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ENHANCEMENT OF IMMUNOSUPPRESSION BY SUBSTITUTION OF FISH OIL FOR OLIVE OIL AS A VEHICLE FOR CYCLOSPORINE[1]

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As previously reported, acute cyclosporine-induced nephrotoxicity is characterized by a decline in glomerular filtration rate and a selective intrarenal production of the vasoconstrictor thromboxane (TxA_2), but not vasodilator prostaglandin E_2 (PGE_2), or prostacyclin (PGI_2), cyclooxygenase metabolites. Fish oils (FO), that are rich in n-3 polyunsaturated fatty acids have a high affinity for cyclooxygenase but serve as poor substrate inhibit TxA2 synthesis. We have shown that when FO replaces olive oil (OO) as the vehicle for CsA, CsA-induced nephrotoxicity and increased TxA2 synthesis are obviated in rodent models. In this study, we demonstrate that the FO vehicle for CsA does not compromise CsA's immunosuppressive properties as deduced from studies of a delayed-type hypersensitivity (DTH) model in BALB/c mice and in a rat heart transplant model. In fact, concurrent FO administration with CsA actually enhances immunosuppression. A dose of CsA incapable of blunting DTH when injected in OO was suppressive when given in FO. Administration of as little as 0.05 ml of FO vehicle potentiated the suppressive action of CsA. In addition, nonconcurrent dietary supplementation of FO in animals receiving CsA caused an increase in the immunosuppressive action of CsA in DTH. FO alone reduced DTH as compared with OO, but was far less effective than CsA plus FO. Furthermore, doses of CsA (5 mg/kg/day or 1.5 mg/kg/day), which are subtherapeutic when administered with OO, prolonged engraftment of Lewis recipients of Lewis × Brown-Norway F_1 hearts when CsA was solubilized with FO. These studies indicate that concurrent administration of CsA and FO potentiates the activity of CsA and thus increases its therapeutic index. Thus, CsA plus FO is potentially a safe, potent antirejection therapy worthy of clinical testing, especially insofar as FO prevents CsA-induced acute nephrotoxicity in the rodent.

Acute cyclosporine nephrotoxicity, characterized by decline in glomerular filtration and renal blood flow, is obviated in rats by concurrent administration of omega-3 (n-3)–rich fish oil (FO)* (*1, 2*). Protection from CsA induced nephrotoxicity when this drug is solubilized in FO as compared with olive oil (OO) can not be accounted for by an absolute differential reduction

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* Abbreviations: DTH(U), DTH units; FO, fish oil; OO, olive oil, PGE_2, prostaglandin E_2; PGI_2, prostacyclin; TNF, tumor necrosis factor; TxA_2 thromboxane A_2.

in CsA blood levels. Thus, the mechanism by which FO prevents acute nephrotoxicity is uncertain. Does FO administration reduce the bioactivity of CsA? The most incisive test of the therapeutic action of CsA solubilized in FO versus OO is to determine the immunosuppressive activity of these two preparations. Clearly, administration of FO to reduce CsA's side effects would be unattractive if FO also reduced overall immunosuppression. The purpose of this study was to compare the immunosuppressive capacity of CsA in its FO vehicle with CsA solubilized in OO. In order to address this important issue, we have tested the effect of the FO vehicle on immunosuppression mediated by CsA using two distinct in vivo models—delayed-type hypersensitivity in mice and a rat heart allograft transplant model. We have determined that FO does not block the immunosuppressive properties of CsA. Indeed, FO administration alone dampens the DTH response. Furthermore, CsA plus FO is a more potent therapeutic immunosuppressive combination than CsA administered in OO, the current vehicle for CsA utilized in clinical practice.

MATERIALS AND METHODS

Murine DTH

Animals. BALB/c mice were obtained from the Jackson Laboratory, Bar Harbor, ME, and were maintained in the animal facilities at Brigham and Women's Hospital, all male and between 5 and 10 weeks of age.

Induction of DTH. Mice were primed with a 10 mM solution of trinitrobenzenesulfonic acid (Nutritional Biochemicals Corp., Cleveland, OH) in 0.5 M phosphate-buffered saline at pH 7.2–7.4., administered by subcutaneous injection of 0.1 ml bilaterally in the dorsum. Six days after immunization these mice were challenged with 25 μl of the same hapten solution into the right footpad.

Footpad thickness was measured bilaterally with a micrometer by an individual lacking knowledge of the immunization protocol 24 hr after rechallenge. One DTH unit (DTHU) was defined as a difference of 0.01 mm in thickness between the injected and noninjected footpad. Injected footpads of nonimmunized mice were measured to determine background values.

Treatment

Groups of mice were treated with CsA (Sandimmune, Sandoz Pharmaceutical Corp., East Hanover, NJ) solubilized in n-3 fatty acid–rich (35%) FO (Max-EPA, R.P. Scherer Inc., Clearwater, FL) or OO, (Progresso Quality Foods Division Ogden Food Products Corp., Rochelle Park, NJ) containing n-9 fatty acid. All groups were treated daily by injection intraperitoneally from the day of immunization through the day of challenge unless specifically stated. Additional groups were given FO or OO alone. The FO was stored under nitrogen until immediately prior to use in order to prevent oxidation. FO treatment protocols utilizing i.p. injection or administration by gavage (p.o.) were analyzed. Statistical significance between groups was determined by rank sum testing.

Rat Cardiac Transplant

Lewis × Brown Norway F_1 or Wistar-Furth donor hearts were transplanted heterotopically into the abdomens of Lewis recipients with standard microvascular techniques. Recipients were treated with CsA intramuscularly for seven days after transplantation at various doses of CsA dissolved in 0.2 ml of FO or OO. Animals also received 1ml/day of the appropriate oil orally by gavage for the same seven days. Graft rejection was defined as loss of a palpable heartbeat in the allograft. Previous studies have determined that the loss of palpable heartbeat correlates with morphologic evidence of rejection (*3*). Statistical analysis between groups was undertaken by use of rank sum testing.

RESULTS

Potentiation of immunosuppression by solubilization of CsA with FO: mouse DTH model. Daily injections of high-dose CsA (25 mg/kg) solubilized in either FO or OO suppressed DTH. (Table 1). At a reduced dose of CsA (8 mg/kg) this drug solubilized in FO (9.7 ± 0.9 DTH[U]) but not OO (61.4 ± 3.6 DTH[U]) was immunosuppressive. In fact, a dose of CsA of 1 mg/kg administered in FO, but not OO, caused a profound reduction in DTH (Table 1). Furthermore, a single injection of CsA (10 mg/kg) in FO vehicle on day 6 alone was immunosuppressive (Table 2).

To determine the amount of FO required to potentiate the suppressive action of CsA, various amounts of FO, alone or together with OO, were used as vehicles for a dose of 5 mg/kg CsA (Table 3). While 0.2 ml of FO markedly reduced DTH—from 44.0 ± 1.1 DTH[U] to 3.2 ± 0.2 ml DTH[U]—0.2 ml of OO was ineffective (44.0 ± 1.1 DTH[U] to 38.4 ± 0.9 DTH[U]). A mixture of FO and OO also potentiated the immunosuppressive action of CsA. An amount of FO as small as 1 μl in 49 μl of OO administered with CsA reduced DTH by 53% that of the uninjected controls (Table 3). A variation of this experiment

TABLE 1. Suppression of DTH by CsA and fish oil[a]

CsA (mg/kg)	DTH(U)[b]	
	FO	OO
Exp. 1:		
25.0	7.5±1.7	12.8±2.1
8.0	9.7±0.9[c]	61.5±3.6
3.5	13.2±2.6	—
1.8	21.4±4.1	—
0.4	26.6±6.6	—
Exp. 2:		
1.0	6.0±1.9[d]	32±5.5
None	12.0±4.2[e]	22.6±4.9

[a] n=4–5 BALB/c mice/group; values are means ± SEM; Exp. 1. daily injections: 0.05 ml., Exp. 2: daily injections: 0.01 ml. injected ip daily.
[b] DTH(U) = difference of 0.1 mm thickness between injected and noninjected footpad.
[c] $P<.005$ (FO vs. OO).
[d] $P<.005$ (CsA 1 mg/kg) + FO vs. (CsA 1 mg/kg + 00).
[e] $P<.05$ (FO vs. OO).

TABLE 2. Suppression of DTH by small doses of CsA and fish oil[a]

CsA (mg/kg)	FO (ml)	DTH(U)[b]
100	0.2	6.6±1.0[c]
10	0.2	9.8±1.5[d]
1	0.2	19.0±5.2
None[e]	—	30.6±8.5

[a] n = 5 BALB/c mice/group; 1 injection i.p. day 6 prior to challenge: values are means ± SEM.
[b] DTH(U) = difference of 0.01 mm thickness between injected and noninjected footpad.
[c] $P<.005$.
[d] $P<.01$ Mann-Whitney U test.
[e] Needle is inserted into peritoneum but nothing is injected.

using several percentages of FO and OO has been repeated yielding similar results.

High doses of CsA given i.p. or p.o. in FO or OO effectively prevents the DTH response (Table 4).

To eliminate the possibility of immunosuppression being related to weight loss, in several sets of experiments mice were weighed before and after treatment. Groups of mice receiving CsA + FO had weights similar to those of mice receiving CsA + OO (data not shown).

Evidence that FO alone is immunosuppressive: mouse DTH model. Administration of FO, not OO, suppressed DTH in a dose-dependent manner (Table 5, Fig. 1). Daily injections of 0.05 ml FO suppressed the DTH response from 50.4 ± 5.2 DTH[U] to 21.8 ± 5.5 DTH[U] (Fig. 1). Increasing the amount of FO injected daily to 0.3 ml dramatically reduced the DTH response (4.0 ± 1.2 DTHU) as compared with administration of an equal volume of OO (50.8 ± 5.3 DTH[U]).

A single injection of 0.2 ml of FO given on day 6 modestly suppressed the DTH response from 54.2 ± 1.1 DTH[U] to 35.8 ± 1.9 DTH[U] (Table 5). Additional injections of FO further reduced DTH. By contrast, even daily injections of OO did not dampen the DTH response.

Groups of mice given FO and those given OO had similar weights at the end of the treatment period (data not shown).

Potentiation of immunosuppression by solubilization of CsA with FO. A subtherapeutic dose of CsA (1.5 or 5 mg/kg) solubilized with FO rather than OO enhanced immunosuppression and prolonged engraftment of LBN hearts into Lewis recipients (Table 6). Graft survival was not extended in this same strain combination when those given FO were compared with those given OO. (Table 6). In a stronger strain combination of Lewis recipients of WF hearts, switching the vehicle solubilizing 1.5 or 5 mg/kg CsA from OO to FO did not potentiate or compromise graft survival.

TABLE 3. Suppression of DTH by fish oil in the presence of olive oil[a]

	FO	OO	DTH(U)[b]
CsA (5 mg/kg)	— (ml)	0.2	38.4±0.9
	0.2	—	3.2±0.4[c]
	0.05	—	11.2±1.4[c]
	0.025[d]	0.025	11.8±1.2[c]
	0.001[d]	0.049	20.8±1.4[c]
None	—	—	44.0±1.1

[a] One i.p. injection day 6 prior to challenge; n = 5 BALB/c mice/group.

[b] DTH(U) = difference of 0.01 mm thickness between injected and noninjected footpad.

[c] $P<0.005$.

[d] One injection of a mixture of FO and OO equaling a total volume of 0.05 ml.

TABLE 4. Reduction of DTH by CsA and fish oil given i.p. or p.o.[a]

	DTH(U)[b]	
	i.p.	p.o.
CsA+FO	4.2±0.9	12.8±1.2
CsA+OO	8.2±0.6	10.8±0.6
Untreated[c]	22.4±0.5	

[a] CsA = 50 mg/kg in 0.1 ml lipid day 6 only. Values are means + SEM, Mann-Whitney *U* test; 5 mice/group.

[b] CsA+FO vs. CsA+OO: $P<.005$; CsA IP vs. CsA PO: $P<.025$; CsA+FO, CsA+OO given i.p. or p.o. vs. uninjected: $P<.005$.

[c] Untreated = No CsA in FO or OO.

TABLE 5. Suppression of DTH by fish oil[a]

Groups	Injections[b] (day)	DTHU[c]
—	None	54.2±1.1
OO	0,1,2,3,4,5,6	54.6±10.7
FO	6	35.8±1.0[d]
FO	4,5,6	29.2±1.4[d]
FO	0,1,2,3,4,5,6	12.2±0.7[d]

[a] 5 BALB/c mice/group; each i.p. injection was 0.2 ml oil on the indicated days; values are means ± SEM.

[b] From challenge (day 0) through challenge (day 6).

[c] DTHU = difference of 0.01 mm thickness between injected and noninjected footpad.

[d] $P<.005$, Mann-Whitney *U* test.

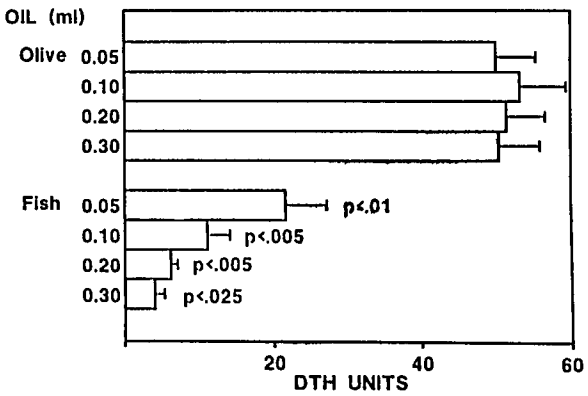

FIGURE 1. FO suppresses DTH in a dose-dependent manner. BALB/c mice were injected i.p. with several different doses of OO or FO ranging from 0.30 to 0.05 ml daily, and DTH was quantitated by measuring differences of 0.01 mm in thickness of the injected and noninjected footpad. *P* values were calculated by the Mann Whitney *U* test.

DISCUSSION

These preclinical studies suggest that solubilization of CsA in FO may prove advantageous in clinical practice. We have previously shown that FO vehicle prevents CsA nephrotoxicity. We now demonstrate that FO potentiates the immunosuppressive activity of CsA in several mouse models. Administration of FO, but not OO, alone potently suppresses DTH in a dose-dependent manner. By solubilizing CsA in FO rather than OO (the lipid vehicle administered in clinical practice) the dose of CsA required to suppress DTH is markedly reduced. Concurrent administration of FO and CsA potentiated the immunosuppressive effects in a parent-to-hybrid rat allograft model. It now seems likely that administration of CsA plus FO will increase the therapeutic potency of CsA.

N-3 polyunsaturated fatty acids are a poor substrate for cyclooxygenase metabolites. These fatty acids have a higher affinity for cyclooxygenase than n-6 or n-9 (OO) fatty acids, and therefore are preferentially selected. However, since n-3 are a poor substrate, the synthesis of prostanoids is reduced (*4–6*). Previous studies from our laboratory indicate that CsA selectively increases intrarenal TxA_2 synthesis and modestly reduces synthesis of the vasodilator prostanoids—e.g., PGE_2 and PGI_2 (*1, 2*). We have suggested that these CsA-induced

TABLE 6. Prolongation of cardiac engraftment by CsA and fish oil[a]

CsA (mg/kg)	OO	FO
LBN into Lew:		
None	5,5,6,8,8,	6,6,6,7,8,
1.5	8,8,8,10,13	10,21,21,23,27[b]
5	33,38,41,42 >50	>50,.50,>50,>50, >50[b]
15	>50,>50,>50,>50,>50	>50,>50,>50>50
WF into Lew:		
None	5,5,5,5,8	4,5,6,6,6,
1.5	9,10,1,12,14	9,10,13,13,14,15,34
5	37,41,41,43,44,49	29,34,42,>40
15	31,33,33,46 >50	33,34,35,37,50

[a] Treatment for 7 days with daily injections of CsA intramuscularly and 1 ml/day p.o. of the appropriate lipid.

[b] $P<0.05$, Mann-Whitney U test.

renal prostanoid alterations reflect the CsA alterations in the macrophage cyclooxygenase profile (*2*). Therefore FO was selected as the lipid vehicle for the hydrophobic CsA based on the ability of these molecules to reduce synthesis of the vasoconstrictor TxA_2. Solubilization of CsA in FO as compared with corn oil (n-6 fatty acids) or OO (n-9 fatty acids) obviates CsA-induced nephrotoxicity and maintains a balanced production of renal vasodilator and vasoconstrictor prostanoids.

Our previous studies did not explore the possibility that FO might alter the immunosuppressive activity of CsA and therefore compromise immunosuppression. While detailed pharmacodynamic studies have not been undertaken, we clearly demonstrate that immunosuppression is not lost when CsA is given with FO. Detailed studies of this nature are desirable but tissue partitioning of this intensely hydrophobic molecule makes such a study a major undertaking. We addressed a simpler clinically relevant issue: Does administration of FO, which reduces nephrotoxicity in rodents, obviate CsA's immunosuppressive effects? Several studies indicate that administration of n-3 fatty acids suppresses immunologic responses. In two distinct murine models of autoimmune lupus, the MRL-1pr and NZB/NZW F_1 hybrids, prophylactic dietary supplementation of FO protected from lupus nephritis and prolonged survival (*7, 8*). Although T cell functions, macrophage activation, and autoantibody titers were reduced, it is unclear if protection from renal injury is related to changes in the immune features or to a direct effect of n-3 on renal tissue (*7–10*). Recent studies in human volunteers indicate that n-3 fatty acid supplementation reduces the capacity of mononuclear cells to elaborate IL-1 and TNF (*11*). IL-1 and TNF are potent proinflammatory peptides (*12, 13*); amplified intrarenal synthesis of these cytokines occurs in autoimmune mice with lupus (*14*) and in the synovium of rheumatoid arthritis patients (*15, 16*). Since FO administration in these autoimmune disorders reduces the severity of disease, the antiinflammatory effect of n-3 fatty acids may be mediated, at least in part, by decreasing IL-1 and TNF. It is possible that n-3's ability to reduce cytokine production of IL-1 and TNF suppresses DTH. By extension, the immunosuppressive benefits of CsA in FO may be related to its reduction of different cytokines. CsA prevents the transcription of IL-2, which in turn reduces the clonal expansion of T cells required to drive an immune response (*17*). FO reduces IL-1 and TNF production, so the combination of CsA and FO may be a more potent immunosuppressant since the immune reaction is blocked at two distinct pathways.

Currently, attempts at minimization of CsA nephrotoxicity often rely on monitoring of blood levels of this drug—however, the effective immunosuppressive and nephrotoxic doses often overlap. Solubilization in FO rather than OO might broaden the therapeutic range of CsA. The present studies suggest that CsA plus FO will allow a reduction of the dose of CsA needed to prevent rejection while reducing toxicity. Increased immunosuppression was observed with concurrent use of FO and CsA in a mouse DTH mode. To further examine the potential therapeutic benefit of solubilizing CsA in FO, we evaluated this combination in a heterotopic rat heart allograft model. CsA in FO, as compared with CsA in OO, prolonged engraftment of a hybrid-into-parent heart transplant. Additional studies using CsA in FO to treat a parent-to-parental strain combination did not increase immunosuppression, but neither did it diminish the immunosuppressive action of CsA. Further studies probing the potential synergy of CsA and FO are underway. These preliminary results suggest that combined CsA and FO therapy may create a broader therapeutic range for CsA. In conclusion, CsA plus FO is a safe, potent antirejection therapy in the rodent worthy of clinical testing.

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RENAL CORTICAL MITOCHONDRIAL INTEGRITY IN EXPERIMENTAL CYCLOSPORINE NEPHROTOXICITY[1]

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The function of renal cortical mitochondria isolated from rats with cyclosporine nephrotoxicity was studied. Renal cortical mitochondria were isolated from 5 male Fischer rats after 14 days of daily intraperitoneal administration of CsA, 25 mg/kg body wt. Compared with the mitochondrial function of 5 pair-fed control rats receiving vehicle alone, state 3 respiration (ADP-dependent) using several substrates was mildly depressed only with pyruvate-malate supported respiration (27±3 vs. 36±2 nmol O_2/min/mg protein; $P<0.05$). The Ca^{2+} accumulation rate was slightly reduced (354±14 vs. 416±18 nmol/min/mg protein; $P<0.025$) while the cytochrome enzyme concentrations were not different from controls. Respiratory control ratios were not affected (CsA group: 9.5±2.8, control group: 8.9±2.3; glutamate-malate as substrates). These minor alterations in mitochondrial function occurred in the presence of severe depression in the glomerular filtration rate and renal morphologic changes commonly seen with CsA administration. Moreover, there was no increase in enzymuria. These results indicate that CsA has minor effects on the respiratory function of renal cortical mitochondria. The severe depression in the glomerular filtration rate is out of proportion to these minor alterations in mitochondrial function. These findings argue against a prominent role for a direct toxic action of CsA on tubular cells in the pathogenesis of acute cyclosporine-induced renal dysfunction.

Use of the potent immunosuppressive drug cyclosporine is associated with nephrotoxicity in a high percentage of patients (*1*), limiting the clinical usefulness of this valuable agent. Despite intensive investigation, controversy exists regarding the nature of cyclosporine's nephrotoxic effect. Several studies have indicated that functional alterations in renal hemodynamics, which result in reduced renal blood flow and a consequent fall in the glomerular filtration rate, are the basis for cyclosporine's acute nephrotoxicity (*2–4*). Other authors have interpreted abnormalities in renal tubular cell morphology observed in CsA-treated patients (*5*) and animals (*6*) to suggest a direct toxic action of CsA on renal tubular epithelium. These structural changes are most prominent in the proximal tubular epithelial cells and include cytoplasmic vacuolization, an increased number of lysosomes, and the presence of giant mitochondria (*6*). While tubular cell necrosis is infrequent, the presence of lysosomal enzymuria observed by some workers in CsA-treated animals (*6*) is consistent with an action of CsA to produce direct toxicity to tubular cells. Moreover, functional derangements in isolated renal cortical mitochondria (RCM)* following in vitro (*7*) and in vivo (*8*) exposure to CsA have been proposed by one group as a possible pathogenetic mechanism. Other workers, however, have not been able to confirm the presence of enzymuria (*4*) or mitochondrial dysfunction (*9*) with CsA treatment.

In order to more clearly define the nature of CsA nephrotoxicity, the present studies were performed to examine the res-

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* Abbreviations: EGTA, ethyleneglycol bis (aminoethylether) tetraacetate; γGT, gamma glutamyl transferase; NAG; N-acetyl-β-glycosaminidase; RCM, renal cortical mitochondria; RCR, respiratory control ratio.