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THE EFFECT OF DIETARY POLYUNSATURATED FATTY ACIDS (PUFA) ON ACUTE REJECTION AND CARDIAC ALLOGRAFT BLOOD FLOW IN RATS

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For six weeks, recipient (Lewis RT1^l) and donor rats (LBNF1$^{l/n}$) were fed three diets that varied only in their lipid content. Diet A (MO) contained 19.5% menhaden oil and 0.5% safflower oil and was rich in ω3 PUFA; diet B (SO) was 20% safflower oil rich in ω6 PUFA; and diet C (BT) was 20% beef tallow rich in ω9 monounsaturated fatty acids and saturated fat. In the first set of graft survival studies a group fed laboratory chow was included (CHOW). Heterotopic cardiac transplantation from donor to recipient animals was performed after the six-week feeding period. The effect of these diets on cardiac allograft survival, mixed lymphocyte response, and blood flow in the rejecting grafts was investigated. The median graft survival in days was significantly prolonged in the rats maintained on either MO (12 days) or SO (14.5 days) compared with the BT (8 days)–or lab chow (7.5 days)–fed animals ($P<0.05$). Cyclosporine (CsA) administered at subtherapeutic levels further increased the differences between the PUFA-fed animals and the BT-fed group. The myocardial blood flow of the rejecting allografts was measured using an ^{85}Sr-labeled microsphere technique on the fifth posttransplant day. Flow was greatest in the MO-fed group, and both MO and SO groups had significantly higher myocardial blood flow than BT-fed rats ($P<0.05$) or those bearing isografts. The allogenic mixed lymphocyte responses of peripheral blood mononuclear cells (PBMC) and splenic lymphocytes were suppressed in MO- and SO-fed groups compared with BT-fed animals. The immunosuppressive effect of dietary PUFA warrants further investigation, and their use as a possible adjunctive treatment in organ transplantation should be considered.

Arachidonic acid (AA)* is a substrate for both the cyclooxygenase and lipoxygenase enzyme systems, resulting in the production of prostaglandins (PG) and (LT) (*1, 2*). Consumption of diets rich in linoleic acid (LA) leads to the accumulation of both LA and AA in cell membrane phospholipids. Such loading with ω6 PUFA leads to the production of PGs of the 2-series and LTs of the 4-series. This leads to the production of proinflammatory mediators (*1, 2*) and some authors suggest a proinflammatory state (*3*), while others have found ω6 PUFA to be immunosuppressive (*4–6*). Dietary fish oils are rich in ω3 PUFA and contain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These long chain ω3 PUFA are substrates and competitive inhibitors of cyclooxygenase and lipoxygenase. This leads to the production of the analogous 3-series PGs and 5-series LTs, which are generally biologically inactive (*7, 8*). PGI_3 is the only product of ω3 metabolism that retains biological activity (*2*). The result of this manipulation is thought to be generally immunosuppressive by some authors (*9, 10*), and the use of ω3 PUFA has met with some success in treating differing inflammatory pathologies (*11–15*). Other authors have shown a net vasodilatory antithrombotic state with ω3 PUFA and part of any effect of fish oil diets may be due to protection of the microcirculation (*16, 17*). There is therefore ample theoretical, laboratory, and clinical evidence to suppose that both ω3 and ω6 PUFA will influence the process of acute cardiac allograft rejection. Although the power of dietary PUFA to influence the normal response to organ allografts is not disputed, the dose, class of fatty acid responsible, and time necessary to achieve a response remain controversial (*4–6, 9–11, 18, 19*).

Despite the arrival of FK506 in clinical practice, CsA remains the mainstay of most transplant immunosuppressive protocols. It is not yet clear whether the use of FK506 will lead to a reduction in the side effects of immunosuppression for organ transplantation. For the time being rejection is suppressed with significant cost to the patient in terms of morbidity—and, occasionally, mortality (*20, 21*). The emergence of a safe adjunct to standard immunosuppressive protocols that may have a protective effect on renal function (*19, 22*) is potentially of great clinical benefit. We therefore investigated the effect of dietary supplementation with ω3 and ω6 PUFA on cardiac allograft rejection, on allograft blood flow, and on *in vitro* immune function in rats.

MATERIALS AND METHODS

Animals. Pathogen-free Lewis RT1^l recipients and LBNF1$^{l/n}$ donors were obtained at 100–120 g body weight (Harlan Sprague Dawley, Indianapolis, IN).

Diets. Basic AIN76A diets were supplemented with anti oxidants, 0.05% TBHQ (tert-butylhydroxyquinone) and vitamin E, at 2.5 times the regular level in AIN rat diets, and were then varied only in the fat source of the three test diets. Diet A (MO) was 19.5 wt% menhaden oil + 0.5% safflower oil; diet B (SO) was 20 wt% safflower oil; diet C (BT) was 20 wt% beef tallow (Dyets, Bethlehem, PN). The diets were delivered refrigerated, sealed under nitrogen, and stored until use in the dark at 4°C. In the first graft survival study a fourth group was added and fed regular Purina laboratory chow (CHOW); this diet

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* Abbreviations: AA, arachidonic acid; AIN, american institute of nutrition; BT, beef tallow; CHOW, laboratory chow; CsA, cyclosporine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LDL, low-density lipoproteins; MLR, mixed lymphocyte responses; MO, menhaden oil; PBMC, peripheral blood mononuclear cells; PGE_2, prostaglandins; PGI_3, prostaglandin I_3; PUFA, polyunsaturated fatty acids; SO, safflower oil; ^{85}Sr, radiolabeled strontium; TxB_2, thromboxane B_2; VLDL, very-low-density lipoproteins.

contained 5.5 wt% fat from a mixed source. The diets were fed for 6 weeks prior to transplantation, recommenced the morning after surgery, and continued until graft rejection studies were completed. The animals were allowed free access to water; they were housed in a controlled environment having a 12-hour day/light cycle.

Cardiac allograft transplantation. Following six weeks of feeding, and under ether anesthesia, Lewis rats received abdominal heterotopic cardiac allografts from the $LBNF_1$ donors using a modified version of the technique of Lindsey and Ono (*23*). Briefly the donor heart was removed by ligation and division of the venous connections of the heart, then transection of the arterial outflow. The still-beating organ was then lowered into slushed saline at 4°C while the recipient animal was prepared. Implantation was achieved by anastomosis of the graft ascending aorta to the recipient abdominal aorta below the renal vessels. Then the graft pulmonary trunk was anastomosed to the inferior vena cava of the recipient. During the recovery period the animals were allowed only water—however, diets were recommenced the following morning. In the initial study a group of Lewis-to-Lewis isografts were performed.

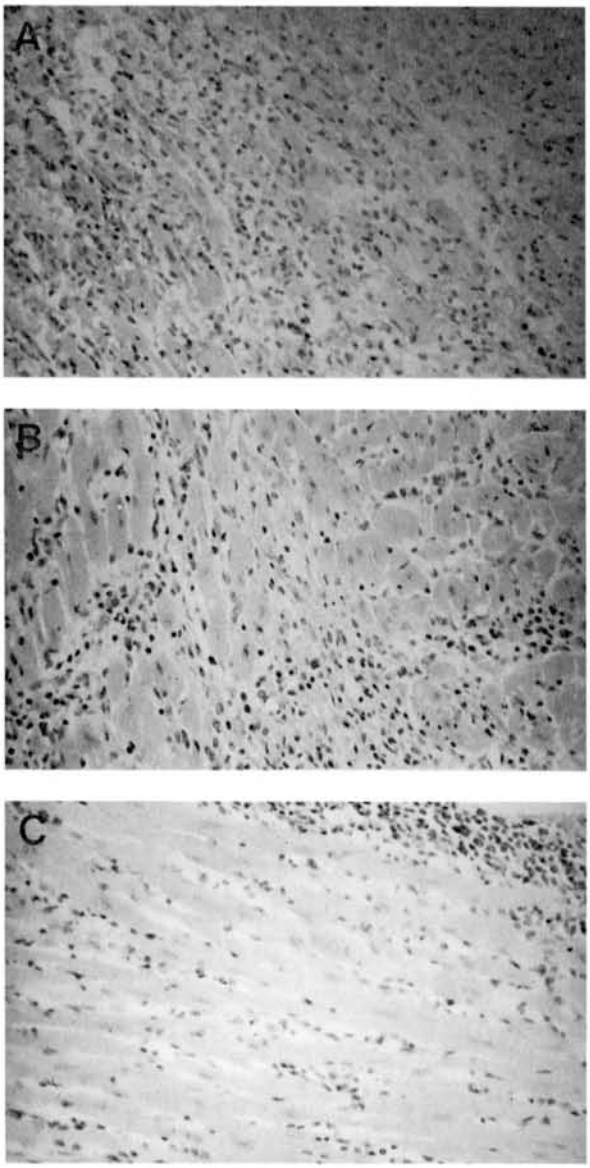


FIGURE 1. Histological sections show the typical appearance of severe allograft rejection in the animals fed diets supplemented with beef tallow (A), safflower oil (B), or fish oil (C).

Following surgery half the animals in each of the groups was administered 5 mg/kg/day CsA intraperitoneally for 10 days. All animals were examined for signs of ill health, and daily weights were taken. Graft survival was assessed by abdominal palpation, and rejection was diagnosed as cessation of abdominal heartbeat. The animal was then killed by ether anesthesia and the cardiac allograft and liver removed for histological examination. Graft survival was expressed as whole days by a life table analysis and differences were analyzed by the Kruskal-Wallis test.

In vitro immune function. Graft-bearing animals were treated with CsA as described above for 10 days and euthanized by ether anesthesia on posttransplant day 15. Spleens were removed aseptically and single-cell suspensions were prepared in RPMI 1640 medium (Grand Island Biologicals) supplemented with L-glutamine, penicillin-streptomycin, 5% fetal calf serum, and 0.05 mM 2-mercaptoethanol (complete medium). Also 5 ml venous blood was collected into heparinized tubes. Mononuclear cells were collected over a ficoll gradient at 500 ×g for 20 min. Cells were washed twice at 500 ×g for 10 min, then adjusted to 5×10^6/ml in complete medium. PBMC were collected from $LBNF_1$ rats in a similar way, but the final concentration was adjusted to 5×10^5/ml. These donor cells were then irradiated at 1500 rads over 2.7 min. The MLR was set up between Lewis responder cells and $LBNF_1$ stimulator cells in 96-well flat-bottomed microtiter plates. Each MLR was performed in triplicate, with triplicate background cultures of responder cells only, and triplicate control cultures of stimulator cells only. The final volume of each MLR was 200 μl of complete medium with 5×10^4 stimulator cells and 5×10^5 responder cells. Cultures were incubated at 37°C in a 7% CO_2 humidified incubator for 72 hr.

Cells were then pulsed with 2 μCi/well of ^{3}H-thymidine during the last 8 hr of the culture period, harvested using an automatic cell harvester, transferred onto a filter paper, dried, and stored in scintillation fluid (Optifluor). The amount of radioactivity (disintegrations per minute [dpm]) was measured in a liquid scintillation counter (Beckton-Dickinson), the results were expressed as mean ± SD, and the significance of differences among the groups was determined by Student's *t* test applying Bonferroni's correction.

Cardiac allograft blood flow. To measure cardiac allograft blood flow during rejection, animals were studied on day five following transplantation without any treatment with CsA. A group of 6 Lewis-to-Lewis isografts was also studied. On the fourth posttransplant day under ether anesthesia the animals had a PE10 catheter placed from the right common carotid artery to the left ventricle, and a PE50 catheter across the left common carotid artery to the arch of aorta. The next day animals were injected through the PE10 catheter with ^{85}Sr-labeled microspheres over 60 to 75 secs. During the injection blood was withdrawn from the PE50 catheter at a standard rate. At the end of the run, the animals were killed by a lethal injection of sodium pentobarbitone. Each animal then underwent a limited postmortem to ensure that the catheters were within the left ventricle, and at the junction of the common carotid artery and the arch of the aorta. Misplacement of the lines was prevented by leading them off the skin at the back of the neck and through a protective swivel device, and any blockage was avoided by infusing heparinized saline prior to the experiment. Only samples from animals with lines in the correct positions and having no clots were included in the study.

By knowing the total dose of radioactivity injected, and the proportion of the dose that ends up in the reference sample, the cardiac output is calculated as follows:

$$\text{cardiac output} = \frac{\text{total injected radioactivity (cpm)}}{\text{radioactivity in withdrawal sample (cpm)}} \times \text{withdrawal sample flow}$$

Having established the cardiac output, the flow through the rejecting allograft is calculated by;

$$\text{allograft flow} = \frac{\text{radioactivity in allograft (cpm)}}{\text{total injected radioactivity (cpm)}} \times \text{cardiac output}$$

Cardiac output is expressed as flow/kg/min, and flow in the allograft myocardium is expressed as ml/g/min. Results are shown as mean ± SEM, and the statistical analysis is with the Bonferroni correction of the Student's *t* test.

Fatty acid analysis. All diets and the livers taken from animals fed for six weeks were extracted with a mixture of chloroform:methanol (2:1 v/v) and analyzed on a gas liquid chromatograph following thin-layer chromatography according to methods described elsewhere (*24*). The results of the analysis of liver phospholipid pools are expressed as relative molar percent of each fatty acid class. These are shown for each dietary group.

RESULTS

Body weight. The mean gain in body weight did not differ significantly in any of the dietary groups. All animals remained healthy prior to cardiac transplantation.

Graft survival. All diagnoses of graft rejection were confirmed macroscopically at the time of rejection, and there was

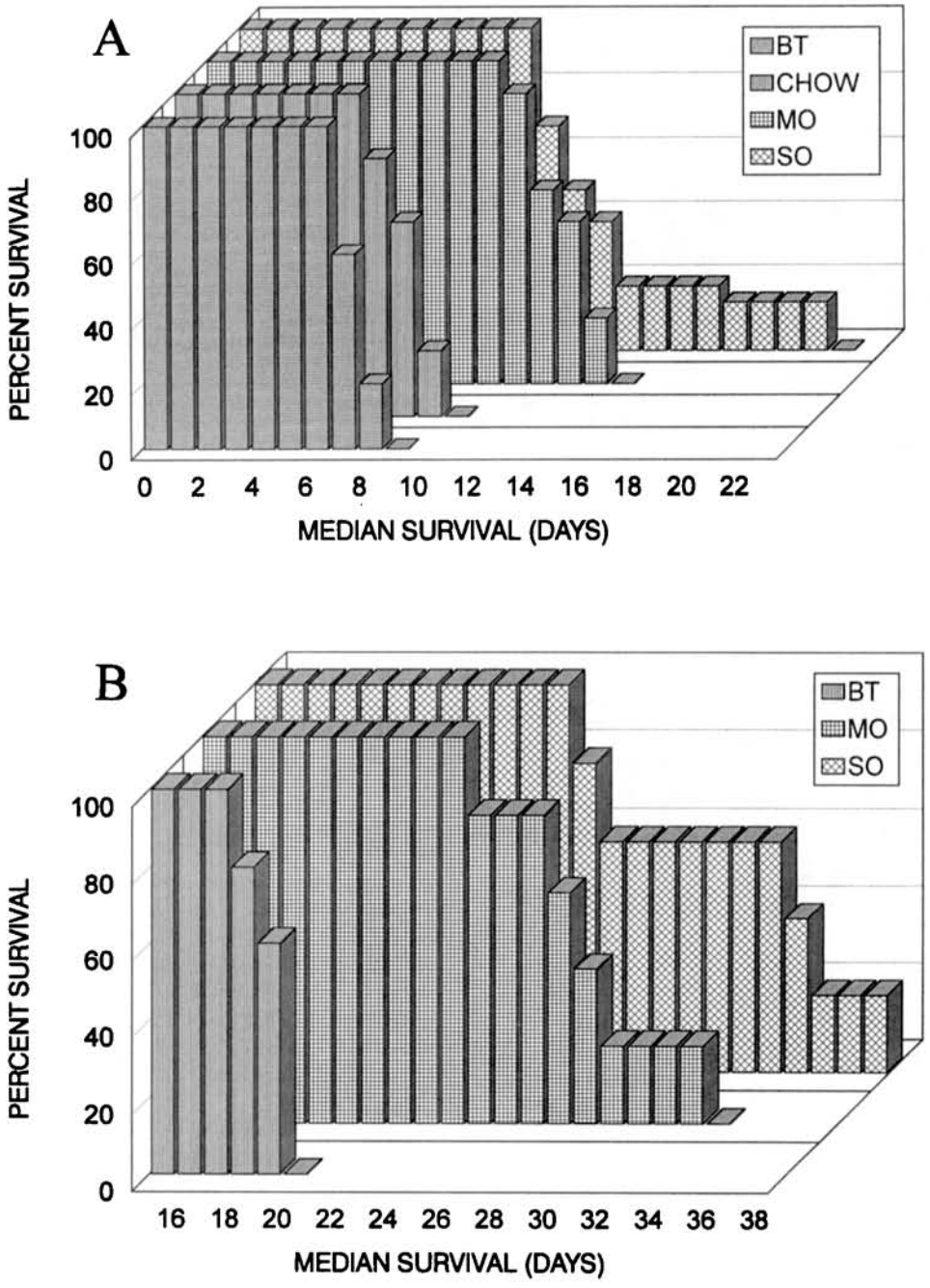
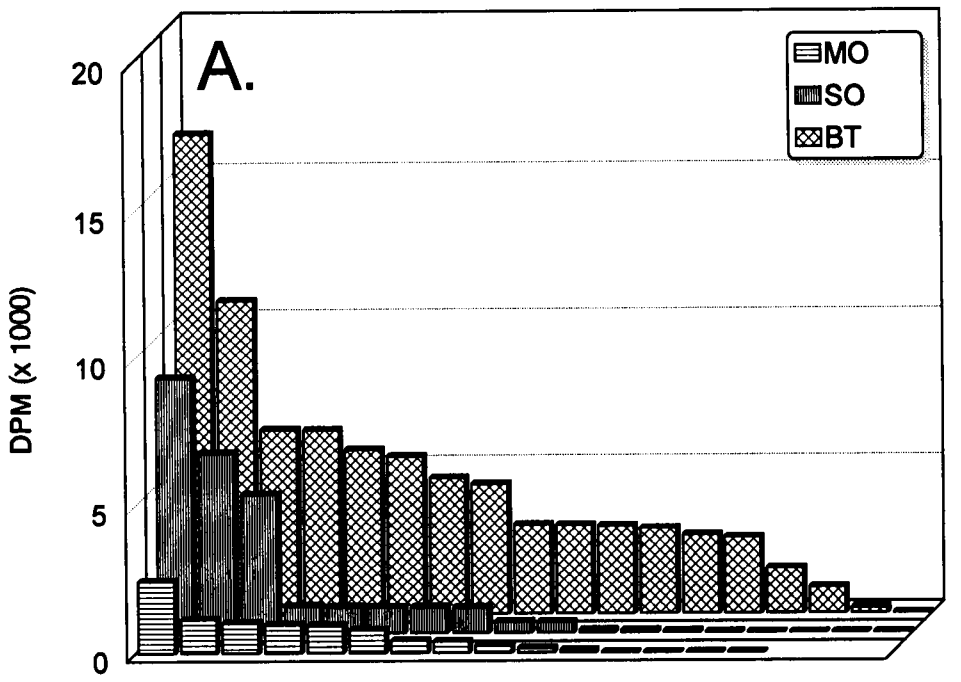
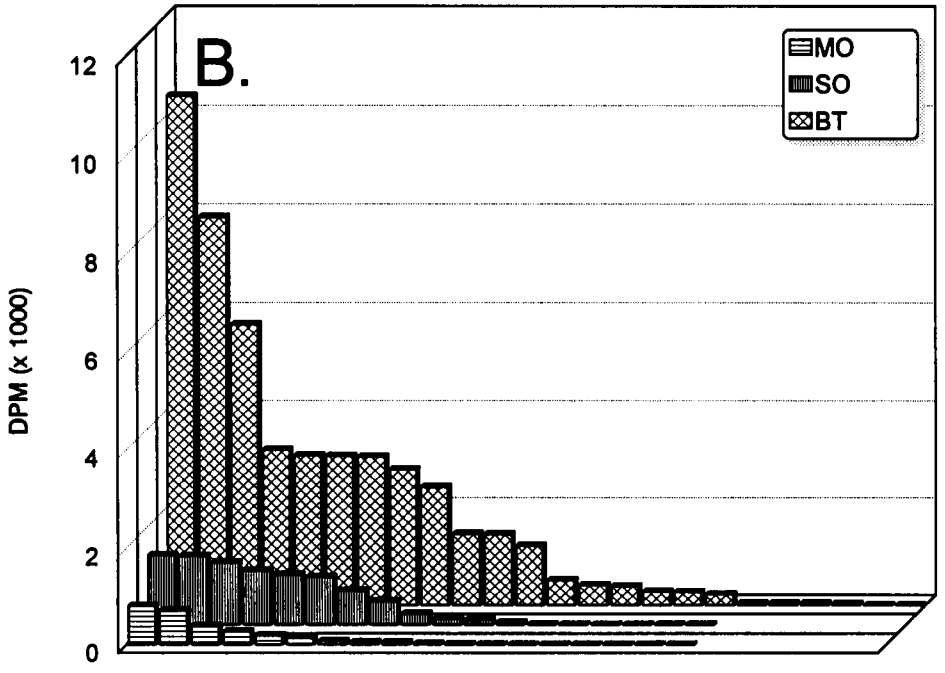


FIGURE 2. The $LBNF_1$ hearts were transplanted into Lewis rats ($n=6$). The median survival times for allografts (panel A) without and (panel B) with CsA administration for the recipient animals is shown. The differences in allograft survival rates for the menhaden oil (MO)- and for the safflower oil (SO)-fed rats were compared with those maintained on beef tallow (BT) or the Purina chow (CHOW) groups, and the levels of significance were determined by Kruskal-Wallis test.



MLR from splenic lymphocytes, 8 days following cardiac transplantation in rats

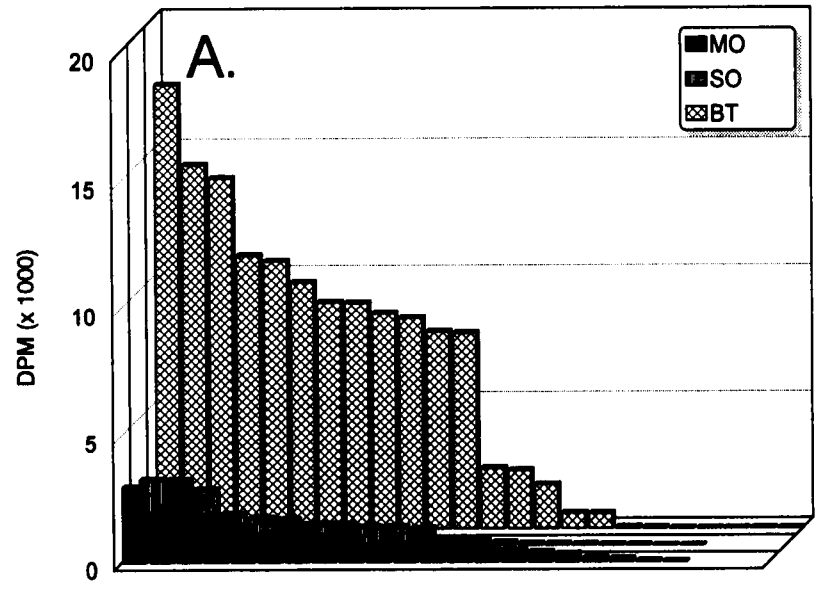


MLR from PBMCs, 8 days following cardiac transplantation in rats

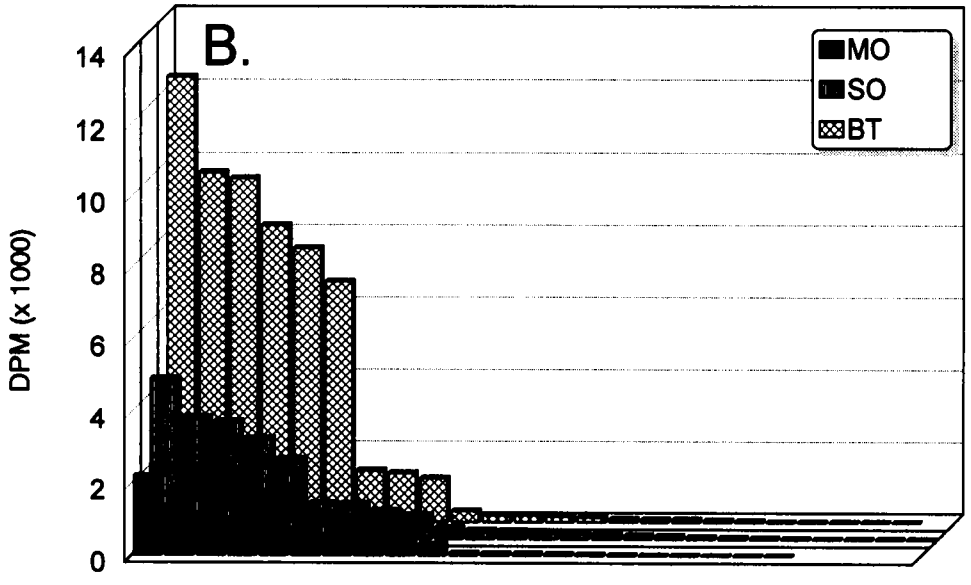
FIGURE 3. The mixed lymphocyte reactions of lymphocytes from spleen and peripheral blood cells from the recipient rats were determined after 8 days of allogenic cardiac transplantation. The allogenic responses for both the groups of rats maintained on menhaden oil (MO) or on safflower oil (SO) were compared with those maintained on beef tallow (BT), and the levels of significance ($P<0.05$) were determined by Kruskal-Wallis test.

no evidence of morphological differences in the histology of the rejected allografts (Fig. 1). Six Lewis-to-Lewis isografts were performed, two in each group, and each was still functioning when the animals were sacrificed 287 days later. The median survival of the allografts in group MO was 12 days (range 10–14 days), and in SO it was 14.5 days (range 10–21 days). This was significantly longer ($P<0.05$) than either BT, 8 days (range 6–9 days), or CHOW, 7.5 days (range 6–9 days) (Fig. 2A). The increase in median survival was more than two-fold following administration of CsA at 5 mg/kg/day for 10 postoperative doses (Fig. 2B). Median survival for MO-fed animals was 28.5 days (range 24–33 days), for SO-fed animals it was 34.5 days (range 26–76 days), and for the BT-fed animals it was 18 days (range 17–19 days). The difference between MO and SO, and the BT group, was again significant ($P<0.05$).

Mixed lymphocyte response. The allogenic response of lymphocytes taken from untreated and CsA-treated recipients during graft rejection was tested. Figures 3 and 4 summarize the effect of the test diets on lymphocyte response to irradiated allogenic PBMC. There is a reduction in ^{3}H-thymidine uptake in the cells from the MO and SO groups compared



MLR from splenocytes, 15 days following cardiac transplantation in CsA treated rats



MLR from PBMC, 15 days following cardiac transplantation in CsA treated rats

FIGURE 4. The mixed lymphocyte responses of the recipient rats were determined as in Figure 3, except that the animals were treated with CsA for 15 days following transplantation.

TABLE 1. Effects of polyunsaturated fatty acids on blood flow in rejecting cardiac allografts[a]

Group[b]		CO (ml/kg/min)	MAP (mmHg)	Ht rate (beats/min)	Blood flow (ml/gm/min)
BT	(6)	351±28	92±4	399±38	1.97±0.18
MO	(6)	345±19	87±6	359±8	2.96±0.25[c]
SO	(6)	417±26	83±3	360±9	2.42±0.21[c]
ISO	(6)	368±33	90±3	385±16	1.88±0.18

[a] Data are shown as mean ± SEM. A significant ($P<0.05$) increase in blood flow per gram of rejecting myocardium was seen in the MO- and SO-fed animals, compared with those maintained on beef tallow (BT) or the animals bearing nonrejecting allografts.

[b] Rats were maintained on experimental diets for six weeks prior to transplantation. Numbers in parentheses represent number of animals in each group.

[c] $P<0.05$ against both BT and ISO using ANOVA.

with BT-fed animals, both after 8 days without CsA (Fig. 3), and after 15 days when treated with CsA (Fig. 4).

Allograft blood flow. Nonrejecting syngeneic isografts had a mean myocardial blood flow of 1.9 ml/g/min. This was not significantly different from the acutely rejecting BT-fed animals on day five after their allograft. The MO-fed group had the highest flow, and both SO and MO had significantly elevated flow compared with both BT and the isograft group ($P<0.05$). The hemodynamic data suggest that the cardiovascular condition of all the groups was stable. These data are summarized in Table 1.

Fatty acid composition. To investigate the effect of the diets on cell lipid composition, we examined the fatty acid incorporation into liver membrane phospholipids. The data are shown in Table 2, and reveal that linoleic acid is increased in the SO-fed animals, and that EPA and DHA are elevated by the MO diet. Both MO and SO groups had a significantly reduced level of oleic acid when compared with the BT group.

DISCUSSION

In the present report we document evidence that consumption of dietary PUFA delays cardiac allograft rejection and further augments the graft survival in the presence of a subtherapeutic dose of CsA. The possible underlying mechanisms are discussed.

The data summarized in Figure 2, A, B demonstrate that consumption of SO- or MO-supplemented diets that contain increased amounts of PUFA for 6 weeks significantly prolongs allograft survival compared with BT or CHOW, both of which represent diets with low PUFA. These data support the hypothesis that consumption of increasing amounts of PUFA is associated with prolonged allograft survival (*10*). It has also been documented that for patients with renal transplants postoperative administration of 6 g fish oil along with cyclosporine for a year results in significantly fewer rejection episodes compared with those given coconut oil (*25*). However, it should be noted that in the fish-oil-treated group graft survival was not markedly better than in the controls (*25*). Consistent with the ability of PUFA to delay graft rejection, the responsiveness of lymphocytes in a one-way MLR, a measure of allogenic recognition (*26*), was markedly reduced for recipients fed MO or SO diets (Figs. 3 and 4). The increase in graft survival and the decrease in MLR response suggest that dietary manipulation alters allogenic recognition. Taken together, all these data indicate that dietary intake of ω3 or ω6 PUFA suppresses immune function, and the observation that a subtherapeutic dose of CsA (5 mg/kg/day) is additive to the dietary effect suggests that adjunctive therapy might be effective.

Consumption of diets enriched with ω3 or ω6 PUFA alters the production of inflammatory mediators (*3*), which themselves are involved in the control of blood flow through acutely inflamed tissue. We evaluated the effect of MO and SO on the microcirculation of rejecting allografts on day 5 posttransplantation. The expected result was that nonrejecting isografts would have the highest flow and there would be a stepwise diminution of flow from MO- to SO- to BT-fed animals. It is surprising to observe that nonrejecting isografts and acutely rejecting grafts of BT-fed rats had the same myocardial flow (Table 1). There was significantly increased flow in the allografts of MO- and SO-fed animals but, although the flow in the grafts from the MO-fed animals was the greatest, there was no statistically significant difference between SO- and MO-fed rats. Both PUFA diets were able to increase flow in a rejecting organ. The relatively low flow through the isografts may represent the lack of local inflammation in these grafts. It is interesting to speculate about the mechanism responsible for the observed effects. Although we did not measure prostaglandins in our animals, the differential effects of consumption of increasing amounts of ω3 and ω6 PUFA on eicosanoid production probably played an important role in the improved hemodynamics compared with

TABLE 2. Fatty acid composition of liver phospholipid pools[a]

Fatty acids	Experimental groups (relative mol%: mean ± SD)		
	Beef tallow	Safflower oil	Fish oil
Palmitic acid (16:0)	21.5±2.2	23.3±1.7	32.6±0.9
Stearic acid (18:0)	27.2±3.3	23.2±1.1	16.0±1.6
Palmitoleic acid (16:1 ω7)	1.3±0.5	0.4±0.1	2.9±0.4
Oleic acid (18:1 ω9)	11.1±1.6	2.4±0.1	4.3±1.0
Linoleic acid (18:2 ω6)	6.6±1.2	15.9±2.3	4.5±1.0
Arachidonic acid (20:4 ω6)	21.6±1.9	29.6±3.0	7.9±1.0
Eicosapentaenoic acid (20:5 ω3)	0.8±0.6	N.D.[b]	14.2±2.6
Docosapentaenoic acid (22:5 ω3)	0.4±0.4	N.D.	1.9±0.1
Docosahexaenoic acid (22:6 ω3)	6.1±0.4	2.3±1.0	13.2±0.8

[a] Rats were maintained on experimental diets for 6 wk and the fatty acid composition of liver phospholipids were analyzed on a gas chromatograph as described elsewhere (*16*).

[b] N.D., not detected.

those maintained on BT. A local release of PGE_2 may have a vasodilatory effect and mimic prostacyclin (*27*), thus increasing the flow in the SO-fed group. EPA and DHA have been shown to reduce the production of TXA_2 in models of inflammation; the prostacyclin analogue PGI_3 also has some physiological activity as a vasodilator. In addition platelet function is inhibited, with a reduction in adhesiveness and a reduction in the production of the fatty di-acyl derivative platelet-activating factor (*17, 28*). This net antiaggregatory and vasodilatory state may explain part of the effect of the MO diet.

Allogenic graft rejection has been positively correlated with an increase in tissue levels of linoleic acid, and inversely correlated with oleic acid (*29*). Since the relative amounts of LA in the tissues were higher for the SO group and lower for the MO group, the difference in LA levels (Table 2) cannot explain an increase in graft survival in the MO and SO groups (Fig. 2, A, B). However, the oleic acid content was significantly lower in the MO and SO groups compared with the BT-fed animals. These data are consistent with the finding that substituting olive oil for fish oil as a vehicle for the s.c. injection of CsA results in a delay in allograft rejection (*9*). The reduction in the dose of oleic acid may be responsible for the observed effects. Based on these data we speculate that a reduction in the tissue levels of oleic acid may enhance allograft survival.

The augmented response to CsA seen in the groups fed MO and SO could also be explained by the effect of dietary PUFA on lipoprotein levels (*30, 31*). An increase in the level of cholesterol and VLDL may increase binding to CsA and reduce its therapeutic effect in the BT-fed animals. Conversely the PUFA diets lead to reduction in cholesterol and VLDL and LDL (*32*), thus reducing CsA binding and increasing therapeutic efficacy.

In summary, consumption of $\omega 3$ and $\omega 6$ PUFA prolonged allograft survival, in association with a marked reduction in the MLR in recipient animals and an increased allograft myocardial blood flow. It is clear from these studies that a measurable and consistent manipulation of allograft rejection and augmentation of the action of CsA can be achieved with dietary PUFA. Although the molecular mechanisms remain unclear, we believe that the use of PUFA in transplantation remains of potential clinical significance.

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PATTERNS OF INFLAMMATORY VASCULAR ENDOTHELIAL CHANGES IN MURINE LIVER GRAFTS[1,2]

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We have investigated the vascular endothelial phenotypes found at various times posttransplant in murine B10→C3H liver grafts. In this model, liver allografts are spontaneously accepted, and survive indefinitely unless the recipient is first allosensitized with a skin allograft, in which case the liver allografts are rejected within five days. In our previous studies, allograft inflammation was associated with the development of vascular endothelial reactivity with the mAbs MECA-32 and M/K-2 (anti-VCAM-1). We observed that vascular endothelia in both liver isografts and allografts develop reactivity with MECA-32 mAb within two days of transplantation, indicating endothelial activation in both situations. In contrast, only the endothelia in liver allografts develop VCAM-1 expression, as detected with M/K-2 mAb. VCAM-1 was expressed in both rejecting and accepting liver allografts, demonstrating that endothelial VCAM-1 expression is indicative of ongoing graft inflammation but not necessarily graft rejection. Liver parenchymal cells did not appear to develop reactivity with either antibody under any of the conditions tested. In contrast, bile duct epithelia developed M/K-2 reactivity (VCAM-1 expression), but not MECA-32 reactivity in liver allografts, but not isografts. These data demonstrate alloantigen-dependent and alloantigen-independent patterns of endothelial behavior in murine liver grafts that are quite similar to those found in murine cardiac grafts. Further, they demonstrate that the expression of VCAM-1 by graft endothelia is not diagnostic for acute rejection of liver allografts.

Vascular endothelial cells play a major role in inflammatory responses, including the inflammation occurring in transplanted organ grafts. However, the specific endothelial responses that develop in rejecting allografts remain under investigation. Among the endothelial changes that have been clinically associated with graft rejection are the increased expression of major histocompatibility complex (MHC)* molecules, and the increased expression of various molecules, such as ICAM-1 and VCAM-1. VCAM-1 is of particular interest because it is normally not expressed by vascular endothelia in most tissues, but has been widely reported to develop on endothelia in rejecting cardiac (*1*), liver (*2*, *3*), and pancreas grafts (*2*). In rejecting grafts, ICAM-1 expression is also increased on vascular endothelial cells, as well as other cells in the graft tissues (*4*). In contrast, the endothelia of nonrejecting grafts do not express endothelial VCAM-1 (*5*), but may display significantly increased ICAM-1 expression.

Similar observations have been made in experimental transplantation models. The most widely studied of these has been the murine heterotopic cardiac graft model, where it has been observed that endothelial VCAM-1, as detected with the mAb M/K-2, is expressed in rejecting cardiac allografts after

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* Abbreviations: MHC, major histocompatibility complex; OLTX, orthotopic liver transplantation; VCAM-1, vascular cell adhesion molecule.