The Role of Apoptosis in the Suppression of Lymphoproliferation which is Observed in Animals Fed Diets Rich in n-3 Polyunsaturated Fatty Acids

by

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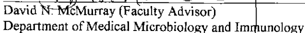

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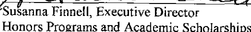

Susanna Finnell, Executive Director
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Fellows Group: Biology

Abstract

The Role of Apoptosis in the Suppression of Lymphoproliferation which is Observed in Animals Fed Diets Rich in n-3 Polyunsaturated Fatty Acids

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Dietary fish oil has been shown to be anti-inflammatory, and to relieve the symptoms of some inflammatory diseases in humans. It is thought that n-3 fatty acids found in fish oils may act to suppress T cell activation, which is a prerequisite for many types of inflammation. Previously, we demonstrated that splenocyte cultures of mice fed diets enriched in the n-3 fatty acid eicosapentanoic acid (EPA) undergo a higher degree of apoptosis, or programmed cell death, upon stimulation, than cells from mice fed the n-6 fatty acid control diet, and that increased apoptosis was correlated with decreased proliferation in those cultures. In the present study we show that T cells from animals fed diets enriched in fish oils have a lower proliferative response and a higher incidence of apoptosis when stimulated with α-CD3/α-CD28 than similarly stimulated T cells from mice fed the arachidonic acid and corn oil (n-6) enriched diets. This indicates that an α-CD3/α-CD28 activation induced mechanism of apoptosis is modulated by dietary fatty acid, and could explain diet-induced alterations in T cell function.

The Role of Apoptosis in the Suppression of Lymphoproliferation which is Observed in Animals Fed Diets Rich in n-3 Polyunsaturated Fatty Acids

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Dietary fish oil has been shown to be anti-inflammatory, and to relieve the symptoms of some inflammatory diseases in humans. It is thought that n-3 fatty acids found in fish oils may act to suppress T cell activation, which is a prerequisite for many types of inflammation. Previously, we demonstrated that splenocyte cultures of mice fed diets enriched in the n-3 fatty acid eicosapentanoic acid (EPA) undergo a higher degree of apoptosis, or programmed cell death, upon stimulation, than cells from mice fed the n-6 fatty acid control diet, and that increased apoptosis was correlated with decreased proliferation in those cultures. In the present study we show that T cells from animals fed diets enriched in fish oils have a lower proliferative response and a higher incidence of apoptosis when stimulated with α-CD3/α-CD28 than similarly stimulated T cells from mice fed the arachidonic acid and corn oil (n-6) enriched diets. This indicates that an α-CD3/α-CD28 activation induced mechanism of apoptosis is modulated by dietary fatty acid, and could explain diet-induced alterations in T cell function.

Arthritis, an inflammatory disease of the joints, affects one in seven Americans and is the primary cause of disability in this nation [1]. Dietary fish oil has been shown to be anti-inflammatory, and to relieve the symptoms of some inflammatory diseases in humans [1]. These potential medical benefits must, however, be weighed against the possible immunosuppressive effects of dietary therapies, thus necessitating a more detailed understanding of the mode of action of fish oils on the immune system. It is thought that n-3 fatty acids, found in fish oils, may act to suppress T lymphocyte activation, a prerequisite for many types of inflammation. One hypothesized mechanism for this dietary suppression of lymphoproliferation is that n-3 fatty acids somehow act to induce apoptosis, or programmed cell death, in activated T cells that should otherwise be proliferating.

T cell activation can be induced *in vitro* by culturing murine splenocytes with either concanavalin A (Con A)[‡], a plant lectin that causes polyclonal T cell proliferation, or hamster anti-mouse CD3 and hamster anti-mouse CD28 (Ab), monoclonal antibodies to the T cell receptor accessory molecules CD3 and CD28 that, when bound, induce polyclonal T cell activation [2]. We have demonstrated that the dietary n-3 fatty acids eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) result in suppressed T cell proliferation when induced with Con A [1]. In a preliminary study, we determined that splenocytes of mice fed diets rich in EPA underwent a larger degree of apoptosis than the control group fed safflower oil (an n-6 polyunsaturated fatty acid) when stimulated to proliferate with Ab. This preliminary study also demonstrated a correlation between increased apoptosis and decreased proliferative response. No significant change was found, however, in the degree of apoptosis for the Con A stimulated splenocytes of animals fed EPA or DHA or for the Ab stimulated splenocytes of animals fed DHA.

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‡ Abbreviations used in this paper: 2-ME, 2-mercaptoethanol; α-CD3, fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3e monoclonal antibody; Ab, hamster anti-mouse CD3/hamster anti-mouse CD28 antibody stimulus; BSA, bovine serum albumin; Con A, concanavalin A; Dnase I, deoxyribonuclease I; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IL-2, interleukin-2; PBS, phosphate buffered saline; RPMI, complete tissue culture media.

TABLE 1. Experimental design.

Day	Animal numbers	Diet treatment group	Splenocyte culture stimulus	Assay performed	Number of wells per assay
1	1-5	Arachidonic Acid	RPMI		
			Con A		
			Ab		
	6-10	Corn Oil	RPMI		
			Con A		
			Ab		
	11-15	Fish Oil	RPMI		
			Con A	Proliferation at 24 hours	3
			Ab	Proliferation at 96 hours	3
2	16-20	Arachidonic Acid	RPMI	% T cells at 24 hours	6
			Con A	% Apoptosis at 24 hours	6
			Ab		
	21-25	Corn Oil	RPMI		
			Con A		
			Ab		
	26-31	Fish Oil	RPMI		
			Con A		
			Ab		

Additionally, since whole splenocyte populations were used, these preliminary results could not be attributed to the T cells in culture.

To determine levels of apoptosis in the T cell population, we developed two flow cytometric analyses using fluorescent antibody markers for T cells and apoptotic cells. The first identifies T cells based upon expression of the T cell specific marker CD3; the second tags the free 3'-OH groups of cellular DNA cleaved during apoptotic DNA fragmentation. We then used these cytofluorometric analyses in conjunction with an assay of proliferation based upon uptake of tritiated thymidine to determine if diet plays a significant role in the apoptotic death of stimulated T cells. Here we demonstrate that Ab stimulated T cells from mice fed diets enriched in fish oil have a lower proliferative response and a higher incidence of apoptosis than T cells from arachidonic acid and corn oil (n-6) controls. This reveals an α-CD3/α-CD28 activation induced mechanism of apoptosis which is modulated by dietary fatty acids.

MATERIALS AND METHODS

Experimental animals and diets. Thirty-one pathogen-free female C57BL/6 mice were housed five per cage (6 per cage for one group) in polycarbonate cages on stainless steel mesh floors and provided free access to commercial chow and tap water. Following a one-week acclimation period the mice were fed diets with 4% corn oil + 1% arachidonic acid-containing triglyceride. After three weeks, each cage of mice was assigned randomly to one of three diets differing only in lipid source. One group continued on the 4% corn oil + 1% arachidonic acid diet and is heretofore referred to as the arachidonic acid diet treatment group. Animals in the second group were fed a 5% corn oil diet and are referred to as the corn oil diet treatment group. Animals in the third group were fed a diet of 4.5% fish oil + 0.5% corn oil and are referred to as the fish oil diet treatment group. The extra mouse was placed in the fish oil group. These diets were continued for two weeks. Throughout the course of the diet treatments mice were allowed unrestricted access to food.

Isolation and preparation of splenic lymphocytes. Mice were CO_2 asphyxiated, and each spleen was aseptically removed and placed in 3mL complete tissue culture medium (RPMI 1640 + 10% FBS + 1% glutamine + 0.01mM 2-ME), hereafter designated RPMI. A single cell suspension was produced using a sterile glass homogenizer and wire mesh filter and brought to a concentration of 2×10^6 viable cells/mL; viability was determined by trypan blue exclusion.

Lymphocyte stimulation. Splenocyotes were treated as above and transferred to 96 well microtiter plates at a concentration of 2×10^5 viable cells/well. Cells were then stimulated with either hamster anti-mouse-CD3 (α-CD3) and hamster anti-mouse-CD28 (α-CD28) or concanavalin A (Con A). For α-CD3/α-CD28 (Ab) stimulation, the wells of a round bottomed 96 well microtiter plate were first coated with 50μL of a 1μg/mL α-CD3 solution in PBS. After an overnight incubation at 4°C, the plates were rinsed with RPMI and filled with 100μL of a 10μg/mL solution of α-CD28 in RPMI. The wells that were to contain the Con A stimulated cells received 100μL of a 5μg/mL solution of Con

A in RPMI. Wells containing only RPMI and cells served as the negative stimulation controls. Triplicate wells were made per animal per stimulus for each of the proliferation assays; 6 wells per animal per stimulus were made for each flow cytometric analysis. (See Table 1 for experimental design.)

Proliferation analysis. Cells in triplicate wells were incubated for either 24 hours or 96 hours at 37°C in a humidified 5% CO_2 environment. Six hours before the end of the incubation period, cells were radiolabeled with a 1μCi solution of tritiated thymidine in RPMI. Cells were harvested at 24 and 96 hours onto glass micro filter paper, resuspended in 10mL of Ecoscint A scintillation fluor, and counted on a Beckman β scintillation counter.

Fixation and permeabilization of cultured cells for flow cytometric analysis. Cells were incubated for 24 hours at 37°C in a humidified 5% CO_2 environment. Cultured splenocytes of each animal under different stimuli were harvested from their 6 wells into 1.5mL microfuge tubes and centrifuged at 300 x g for 10 minutes to remove the culture media. Cell samples were resuspended in 100μL PBS + 1% BSA and transferred into separate wells of a 96 well V-bottomed microtiter plate. Samples were centrifuged as before, washed with 200μL/well PBS + 1% BSA, and fixed with 100μL/well 4% paraformaldehyde in PBS for 30 minutes. Fixative was removed by centrifugation, and cells were washed in PBS and permeabilized with 100μL/well 0.1% Triton® X-100 + 0.1% sodium citrate for 2 minutes on ice. Samples were centrifuged to remove the permeabilization solution, resuspended in 200μL/well PBS, and stored at 4°C until staining was begun.

Staining of T cells and apoptotic cells. Fixed and permeabilized cells in PBS were centrifuged at 300 x g for 10 minutes and resuspended in 50μL of either 1μg/mL fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3e monoclonal antibody (PharMingen, San Diego, CA) to detect T cells or a 9:1 dilution of TUNEL enzyme solution (calf thymus terminal deoxynucleotidyl transferase, 10 x conc.) to TUNEL label solution (nucleotide mixture in reaction buffer, 1 x conc.) (In Situ Cell Death Detection Kit, Fluorescein: Boehringer Mannheim) for detection of apoptotic cells. Staining was allowed to proceed for 60 minutes at 37°C in a humidified incubator, then samples were washed and resuspended in PBS for analysis by flow cytometry. Fixed and permeabilized unstained cells from each animal, cultured with RPMI, Con A, or Ab, served as negative controls for the α-CD3 stain. A positive control for the apoptosis assay was generated by incubating cultured cells from one animal, chosen at random, with 1mg/mL Dnase I (Sigma, St. Louis, MO) in 50mM Tris-HCl, pH 7.5, 1mM $MgCl_2$, 1mg/mL BSA for 10 minutes at room temperature. Fixed, permeabilized cells from one animal of each diet group, cultured with either RPMI, Con A, or Ab, and treated with TUNEL labeling solution, but no terminal transferase at the staining step, served as the negative controls for the apoptosis assay.

Cytofluorometric analysis. Light scatters and fluorescence histograms were obtained for both α-CD3 and TUNEL stains of each sample (Figure 1). Positive fluorescence, M1, was set to less than 2% on the TUNEL negative control. A regional gate, R3, was assigned to the positive fluorescence area of the histogram of cells stained with α-CD3. The R3 gate was then applied to the α-CD3 light scatter to generate a light scatter of the T cells alone. Another regional gate, R4, was assigned to the portion of the light scatter with the highest density of T cells. This second regional gate was applied to the histograms of the TUNEL stained samples to determine percent apoptosis in the population of cells with the highest T cell density.

Statistical analysis. Triplicate results for proliferation for each animal under each type of stimulus were averaged,

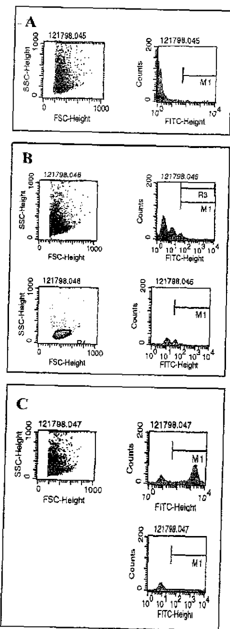
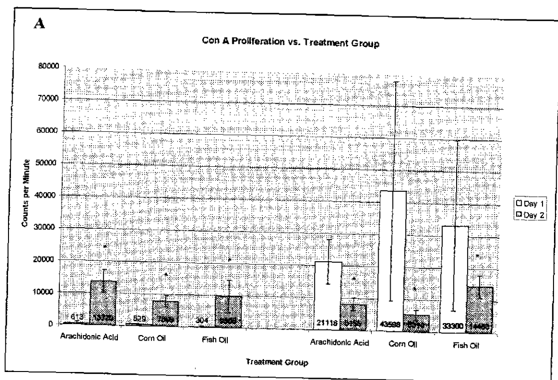


FIG. 1. Sample light scatters and fluorescence histograms from the cytofluorometric analysis of cultured splenocytes. Cells from Animal 5 (in the arachidonic acid diet group) were cultured for 24 hours at a concentration of $2x10^5$ cells/well with 2.5μg/mL Con A in RPMI. Though histogram statistics differed between animals and stimuli, all data from cytofluorometric analysis had an overall appearance similar to the graphs in this figure. **(A)** M1 was set at < 2% fluorescence on the TUNEL negative control. **(B)** Cells stained with α-CD3 (T cells) were selected for by applying gate R3 to the α-CD3 light scatter (bottom left). Regional gate R4 was assigned to the area of highest T cell concentration, and applied to the fluorescence histogram (bottom right), eliminating a significant portion of the other cell types. **(C)** Regional gate R4 was assigned to the TUNEL histogram for all cells (top right), almost eliminating apoptotic cells from the gated histogram (bottom right.)



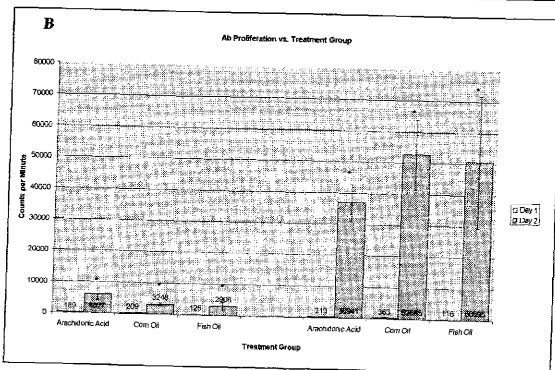


FIG. 2. Comparison of proliferative response by day in whole splenocyte populations. Animals were fed diets with arachidonic acid as the sole lipid source for five weeks then split into three diet test groups: arachidonic acid, corn oil, and fish oil. Five mice from each diet group (six from the fish oil – day 2 group) were necropsied on separate days. Cells were cultured on microtiter plates at 2×10^5 cells/well for 24 hours and 96 hours with 100μL of different culture stimuli; RPMI served as a negative control for proliferation (not shown), 5μg/mL Con A in RPMI and adherent α-CD3 + 10μg/mL α-CD28 in RPMI served as proliferative tests. Cells were incubated with 1μCi tritiated thymidine in RPMI 6 hours prior to harvesting. Radioactivity for each sample was determined on a Beckman β scintillation counter and reported as scintilla counts per minute. Results for the animals in each treatment group were averaged. Significant differences between days are noted with an asterisk ($p<0.05$). (**A**) Con A proliferation for each diet grouped by length of stimulation. (**B**) Ab proliferation for each diet.

and standard deviations were found. Proliferation and apoptosis results from each animal at each stimulus were averaged by diet treatment group and standard deviations were calculated. The Dixon test for outlying observations was used to remove outliers with a 95% confidence level before all averagings. P-values were obtained using numbers of observations, averages, and standard deviations. Statistical significance was assigned to those observations having a p-value less than 0.05.

RESULTS

Experimental controls. Thirty-one animals, assigned randomly to three diet groups differing only in lipid composition were stimulated *in vitro* with Con A or Ab as described above. RPMI served as the negative control for stimulation. Cultures from animals fed arachidonic acid alone served as the background control; cultures from animals in the corn oil diet group served as the n-6 positive control for proliferation and negative control for apoptosis; cultures from animals fed fish oil served as the n-3 test group for proliferation and apoptosis.

Day effects on proliferative response. Whole splenocyte cultures from each diet group were radiolabeled, harvested, and counted as described above. Due to the time limitations involved in culturing the cells from 31 animals, necropsies were performed on two separate days. Though care was taken to ensure the similarity of experimental conditions between the two days, the proliferative responses for both Con A (Fig. 2a) and Ab (Fig. 2b) stimuli in all of the diet groups differed significantly ($p<0.05$) from day 1 to day 2 at both 24 and 96 hours. In all subsequent discussions in this paper, cells cultured on day 1 and day 2 are treated as two separate populations.

Culture stimulus effects on proliferative response. Day 1 proliferation at 24 hours (Fig.3a) reveals only low level stimulation in all diet treatment groups. After 96 hours of culture (Fig. 3b), Con A stimulated cells surged in proliferative response while Ab stimulated cells did not proliferate significantly over the RPMI

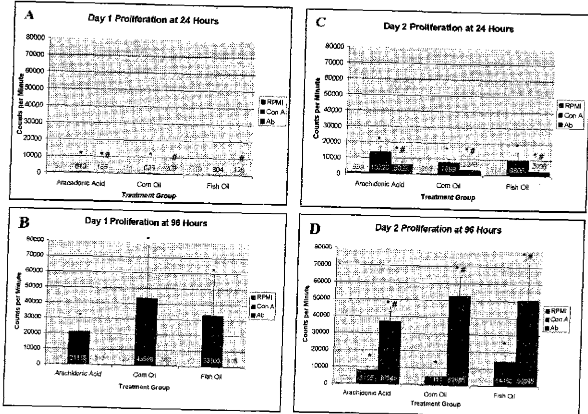


FIG. 3. Comparison of proliferative response by stimulus. Animals were assigned randomly to one of three diet groups: arachidonic acid, corn oil, or fish oil. Whole splenocytes were cultured on microtiter plates at $2x10^5$ cells/well on two separate days for 24 hours and 96 hours with either RPMI, 5μg/mL Con A, or 10μg/mL Ab. Cells were incubated with 1μCi tritiated thymidine in RPMI 6 hours prior to harvesting, and radioactivity for each sample was reported as scintilla counts per minute. Results for the animals in each treatment group were averaged by stimulus for each day. Significant differences over RPMI stimulation controls are noted with an asterisk ($p<0.05$). Significant differences between Con A and Ab stimuli are noted with a pound sign ($p<0.05$). **(A)** Day 1 proliferation at 24 hours for each stimulus grouped by diet treatment. **(B)** Day 1 proliferation at 96 hours. **(C)** Day 2 proliferation at 24 hours. **(D)** Day 2 proliferation at 96 hours.

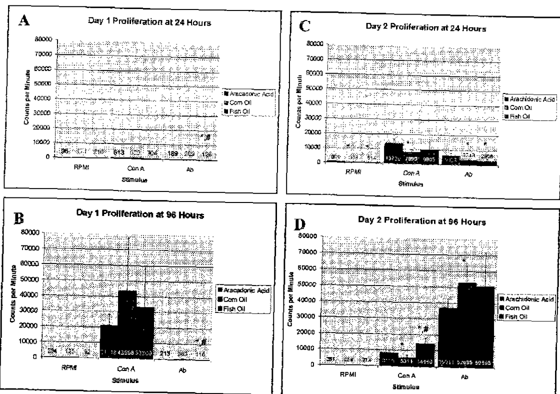


FIG. 4. Comparison of proliferative response by diet treatment. Results for the animals in each treatment group were averaged by stimulus for each day. Significant differences over the arachidonic acid diet control group are noted with an asterisk ($p<0.05$). Significant differences between corn oil and fish oil diet treatment groups are noted with a pound sign ($p<0.05$). (**A**) Day 1 proliferation at 24 hours for each diet treatment grouped by stimulus. (**B**) Day 1 proliferation at 96 hours. (**C**) Day 2 proliferation at *24 hours*. (**D**) *Day 2 proliferation* at 96 hours.

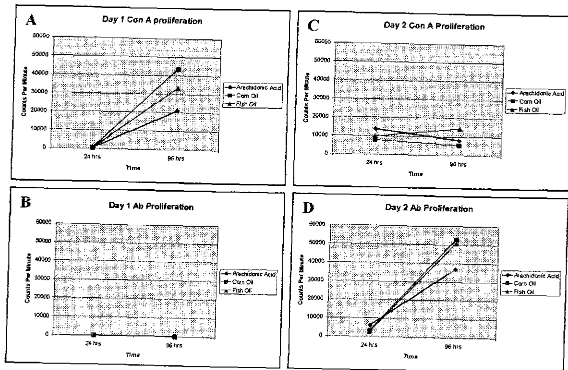


FIG. 5. Kinetics of the proliferative response. Results for the animals in each treatment group were averaged by stimulus for each day. RPMI control data are not shown. (**A**) Day 1 Con A proliferation over time for each diet treatment group. (**B**) Day 1 Ab *proliferation*. (*C*) Day 2 Con A proliferation. (**D**) Day 2 Ab proliferation.

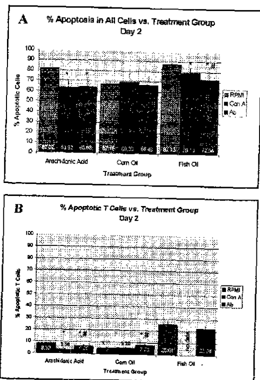


FIG. 6. Comparison of percent apoptosis in all cultured splenocytes and in T cells alone by stimulus. Animals were assigned randomly to one of three diet groups: arachidonic acid, corn oil, or fish oil. Splenocytes were cultured on microtiter plates at $2x10^5$ cells/well on two separate days for 24 hours with either RPMI, 5μg/mL Con A, or 10μg/mL Ab. (Apoptosis data for Day 1 was lost and will not be referred to subsequently.) Cells for T cell and apoptosis detection were separately fixed, permeabilized, and stained with FITC-conjugated α-CD3 or TUNEL reaction mixture and analyzed cytofluorometrically. Results for the animals in each treatment group were averaged. Significant differences over RPMI stimulation controls are noted with an asterisk. Significant differences between Con A and Ab stimuli are noted with a pound sign. **(A)** Percent apoptotic cells in the total splenocyte culture grouped by diet treatment. **(B)** Percent apoptotic T cells. This measure of apoptosis was generated mathematically by combining percent T cells from α-CD3 flow data and percent apoptosis from TUNEL experiments.

control at all. Day 2 animals exhibited the converse. Proliferation at 24 hours (Fig. 3c) shows that cells from all diet groups stimulated with Con A proliferated to a significantly higher degree than those stimulated with Ab. By 96 hours (Fig. 3d), however, Ab proliferation surged in all diet treatments while Con A proliferation dropped off in the arachidonic acid and corn oil control diet treatment groups. Only in the fish oil test group did proliferation on day 2 increase in the Con A stimulated cultures.

Diet effects on proliferative response. When grouped by stimulus to compare diet treatment effects, proliferative data for day 1 animals (Figs. 4a,b) reveal the surge of proliferation in Con A treated cells at 96 hours. At both time points, splenocytes from animals cultured under Ab stimulus proliferated to a significantly lower extent ($p<0.05$) that those from arachidonic acid or fish oil control animals. Day 2 data, on the other hand, reveal a marked diet effect for both stimuli. At 24 hours (Fig. 4c) Con A stimulated whole splenocytes from the corn oil n-6 group lagged behind the arachidonic acid group while the fish oil group did not differ significantly from either diet control. Ab stimulated cells from both the corn oil and fish oil diet treatments at this same time point also proliferated less extensively than cells from the arachidonic acid group. By 96 hours, (Fig. 4d) Ab-induced proliferation in day 2 animals for all diet groups increased, showing no significant differences between diet treatments. Con A proliferative response for splenocytes of corn oil fed animals at this same time point were significantly lower than cells from arachidonic acid control animals. Splenocyte cultures of fish oil treated animals, on the other hand, demonstrated a significantly higher level of proliferation than either arachidonic acid or corn oil diet control groups.

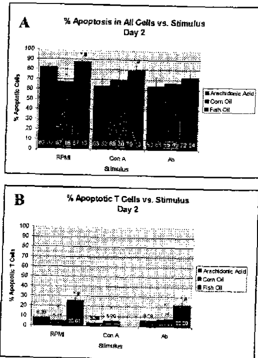


FIG. 7. Comparison of percent apoptosis in all cultured splenocytes and in T cells alone by diet treatment. Cells for T cell and apoptosis detection were separately fixed, permeabilized, and stained with FITC-conjugated α-CD3 or TUNEL reaction mixture and analyzed cytofluorometrically. Results for the animals in each treatment group were averaged. Significant differences over the arachidonic acid diet control group are noted with an asterisk ($p<0.05$). Significant differences between corn oil and fish oil diet treatment groups are noted with a pound sign ($p<0.05$). **(A)** Percent apoptotic cells in the total splenocyte culture grouped by culture stimulus. **(B)** Percent apoptotic T cells.

The kinetics of diet effects on proliferation are summarized in Fig. 5. Proliferation in all three diet treatments stimulated with Con A increased over time in the day 1 animals (Fig. 5a) but remained low on day 2 (Fig. 5c). The converse was true for Ab stimulation between days (Figs. 5b,d). Comparison of Figs. 5a and 5d reveals that for both Con A and Ab stimuli, proliferation in the corn oil control group increases at the fastest rate. Differing rates of proliferation in the Con A stimulated corn oil and fish oil diet groups (Fig. 5a) accounts for the differences in their 96 hour counts. Ab stimulated cells in these two diet groups (Fig. 5d), however, had the same proliferative rate.

Culture stimulus effects on apoptosis in the whole splenocyte culture and the T cell subpopulation. Cells from each diet group were harvested and stained at 24 hours for cytofluorometric analysis as described above. The majority of day 1 α-CD3 and TUNEL data was lost in a centrifuge accident and, consequently, will not be addressed. Apoptosis was measured after 24 hours of culture only.

Significantly lower levels of apoptosis ($p<0.05$) were seen in stimulated cultures of splenocytes in the arachidonic acid and fish oil treatment groups, while no difference was noted in the corn oil diet group (Fig. 6a). When the splenocyte population was reduced mathematically to T cells alone (% T cells from the α-CD3 experiment times % apoptosis from the TUNEL experiment), Ab treated corn oil diet group T cells exhibited a significant increase in apoptosis ($p<0.05$) over Con A stimulated T cells from the same animals (Fig. 6b). There was no difference between stimuli in the fish oil diet group, although the overall degree of T cell apoptosis was higher in both stimulated and unstimulated T cells from this group.

Diet effects on apoptosis in both whole *splenocyte culture and the T cell* subpopulation. When percent apoptosis was measured across all cells in culture (Fig. 7a), there was a trend toward increased apoptosis in the fish oil diet group for both stimuli. Percent apoptosis in Con A stimulated splenocytes differed significantly ($p<0.05$) between the fish

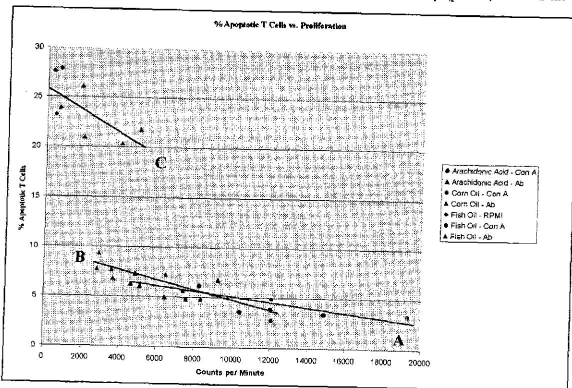


FIG. 8. Correlation between decreased proliferation and increased apoptosis in the T cell population. Both proliferation and apoptosis were measured after 24 hours of culture. Proliferative response, reported in counts per minute, was determined by incubating cells with 1μCi tritiated thymidine in RPMI 6 hours prior to harvesting. Cells for T cell and apoptosis detection were separately fixed, permeabilized, and stained with FITC-conjugated α-CD3 or TUNEL reaction mixture and analyzed cytofluorometrically. Percent apoptotic T cells was determined mathematically by multiplying the overall percent apoptosis in the splenocyte culture (TUNEL) by the percentage of T cells in culture (α-CD3). Results for each animal are reported separately. Low cell count for animals in the corn oil and fish oil diet treatment groups rendered many samples statistically insignificant. These are not reported here. (**A**) Linear least squares line for the arachidonic acid data points. $R^2 = 0.6156$ with 99% confidence. (**B**) Linear least squares line for the corn oil data points. $R^2 = 0.6852$ with 99% confidence. (**C**) Linear least squares line for the fish oil data points. (No Con A data was significant for this diet group.) $R^2 = 0.4327$ with 92% confidence.

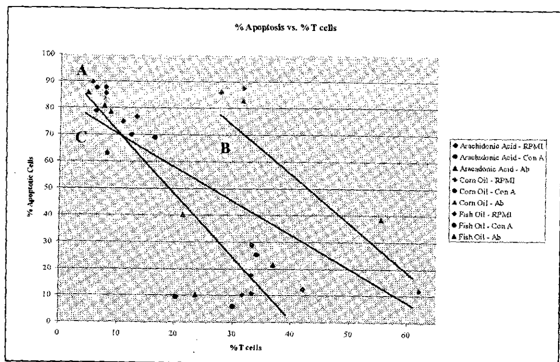


FIG. 9. Correlation between increased T cell density in the light scatter of α-CD3 staining cells and decreased percentage of TUNEL staining (apoptotic) cells. Cells for T cell and apoptosis detection were separately fixed, permeabilized, and stained with FITC-conjugated α-CD3 or TUNEL reaction mixture and analyzed cytofluorometrically. Results for each animal are reported separately. Low cell count for animals in the corn oil and fish oil diet treatment groups rendered many samples statistically insignificant. These are not reported here. **(A)** Linear least squares line for the combined arachidonic acid and corn oil data points. $R^2 = 0.8166$ with 99% confidence. **(B)** Linear least squares line for the fish oil data points. $R^2 = 0.4444$ with 85% confidence. **(C)** Linear least squares line for all data points. $R^2 = 0.4774$ with 99% confidence.

oil diet treatment group and both diet control groups. This trend became significant for Ab stimulated splenocytes ($p<0.05$) when T cells alone were considered (Fig. 7b). For both RPMI and Ab treated cultures, T cells from the fish oil diet group underwent a significantly higher degree of apoptosis than either diet control. T cells from corn oil treated animals also exhibited a significantly higher level of apoptosis ($p<0.05$) that cells from the arachidonic acid control group, however the difference was not as marked as that seen in the cells of fish oil test group animals.

Correlation between decreased proliferation and increased apoptosis in the T cell subset. Mathematically derived percent apoptotic T cells for each animal following Con A and Ab stimulus were plotted against proliferative response at 24 hours measured in counts per minute (Fig. 8). Low cell count on the flow cytometer for animals in the corn oil and fish oil diet treatment groups rendered many samples statistically insignificant. These results were not included in linear regression analysis. Cells fell along two obvious lines, differing by diet; arachidonic acid and corn oil controls spread out along the lower portion of the graph, while samples from the fish oil test group clustered about the upper left-hand portion of the graph.

Linear least squares analysis of arachidonic acid samples yielded an R-squared value of 0.6156 with 99% confidence in the model (Fig. 8a). Fit along the least squares line for the corn oil control group (Fig. 8b) was slightly tighter with an R-squared value of 0.6852 at the same confidence level. The low number of statistically significant samples in the fish oil group necessitated including RPMI values in the least squares analysis (Fig. 8c). The y-axis spread created by including these values produced an R-squared value of 0.4327, however the confidence level remained a high 92%.

Correlation between T cell density in the light scatter of α-CD3 staining cells and apoptosis. Percentage of α-CD3 staining cells before and after gating for the highest density of T cells on the α-CD3 light scatter (Fig. 1) was plotted against percentage of apoptotic cells before and after the same gate was applied to the TUNEL light scatter (Fig. 9). Least squares analysis was performed for arachidonic acid and

corn oil combined data (Fig. 9a), fish oil data alone (Fig. 9b), and combined data from all diet groups (Fig. 9c). R-squared values were found to be 0.8166, 0.4444, and 0.4774 with confidence levels of 99%, 85%, and 99% respectively, indicating a high level of correlation between increased T cell density and decreased apoptosis in the diet control groups. A clean correlation was not found in the fish oil diet group, however the number of data points was limited.

DISCUSSION

The membrane composition of lymphocytes reflects the composition of fatty acids in the diet and in culture [3,4]. These membrane fatty acids regulate membrane permeability, fusion, and fluidity, an important parameter for enzyme and receptor behavior [5,6]. Fatty acid analysis of lymphocyte membranes from DHA- and EPA-fed mice in this laboratory have demonstrated an enrichment of the membrane composition with these n-3 polyunsaturated fatty acids [6]. We assume that the splenocytes of arachidonic acid-, corn oil-, and fish oil-fed mice used in this study demonstrated a similar enrichment of membrane lipids with their respective diets. Because this membrane lipid composition can take on the character of the culture media, and the media used for culture of each diet treatment group in this study were identical, we believe that cells from different diet groups began to take on similar membrane character after several days of incubation. Thus, we propose that proliferative responses at 96 hours for each stimulus did not always vary significantly by diet due to similarities in media lipid composition. Repeating this study with media differing in lipid concentration to reflect the lipid composition of the diet could confirm this hypothesis.

The effects of dividing the study over a two day period were unexpected given the otherwise identical treatment of the animals and cell cultures. In order to ensure the statistical significance of results in the proliferation and apoptosis experiments, 31 mice were assigned to the three diets – 10 arachidonic acid control mice, 10 corn oil, n-6 polyunsaturated fatty acid mice, and 11 fish oil, n-3 polyunsaturated fatty acid mice. Due to the relatively lengthy process of setting up so many samples in culture, the necropsies were divided between two days; half of each diet treatment group was performed each day. These two treatment group subsets were managed identically from initiation of the diet treatments to scintillation and cytofluorometric analyses of cultured cells with the exception of the slight variations inherent in any experiment. The cumulative results of these seemingly innocuous differences were, however, staggering. The most noteworthy difference in culture treatments was that the cells from day 1 necropsies required three hours longer to put them into culture than the cells from day 2 due to some logistical problems. Though this is a seemingly minor difference, it is unknown what potentially important cellular processes occur directly after necropsy that may have been altered in cells which sat for an additional three hours in culture medium.

Comparison of proliferative response by stimulus revealed that day 1 cells cultured in Ab did not proliferate as expected based on previous observations in this laboratory (unpublished data). Additionally, none of the results obtained from the day 1 procedure produced useful statistical significance. Results for day 2 Con A proliferation for both time points and all diet treatment groups were unexpectedly low. Since day 1 Con A results were near the expected range for that stimulus [1], we assume that the low proliferation at day 2 was a result of some experimental difference between the two days and not a function of differential overall proliferative response to mitogen and antibody induction.

Con A T cell induction in previous diet studies revealed that lymphocytes from animals fed EPA have significantly higher mitogenic responses than lymphocytes from animals fed n-6 fatty acid controls [7, 8], but another group reported the opposite effect [1]. The former observation was confirmed for the unpurified fish oil diet group here. Because of the lack of Con A apoptosis data for the fish oil diet group, we do not know if this increased proliferation can be correlated to apoptosis, and we cannot speculate about a mitogen induced mechanism of apoptosis. Significant correlation between Ab induced proliferation and apoptosis in each diet group, however, allows us to put forth several more hypotheses regarding n-3 fatty acids and the Ab induction of apoptosis.

While there was a significant decrease in proliferative response ($p<0.05$) for the 24 hour Ab stimulated splenocytes of day 2 animals in the corn oil and fish oil diet treatment groups compared to the arachidonic acid control group, there was no significant difference in proliferative response ($p>0.05$) between the corn oil and fish oil groups themselves. Despite similarities in proliferative response between these two groups, the percent apoptotic T cells was significantly higher ($p<0.05$) in Ab stimulated cells from fish oil fed mice than those

cells from mice fed corn oil. To account for these findings we hypothesize that T cells from mice fed fish oil are more responsive to Ab stimulation than T cells from corn oil fed mice, but increased apoptosis in the T cells of the fish oil group keeps the overall proliferative response at the same level that of T cells from animals in the corn oil control group. If rates of proliferation do differ between diet groups, we would expect to see differences in the proliferation kinetics; we do not. A reason for the apparent similarities in rates of proliferation between the two groups, however, has already been addressed. Similarities in media lipid compositions may falsely produce similar proliferative responses as the culture time is extended due to incorporation of the lipids from the culture media into the cellular membranes of the test cells. Two possible explanations for the higher antibody induced levels of apoptosis in cells from fish oil fed animals are possible - the diet-induced heightened rate of cell division triggers activation of apoptotic pathways or diet lipid components alter the structure of the cellular membrane sufficiently to induce Ab activated T cells to undergo apoptosis rather than cell division. Since Ab stimulation requires the binding of surface molecules, and the lipid composition of the T cells from the test group differs from that of the control group, we support the latter hypothesis. Regardless of which theory proves to be correct, higher levels of apoptosis in the Ab stimulated cells of the fish oil group suggest an activation-induced mechanism of apoptosis.

Early proliferative response was significantly ($p<0.05$) lower in the Ab stimulated fish oil and corn oil diet groups than the arachidonic acid control, but at 96 hours Ab stimulation in the control group had been induced, eliminating any significant difference in response by diet. One possible reason for this difference in proliferative response over time that was previously discussed is increased homogeneity of membrane lipid composition between diet groups over time. Another possible explanation is that over the course of the three days between analyses of proliferation there was an upregulation of interleukin-2 (IL-2) production and receptor expression in the arachidonic acid and corn oil control cultures, inducing late proliferation. This line of reasoning is consistent with Fernandes, et. al. who reported decreased IL-2 production and IL-2 receptor expression in corn oil fed lupus-prone mice with respect to fish oil fed mice [8]. Jolly, et. al., on the other hand, found suppression of IL-2Rα mRNA levels in animals fed EPA and DHA (n-3) relative to safflower oil (n-6) and arachidonic acid controls [1]. This study, however, also reported decreased levels of proliferation in the EPA and DHA diet groups, a finding that was not supported by this study or Fowler et. al. [7].

Our measure of % apoptotic T cells was made by multiplying % apoptosis data from the TUNEL assay by % T cells data from the α-CD3 assay. Taking this measurement as an absolute determinant of the fraction of apoptotic T cells in culture assumes that apoptosis occurs with equal likelihood in all subpopulations of cells. We believe apoptosis does occur in the T cell subpopulation, however that does not mean it does not occur in other subpopulations in culture. The high percentages of apoptotic cells overall may indicate that other subpopulations are indeed undergoing apoptosis. A simultaneous analysis of the α-CD3 and TUNEL stains, which allows the unambiguous assignment of both T cell identity and apoptotic status to each individual cell, is needed to determine absolutely which cells are undergoing apoptosis in culture.

There is a strong inverse correlation between percent apoptosis and T cell density on the flow cytometer light scatters. This might initially indicate that most of the apoptosis is occurring in another subpopulation of cells in the whole splenocyte culture. It is possible, however, that this is an experimentally introduced relationship. Gating α-CD3 light scatters on the regions of highest T cell density and applying that gate to TUNEL histograms assumes that the T cells gated are representative of the overall T cell population and that apoptosis has no effect on light scatter. In fact, positive controls for apoptosis in the TUNEL assay had significantly reduced forward scatter. The same results, however, are seen with necrotic cells (data not shown). Broad interpretation of this data is therefore unwise until light scatter by apoptotic cells can be confirmed.

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