TXA₂ and TXA₃ respectively) are given as means with their standard errors represented by vertical bars. Mean value was significantly different from that for soyabean oil group: **P* < 0.05. Mean value was significantly different from that for saline group: †*P* < 0.05. For details of animals and procedures, see pp. 577–579.

supplemented fat, which is well within the range of parenteral nutrition standards (Nakagawa *et al.* 1991).

Among other mechanisms, *n*-3 and *n*-6 fatty acids modify the immune response by virtue of being precursors of immunomodulating eicosanoids (Foegh, 1988). A high percentage (approximately 50 %) of the total fatty acid composition in soyabean oil and fish oil is polyunsaturated *n*-3 and *n*-6 fatty acids. While in soyabean oil, which represents the fatty acid composition of a standard Western diet (von Schacky & Weber, 1985), *n*-6 lipids predominate 8-fold over *n*-3 lipids, the *n*-3 : *n*-6 fatty acid value for fish oil (1 : 8) is exactly the reverse of that for soyabean oil. In contrast to *n*-3 products, *n*-6 eicosanoids have a net rejection effect: prostaglandin E₂ inhibits the cell-mediated immune response (Goodwin & Webb, 1980; Knudsen *et al.* 1986; Kunkel *et al.* 1986) and both TXA₂ and LTB₄ have antagonizing effects (Foegh, 1988).

The process of rejection is accompanied by a progressive, stereotypical accumulation of mononuclear cells in the graft (Tilney *et al.* 1976; Forbes *et al.* 1983), with the amount of T-cells, especially T-helper cells, characterizing the intensity of the rejection process (Häyry *et al.* 1981; Platt *et al.* 1982). The immunohistological profile of our acutely rejecting grafts harvested on day 4 after transplantation showed a high correlation with the modification of graft survival. In the fish oil group the number of infiltrating cells, i.e. polymorphonuclear neutrophils and the particularly important (Häyry, 1984; Mason & Morris, 1986) T4- and T8-cells, were reduced by up to 50 % compared with the controls. In accordance with the moderately prolonged allograft rejection, the infiltration pattern of the soyabean oil group was just marginally altered in comparison with the control group.

Decreased graft infiltration coincided with a diminished fraction of T-cells circulating in the peripheral blood, indicating a reduced T-cell recruitment due to fish oil. One of the initial events preceding graft rejection is the migration of immunocompetent cells from lymphocyte compartments into allogeneic tissues (Emeson, 1977). This traffic of lymphocytes before being trapped in allografts (Zatz & Gershon, 1974) alters the profile of PBMC, which is used clinically for cytoimmunological monitoring (May *et al.* 1990). Reduced T-cell recruitment due to fish oil infusion has not yet been reported in the literature, and might be explained by the hypothesis that *n*-3 fatty acid incorporation into the phospholipid bilayer modifies membrane fluidity and cytokine receptor expression, thus impairing the cytokine-cytokine receptor interactions which mediate cell migration from the lympho-reticular system (Kinsella, 1990).

Proliferation and graft infiltration of immunocompetent cells depend on cytokine-regulated cell-cell interactions. Among the variety of cytokines involved in acute rejection, TNF- α , for example, induces the development of cytotoxic T lymphocytes (Ranges *et al.* 1987) and the expression of interleukin-2 receptors on T-cells (Scheurich *et al.* 1987). Mitogen-stimulated TNF- α release by mononuclear cells harvested from our fish oil-treated rats was significantly reduced compared with the controls. Suppression of cytokine secretion in rats on oral administration of *n*-3 fatty acids for 6 weeks has been reported previously, and coincided with a markedly increased incorporation of EPA and docosahexaenoic acid into the membrane phospholipid pool

(Endres *et al.* 1989). The increased presence of these *n*-3 fatty acids in the phospholipid bilayer at the cost of AA might impair signal transduction mediated by the phospholipid-AA (second messenger) system (Abdel-Latif, 1986). The suppression of TNF- α release, as early as 4 d after fish oil infusion, is then explained by the accelerated *n*-3 fatty acid incorporation into the membrane phospholipid pool due to the intravenous route of application (Urakaze *et al.* 1987).

The observed inhibition of proliferation and graft infiltration of immunocompetent cells in our fish oil group is at least partially explained by the reduced cytokine release of mononuclear cells and the diminished recruitment of T-cells from lymphatic compartments. Furthermore, T lymphocyte proliferation has been shown to be inhibited *in vitro* by an increased concentration of free fatty acids via an eicosanoid-independent mechanism (Calder *et al.* 1992). Interestingly, fish oil, but not soyabean oil, led to a 2.5-fold increase in free plasma fatty acid concentration in our model. Artificial lipid aggregates are known to activate the endothelial lipoprotein lipase (EC 3.1.1.34), including translocation of this enzyme from its cellular binding sites into the vascular compartment (Peterson, 1990). A differential impact on lipolytic activity which is dependent on the fatty acid composition of artificial lipid aggregates has not been shown before. However, free plasma fatty acid concentration *in vivo* was far beyond the dose which proved effective *in vitro*, so that a substantial free fatty acid-induced inhibition of T-cell proliferation can be excluded in our model. On the other hand, individual components of the free fatty acid pool might have influenced the rejection process by inhibiting PMN mobilization. When compared with the *n*-6 product LTB₄, which mediates adhesion, diapedesis and chemotaxis of PMN (Goetzl & Pickett, 1980; Dahlsen *et al.* 1981), LTB₅ synthesized from free *n*-3 EPA is almost inactive.

Free AA and EPA derived from an intercellular fatty acid exchange among inflammatory active cells (Chauncey *et al.* 1988) are metabolized to lipid mediators via cooperative eicosanoid synthesis. Tissue areas prone to inflammatory reactions show micromolar concentrations of non-esterified AA (Hammarström *et al.* 1975). The considerable increase in free plasma EPA (> 30 mol/l), exceeding by 2-fold free AA concentration, on fish oil infusion in our model, is assumed to result in a preferential synthesis of 5-series LT by neutrophils stimulated in this natural environment (Grimminger *et al.* 1992). Even after being isolated from their plasma environment, neutrophils synthesized appreciable quantities of EPA-derived 5-lipoxygenase (EC 1.13.11.34) products under our conditions of *in vitro* stimulation, although they lacked the high extracellular concentration of free EPA and were forced to recruit precursor fatty acids from their own EPA-containing membrane lipid pool. On the other hand, this finding demonstrates a rapid re-acylation of the membrane lipid pools in exchange with plasma EPA concentrations.

The shift in LT generation toward EPA-derived products explains the reduced PMN graft infiltration due to fish oil infusion, since LTB₅ possesses a more than 10-fold reduced chemotactic and PMN-activating capacity as compared with LTB₄ (Leaf & Weber, 1988). A reduction in neutrophil action is to be expected due to LTB₅ competition with LTB₄ for receptor occupancy (Kragballe *et al.* 1987) and the inter-

ference with the LTB₄-mediated autocrine loop of PMN activation. Furthermore, LTB₅ interference with the LTB₄ feedback control mechanism is known to have depressing effects on the function of other inflammatory cells, including mononuclear cells and their cytokine production (Endres *et al.* 1989).

Lipid-mediator generation toward EPA-derived products in response to fish oil was even more pronounced in platelets similarly isolated from their plasma compartment. *Ex vivo* stimulation of these thrombocytes led to an approximately 1 : 1 release of TXA₃ and TXA₂, which reflects the highest 3-series to 2-series prostanoid ratio ever reported. By comparison, dietary *n*-3 fatty acid supplementation for several months resulted in 5–15 % generation of TXB₃ in relation to TXB₂ by human thrombocytes stimulated *ex vivo* (Fisher & Weber, 1983). Release of the potent vasoconstrictor and platelet aggregator TXA₂ was shown to be increased during acute rejection in experimental transplantation (Foegh *et al.* 1985). Inversely, prolonged transplant rejection was reported in response to TX synthetase (EC 5.3.99.5) inhibitors and TX antagonists (Coffman *et al.* 1989). The considerable shift from TXA₂ to TXA₃ generation following fish oil infusion must be anticipated to have contributed to the prolonged allograft survival, since TXA₃ lacks the prorejection properties of TXA₂ (Leaf & Weber, 1988; Grimminger *et al.* 1993).

In summary, intravenous fish oil has been demonstrated to reduce the immune response following experimental transplantation, as observed in the prolongation of graft survival. Delayed acute rejection could be correlated immunohistologically with a diminished graft infiltration of specific immunocompetent cells and non-specific inflammatory cells. The *n*-3-based lipid emulsion reduced cytokine release of mononuclear cells and the mobilization of immunocompetent cells from lymphatic compartments. The increased plasma free fatty acid concentration and the shift in the plasma fatty acid profile towards *n*-3 fatty acid eicosanoid precursors is claimed to be responsible for 5-series LT and trienoic TX generation, with their suppressive impacts on PMN mobilization, vasoconstriction and thrombus formation. *n*-3 Lipid-based immunonutrition, which has become increasingly popular during the last years in several clinical situations, proved to be effective as a supportive immunosuppressive rationale in transplantation medicine. Recently, the adjuvant administration of dietary fish oil after kidney transplantation has been shown to reduce both the number of acute rejection episodes and the nephrotoxicity of cyclosporine in human subjects (van der Heide *et al.* 1993).

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