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Consumption of *Aphanizomenon flos-aquae* Has Rapid Effects on the Circulation and Function of Immune Cells in Humans

A novel approach to nutritional mobilization of the immune system

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ABSTRACT

Objective: To examine the short-term effects of consumption of a moderate amount (1.5 grams) of the blue-green algae *Aphanizomenon flos-aquae* (AFA), on the immune system.

Methods: Using a crossover, placebo-controlled, randomized, double-blind design, 21 volunteers were studied, including 5 long-term AFA consumers.

Results: Consumption of a moderate amount (1.5 grams) of the blue-green algae *Aphanizomenon flos-aquae* results in rapid changes in immune cell trafficking. Two hours after AFA consumption, a generalized mobilization of lymphocytes and monocytes, but not polymorph nucleated cells, was observed. This included increases in CD3+, CD4+, and CD8+ T cell subsets and CD19+ B cells. In addition, the relative proportions and absolute numbers of natural killer (NK) cells were reduced after AFA consumption. No changes were observed in the relative proportions of naïve versus memory T cells, neither in the CD4 nor the CD8 fractions. A mild but significant reduction in phagocytic activity was observed for polymorph nucleated cells. When freshly purified lymphocytes were exposed to AFA extract in vitro, direct activation was not induced, as evaluated by tyrosine phosphorylation and proliferative activity.

Discussion: The changes in immune cell trafficking displayed a high degree of cell specificity. Long-term consumers responded stronger with respect to altered immune cell trafficking. In vitro, AFA did not induce a direct activation of lymphocytes. These data support a signaling pathway from gut to CNS to lymphoid tissue. The signals from CNS may be crucial for the rapid changes in the general distribution and specific recruitment we observed. Moderate anti-inflammatory modulation may account for the modification of phagocytic activity.

Conclusion: Consumption of AFA leads to rapid changes in immune cell trafficking, but not direct activation of lymphocytes. Thus, AFA increases the immune surveillance without directly stimulating the immune system.

KEYWORDS: Lymphocyte trafficking, natural killer cells, phagocytes.

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INTRODUCTION

Blue-green algae are among the most primitive living organisms on Earth. Though they are technically classified as bacteria, they share properties with bacteria and with plants. They contain many biologically active substances that have beneficial effects on human health. Thus, a large research interest in the use of blue-green algae for food supplementation has emerged. Several blue-green algae, including *Aphanizomenon flos-aquae* (AFA) have pronounced antibacterial properties[1] and have protective effects in the classical AMES test.[2] The blue-green algae Spirulina has documented antiviral[3,4] and anticancer[5,6] properties. In addition, Spirulina subspecies have effects

on the immune system, by enhancing the phagocytic activity in macrophages[7,8,] inhibiting allergic reactions in rodents,[9-11] and by enhancing antigen-specific antibody production and proliferative responses in chickens.[8] Other algae contain sulfolipids with potent anti-viral properties.[12] Thus, blue-green algae species contain phytochemicals that are potent modulators of certain immune functions.

The trafficking of immune cells between various locations is an important aspect of the healthy immune system, as part of scavenging for invading pathogens, infected or transformed cells. The various cell types that constitute our immune system are present throughout almost all tissues in our body. The absolute and relative amounts of trafficking immune cells in the blood is rapidly altered in response to chemical messenger molecules.The monitoring of these changes are widely used to evaluate the short-term immune changes to various physical, chemical, and psychological stressors. The various populations of immune cells in normal blood is depicted in Figure 1, along with the surface markers used for their identification.

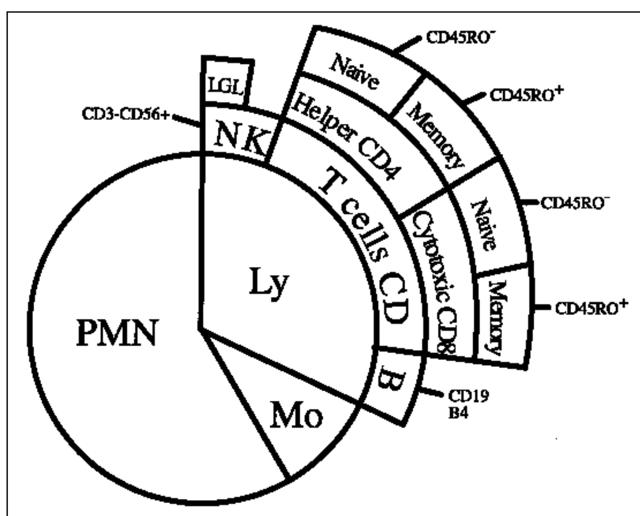

Figure 1: Schematic diagram of the relative proportions of white blood cells and the markers associated with their identification.

Trafficking cells re-circulate between various anatomical locations by entering the blood stream. In order for the cells to exit the blood and enter a new anatomical location, they must be able to adhere and migrate. In almost all tissue (spleen and liver being exceptions), the cells must perform a specific series of tasks in order to transmigrate: 1) Slow down the speed by forming loose adhesion on the vessel wall, and rolling along the endothelial surface, 2) Form a strong adhesion onto the endothelium, and 3) Migrate through the endothelial barrier and the underlying basement membrane.[13-15] These events are mediated by a combination of chemotactic factors and adhesion molecules. The circulating cells are able to “sense” sites of cellular recruitment via chemokines bound to the endothelium or secreted into the lumen of the blood vessel. A large number of chemokines are known, and they are able to activate cell subsets in a highly selective manner.[16] Of interest for our data are the chemokines involved in recruitment of natural killer (NK) cells into tissue. Seven out of 8 tested C-C chemokines induced chemotaxis of NK cells,[17] as well as fractalkine.[18] Another chemokine of interest is lymphotactin, which elicits a migratory response in NK and T cells, while having no effect on monocytes and neutrophils.[19] Thus, mechanisms are in place to mediate highly selective patterns of migration and recruitment of specific leukocyte subpopulations.

The re-circulation pattern of immune cells varies in a circadian pattern, which is dependent on neuro-endocrine signals. In one study, a clear circadian rhythm is seen for T cell subsets, but not for NK cells,[20] whereas another study reported a clear increase of NK numbers and activity in the morning.[21] It is believed that high levels of cortisol in the beginning of the day interfere with interleukin-2 production and enhances the migration of lymphocytes from the blood into tissues. Other mechanisms of inducing high levels of cortisol (stress, exercise),[22-24] as well as injection of hydrocortisone[25] have similar inhibitory effects on lymphocyte migration. Importantly, species variations exist, and stress experiments in rodents cannot directly be compared to human studies. The different physiological responses to various stressors in the human system may be difficult to understand in the light of how apparently similar stressors are perceived in laboratory animals.

The recruitment of NK cells is very sensitive to catecholamines, especially epinephrine.[26] The catecholamines have a negative effect on the adhesion of NK cells to the vessel walls, and causes the NK cells to detach. The changes in NK cell trafficking is not accompanied by changes in adhesion molecule expression on the circulating NK cells. The catecholamine-induced accumulation of NK cells in the blood was identical in normal and splenectomized donors, indicating that the spleen was not the relevant reservoir of NK cells.[27] Several studies have reported a stress-mediated increase in numbers of B and NK cells in blood.[28,29]

Throughout the body, many nerve factors are able to function as chemokines, and immune cells express receptors for neurotransmitter molecules. Only some cytokines are regulated by cortisol, and a hierachy of cortisol-sensitivity has been proposed.[30] The bi-directional relationship between neuronal and immunological systems extends to the lymphoid tissues. In addition to the well characterized central nervous system regulation of adrenals, nerve terminals invade all lymphoid tissue, and synapse-like formations can be seen between nerve endings and immune cells in bone marrow, lymph nodes and spleen.[31] Neuronal control of haematopoiesis has been studied in detail, and a complex feedback system exists, involving multiple cytokines and neurotransmitters.[32,33] Neuropeptide Y is an

example of a neurotransmitter that is directly able to upregulate adhesion molecules on human endothelial cells.[34]

The central nervous system regulation of immune surveillance is of functional importance. In mice, when signaling from the sympathetic nervous system to the periphery was interrupted prior to injection of NK-sensitive tumor cells, the numbers of metastases were significantly increased.[35] As the NK activity was not altered, nor was the ability to respond to tumor antigens, one possible explanation is that the sympathetic nervous system regulates either NK trafficking or matrix deposition in tissue, thereby regulating the ability of NK cells to migrate to the vicinity of tumors. This was partially confirmed by demonstrating that the sympathetic nervous system modulates lymphocyte recruitment into lymph nodes.[36]

Consumption of the blue-green algae AFA has increased, and despite a large number of anecdotal reports on health benefits, studies of the exact mechanisms of AFA's effects on immune function were needed. In a previous brief report, we presented preliminary data to show that AFA induced a rapid induction of NK cell recruitment into tissue in humans.[37] Since then, we have analyzed the migratory patterns of multiple white blood cell types in a total of 21 study subjects. Upon oral consumption of 1.5 grams AFA, we observed immediate changes in several specific immune parameters.

MATERIALS AND METHODS:

Subjects: Twenty-one non-hospitalized volunteers were analyzed in a double-blinded cross-over fashion, upon informed consent. The volunteers had no known acute or chronic infections. Five were long-term AFA consumers, 2 were occasional AFA consumers, and the remaining 14 had never before consumed AFA. Occassional consumers had previously used AFA daily for at least 6 weeks continuously, but were not consuming AFA regularly during the weeks leading up to this study. No volunteer had taken AFA for at least 24 hours prior to being studied. Ten volunteers were male, and eleven were female. The age range was 20-52 years.

Study design: Each volunteer was studied on two separate days. Any volunteer was always studied at the same time on the two study days, to eliminate the circadian influence on the data. The volunteers were asked to consume the same breakfasts at the same times on the two study days, and not to consume any other vitamin preparations or nutraceuticals for at least 12 hours before the study. The volunteers were required to sit quiet for 45 minutes prior to study start, so that any prior walking or other exercise did not affect the relative proportions of leukocytes. The first blood sample was taken, and the substance was given. Until the sampling of the second blood sample 2 hours later, the volunteer was required to remain quiet and avoid any extensive walking.

Consumables and reagents: Both AFA and placebo were provided by Cell Tech (Klamath Falls, Oregon). The dose given to the volunteers was 1.5 grams, which is the recommended dose for daily supplementation. A list of monoclonal antibodies used for immunostaining and flow cytometry is listed in Table 1.

CD#	Clone	Specificity	Source
CD3	SK7	TCR complex	Becton-Dickinson
CD4	SK3	helper/inducer T cells	Becton-Dickinson
CD8	SK1	cytotoxic T cells	Becton-Dickinson
CD11a	25.3	Alpha-L chain (Beta-2 integrin)	Immunotech
CD11b	D12	Alpha-M chain (Beta-2 integrin)	Becton-Dickinson
CD14	MoP9	PI-anchored receptor, binds LPS	Becton-Dickinson
CD18	7E4	Beta-2 subunit (Integrin)	Immunotech
CD19	89B (B4)B	cell surface molecule	Coulter
CD29	3S3	Beta-1 subunit (Integrin)	Serotec
CD44	F1044-2	H-CAM pgp-1	Serotec
CD49d	L25	Alpha-(VLA)-4 chain (integrin)	Becton-Dickinson
CD62L	TQ1	L-selectin	Coulter

Table 1: List of monoclonal antibodies used in this study.

Purification of mononuclear cells: Fourteen ml of heparinized or EDTA blood was drawn from a peripheral vein. The blood was layered onto a Ficoll gradient and centrifuged to purify the peripheral blood mononuclear cells. Cells were washed, and used for direct immunofluorescence labeling. Samples were fixed in 1% formalin and stored cold and dark prior to flow cytometric analysis.

Flow cytometry: Data were acquired and stored on list mode for subsequent data analysis. The CellQuest software (Becton Dickinson) was used for acquisition and analysis. During analysis, electronic gating was used to eliminate red cells and clumps from the analysis.

Data analysis: The relative proportions of monocytes, B and T lymphocytes and T cell subsets were calculated based on positivity for the MoAbs listed in Table 1. The relative proportion of of NK cells was calculated by excluding monocytes and large granular cells from the analysis, then excluding the CD3+ cells, and evaluating the proportion of CD56+ cells in the sample. The number of CD3-CD56+ small lymphocytes was then related to the total number of PBMC. Changes were calculated by comparing the AFA- and placebo-induced values for each volunteer. Figure 1 gives a representation of the various cell types tested, their relationship and the marker used for quantification. By combining the relative proportions with actual cell counts, the absolute numbers of peripheral blood mononuclear cells and PMNs were calculated in 12 volunteers. Also, the changes in absolute numbers of the following subpopulations were calculated: monocytes, CD3+ T

cells, CD19+ B cells, CD4+CD45R0-/+ and CD8+CD45R0-/+ subsets.

Purification of neutrophils: Seven ml of heparinized whole blood was mixed with 1.5 ml of 6% dextran70 in 0.9% saline at room temperature. Sedimentation was allowed for 1 hour. The leukocyte rich supernatant was harvested and the cells pelleted by centrifugation. The pellet was resuspended in 2 ml phosphate buffered saline, which was then layered on top of 3 ml of Ficoll-Hypaque. Gradient centrifugation was performed, and the pellet was resuspended in 0.5 ml of phosphate buffered saline. The remaining red blood cells were lysed by hypotonic shock for 25 seconds, after which isotonicity was restored. Cells were washed, resuspended in RPMI, and kept on ice until use.

Assay for PMN phagocytic activity: The ability of PMN cells to kill Staphylococcus Aureus bacteria was performed as follows: S. Aureus (frozen aliquots) were defrosted and washed. The bacteria were then opsonized with pooled human serum for 30 minutes in a 37°C shaking water bath. PMN cells and bacteria were added to a series of tubes, and incubated in a 37°C shaking water bath. At the following time points: 5, 15, 30, and 45 minutes a tube was removed, immediately placed on ice, and 0.5 ml icecold serum added in order to stop further phagocytic activity. The tubes were centrifuged in the cold for 5 minutes at 3000 rpm, and the supernatant was decanted. The pellet was stained with Acridine Orange (14.4mg/L) for 1 minute. One ml of icecold buffer was added, and cells were washed 3 times. Cells were resuspended in cold buffer and kept on ice until microscopic examination. A wet mount slide was prepared from each tube for examination in a UV microscope at 100 times magnification. The proportion of phagocytic PMN were evaluated by counting 100 PMN, and counting how many of these cells contained at least 3 bacteria (whether bacteria were live or dead). During the examination, the total number of live versus dead bacteria was counted in 50 PMN.

Statistical analysis: Standard statistical analysis was performed using NNCS software. Paired t-test was used to determine statistical significance. Values that were outside two interquartile ranges from the 25th and 75th percentiles were considered extreme outliers and were removed from the analysis. The removal of outliers did not change the actual conclusion.

RESULTS. Immediate mobilization of mononuclear cells into the blood: The absolute cell counts before and after consumption of either AFA or placebo were monitored in 12 volunteers. The consumption of AFA resulted in increased blood cell counts when compared to placebo. The polymorph nucleated cell (PMN) population did not change, whereas the lymphocyte (Ly) and monocyte (CD14) subsets increased (Figure 2A). Within the lymphocyte subpopulation, the increase was observed in all of the following T cell subsets: CD3+, CD4+, CD8+, as well as in

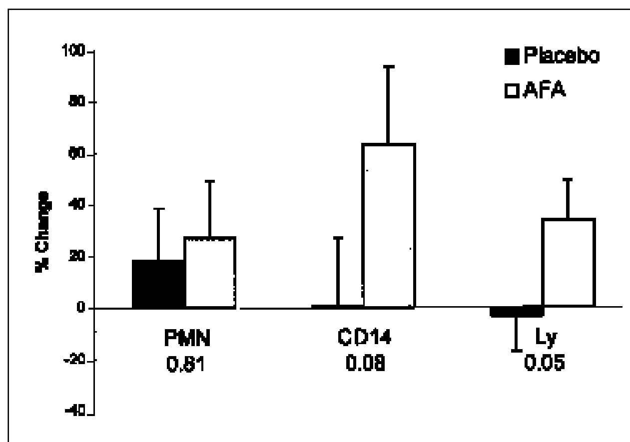

Figure 2A: AFA-induced changes in blood leukocyte populations. The histogram shows the % change of polymorph nucleated cells (PMN), monocytes (CD14), and lymphocytes (Ly). Black columns represent the mean values of placebo, and the white columns represent the mean values of AFA. The bars indicate the standard error of the mean.

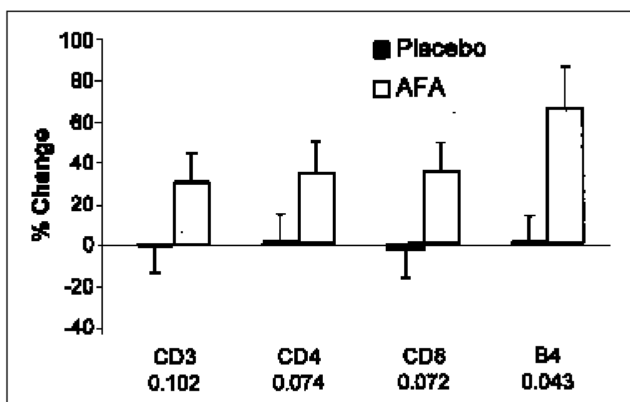

Figure 2B: AFA-induced changes in lymphocyte sub-populations. The histogram shows the % change of total T cells (CD3), T cell subsets (CD4, CD8), and B cell (CD19) lymphocyte populations. Black columns represent the mean values of placebo, and the white columns represent the mean values of AFA. The bars indicate the standard error of the mean.

the CD19+ B cell population (Figure 2B).

The relative proportions between naïve (CD45A+) and memory (CD45R0+) T cells was monitored in all 21 subjects, for both the CD4+ helper and CD8+ cytotoxic T cell subsets. Despite a tendency for a shift towards less naive and more memory T cells in the blood, no significant changes were seen in naive versus memory T cell subsets.

Specific recruitment of CD3- CD56+ small lymphocytes from the blood: In all 21 study subjects, the proportional changes of NK cells was examined. Two hours after AFA consumption, the relative proportion of CD3- CD56+ natural killer cells was decreased, when compared to placebo ($p<0.03$). The effect was specific for small NK cells

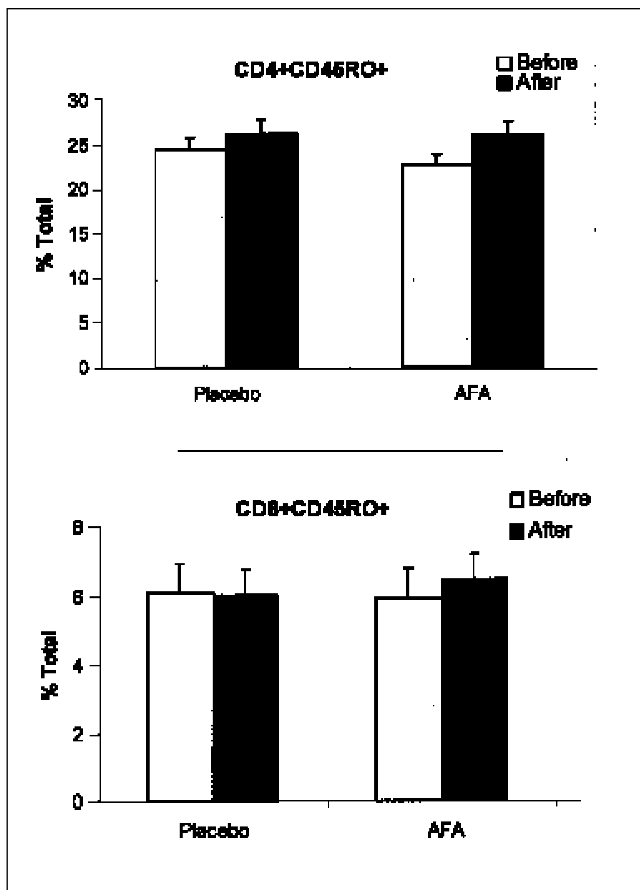

Figure 3: The relative changes in subpopulations of T cells is shown (mean and SEM for 21 volunteers). The helper (CD4+) T cell and cytotoxic (CD8+) T cell populations only showed a slight shift towards less naive and more activated/memory T cells in the circulation, and no statistical significance was reached.

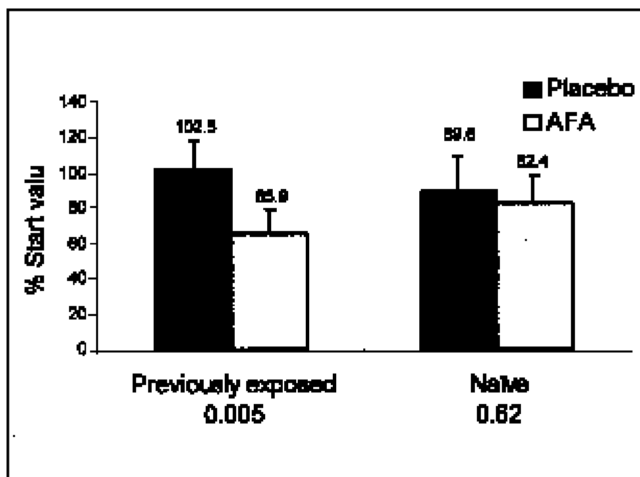

Figure 4: Changes in natural killer cells (NK cells) in % of the starting value. Black columns represent the mean values of placebo, and the white columns represent the mean values of AFA. The bars indicate the standard error of the mean.

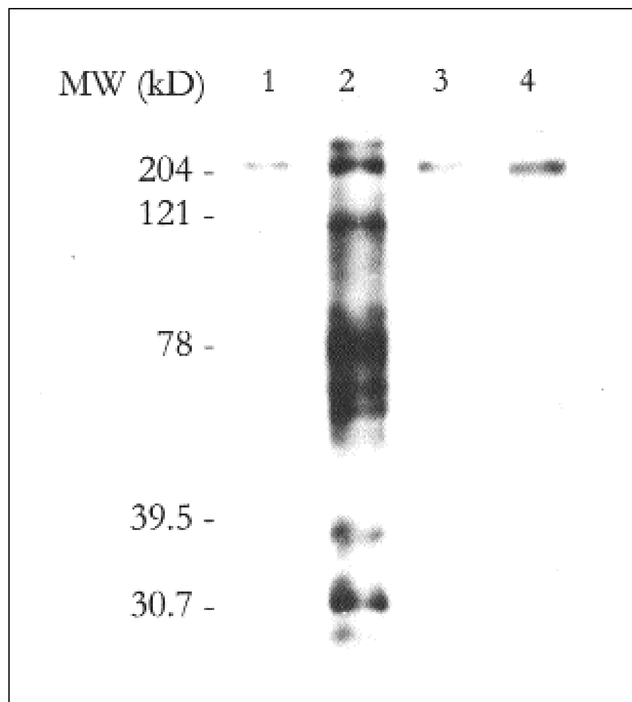

Figure 5: Western blotting of tyrosine phosphorylation of proteins extracted from unstimulated lymphocytes (lane 1) versus lymphocytes incubated with Pokeweed Mitogen (PWM, positive control, lane 2) or AFA (lane 3: extract 1:5, lane 4: extract 1:25). Incubation of freshly purified human lymphocytes with AFA extract did not induce tyrosine phosphorylation. The data are representative of 4 similar experiments.

(low forward/side scatter properties), as the subset of cells defined as large granular lymphocytes (CD14-negative, large granular cells) was not affected (data not shown). Long-term consumers produced a more pronounced response than naive volunteers. When the volunteers were grouped into long-term AFA consumers and naive volunteers, naive volunteers displayed a minor reduction in NK cells after AFA consumption, whereas long-term consumers displayed a pronounced reduction ($p < 0.005$).

Adhesion molecule expression on circulating leukocytes: We examined the expression of a series of adhesion molecules on the surface of monocytes, B and T cells before and after AFA exposure in vivo and in vitro. The following adhesion molecules and subunits were examined: CD62L, CD11a, CD11b, CD18, CD29, CD44, and CD49d. The fluorescence intensity was monitored by % positive, as well as mean and median fluorescence values. Short-term incubation (90 minutes) in vitro with AFA extract resulted in a moderate loss of CD62L on B as well as T cells, and a weak upregulation of CD11b, but no other changes in the expression of the following adhesion molecules: CD11a, CD18, CD29, CD44, and CD49d. Analysis of adhesion molecule expression on lymphocytes from volunteers 2 hours post AFA consumption showed moderate changes in CD62L expression, but no other changes (data not shown).

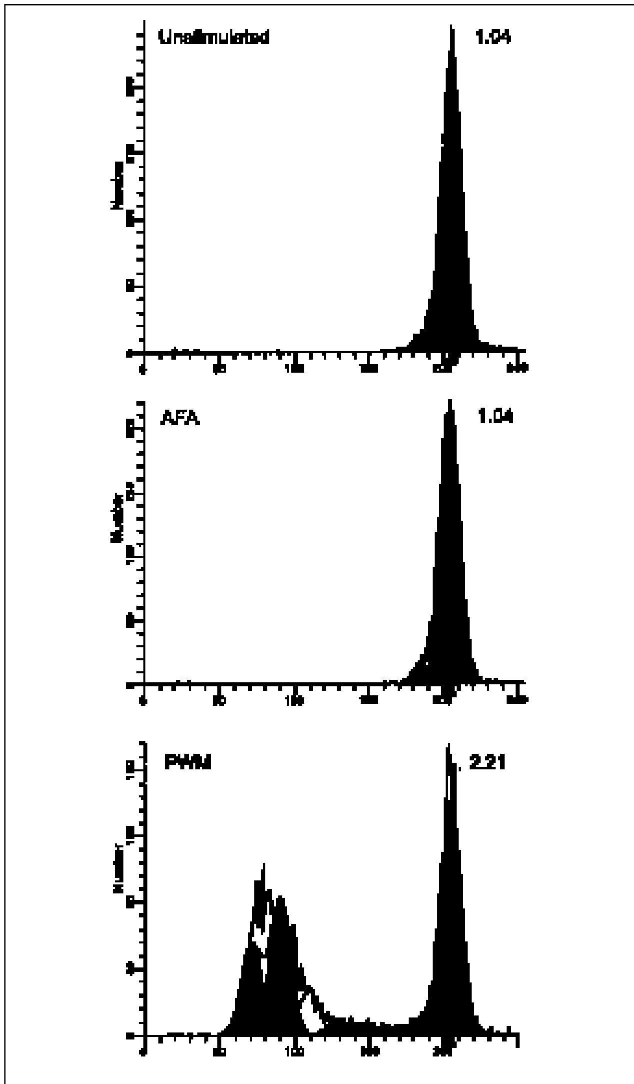

Figure 6: Flow cytometric evaluation of lymphocyte proliferation after 5 days of culture with no stimulation (top), with AFA water extract (middle), and Pokeweed Mitogen (PWM, bottom). The X axis displays fluorescence intensity, where loss of fluorescence corresponds to proliferative activity. The proliferative indexes for each culture condition is displayed in upper right corner of each histogram. The experiment was conducted three times, where all cultures were performed in triplicate.

AFA extract does not activate lymphocytes directly: We tested whether AFA extract could directly activate lymphocytes in vitro. When purified mononuclear cells were incubated with AFA extract, no activation was seen, as examined by tyrosine phosphorylation after 1-20 minutes of AFA exposure (Figure 5) and proliferative responses after 5 days of AFA exposure in vitro (Figure 6).

Modulation of the phagocytic activity of polymorph nucleated (PMN) cells: The phagocytic activity of PMN cells was evaluated, using PMNs from blood samples drawn before and 2 hours after AFA consumption. The phagocytic activity was monitored at different times of incubation. When the study subjects had ingested placebo, no differences on phagocytic activity was seen. In contrast, after consumption of AFA, a mild decrease in phagocytic activity was measured (Figure 7). This effect only reached levels of significance at longer incubation times (see legend to Figure 7).

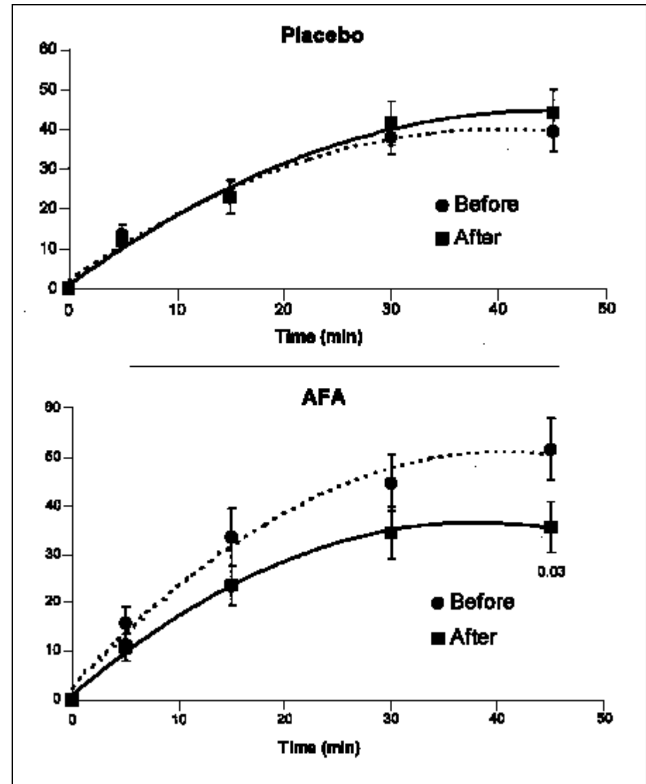

Figure 7: Phagocytic activity of polymorph nucleated cells (PMN) from volunteers before and after placebo or AFA ingestion. The phagocytic activity was unaffected by placebo, but was moderately reduced by AFA, thus resulting in a lower maximum phagocytic capacity, and a lower phagocytic rate.

DISCUSSION

Based on the many case reports on beneficial neurological and immunological effects of consumption of the blue-green algae Aphanizomenon flos-aquae, we studied the immune activation within 2 hours after ingestion of 1.5 grams AFA. This dose is recommended for food supplementation. We examined several aspects of immune cell migration and function. The data presented in this paper indicate a mild, but consistent effect on the human immune system.

The absolute numbers of circulating leukocyte subsets was increased. This effect was limited to lymphocytes and monocytes, whereas polymorph nucleated cells were not affected. This indicates a selective mobilization of lymphocytes and monocytes from primary or secondary lym-

phoid tissues, into the blood circulation. Thus, more monocytes, B and T cells were released into the blood. In the initial preliminary study (involving 1 occasional and 4 regular AFA consumers), AFA consumption induced a substantial transient recruitment of NK cells in all five volunteers, peaking at 2 hours and rapidly declining.[37] In the current analysis of 21 people, there was a specific recruitment from the blood of small NK cells. It could be argued that AFA only leads to margination (i.e. lymphocytes sticking to the vessel walls without transmigration). However, margination is not a permanent phenomenon, and the on/off rate would allow us to sample some cells that have marginated and later released from the blood vessel wall. Such cells would likely demonstrate altered adhesion profiles, which we did not find. In addition, as the recruitment of cells from circulation into lymphoid tissue is highly cell type specific, mediated in part by cell-type specific chemokines, transmigration would provide a more plausible explanation.

Increase in adhesion molecule expression was previously observed in a small number of long term consumers.[37] The present study reports data from a more thorough evaluation. When examining the profile of adhesion molecules on the surface of circulating lymphocyte subsets, we found occasional shifts in adhesion molecule expression, confirming earlier observations, but in this larger study we found no consistent differences induced by AFA in vivo. This evaluation is hampered by the fact that we are not able to directly sample the cells that have left the circulation as a result of AFA. Thus, AFA did not uniformly affect the adhesion profile of all circulating lymphocytes.

The low dose of AFA ingested and the rapidity of the observed effects do not support a direct effect, where bioactive molecules in AFA would be absorbed into the blood, and transported to the bone marrow and spleen, and there result in cellular changes leading to release of cells into the blood. A more plausible model for explanation is that neuro- or immune- active substances in AFA leads to triggering of a gut-to-brain activation. It has been reported that IL-1 beta is able to mediate a gut-to-brain communication via the abdominal vagus nerve.[38,39] Thus, in terms of rapid modulation of leukocyte re-circulation, a gut-to-brain signal would result in brain-to-lymphoid tissue signals, including the rapid release of chemokines. Many neuropeptides are either chemotactic or immuno-modulatory. As nerve terminals wrap around the high endothelial regions of lymphocyte recruitment in the peripheral tissue, a central activation could rapidly amplify and alter cellular recruitment in a highly selective manner. In the bone marrow, nerve terminals come in close contact with developing and maturing cells, and could regulate the volume of cells released into the blood circulation.

The rapid changes in leukocyte re-circulation were stronger in long-term AFA consumers. Since the study design was double-blinded and randomized, the volunteers were not themselves aware of when they were receiving AFA versus placebo. Given the suggested CNS-mediated modulation of the immune system, a conditioning may have been established in which the CNS may recognize the stimulation by AFA and in previously exposed consumers add a conditioned component to the immune activation of cell trafficking.

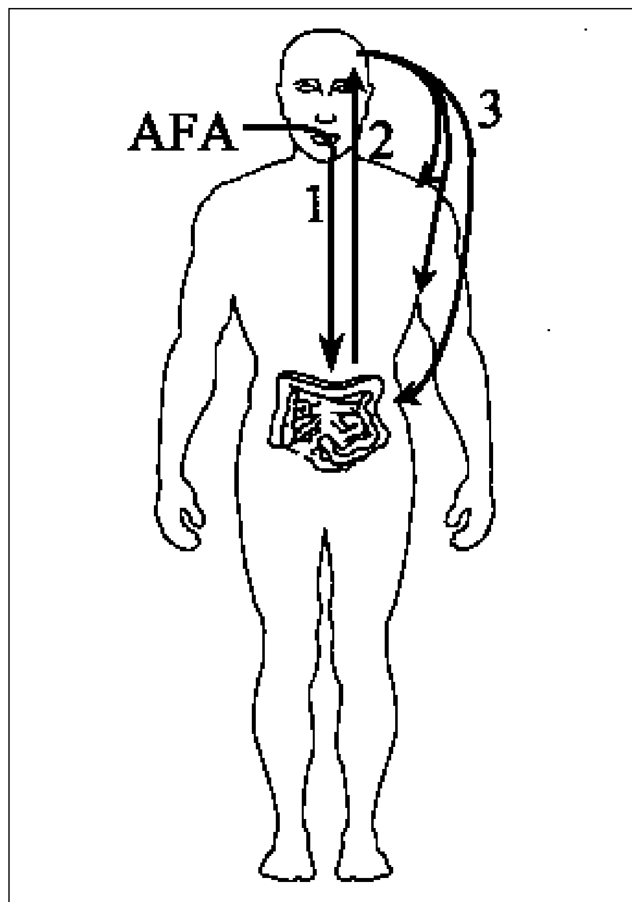

Figure 8: Hypothetical model for AFA-induced immuno-modulation. 1: Ingestion of AFA, and release of bioactive phytochemicals in the stomach and/or upper intestine. 2: Release of cytokine(s) in the gut trigger vagus nerve signals from gut to CNS. 3: Central nervous system signals to the peripheral lymphoid tissues, resulting in altered immune cell trafficking.

During our studies, we have been on guard for observations that could point in the direction of over-activation of the immune system. More is not always better. An over-activation of the immune system could be associated with circulating immune complexes and increase in inflammatory processes that could be detrimental to health. We found no indications of a direct activation of any component of the immune system or a general activation of the immune system as a whole. The increased trafficking of immune cells should translate into a better immune surveillance, i.e. a better and more efficient patrolling of microbial invaders, as well as virus-infected or transformed cells. We see this

as very positive for a potential use of AFA in various clinical situations or as a nutritional support for the prevention of viral infections. This data also points to further research in a potential role for AFA in cancer prevention.

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ORIGINAL RESEARCH

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Favorable Effects of Blue-Green Algae *Aphanizomenon flos-aquae* on Rat Plasma Lipids

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ABSTRACT

Background: Polyunsaturated fatty acids (PUFAs) are essential for human health. There are indications that the lipid fraction of blue-green algae *Aphanizomenon flos-aquae* contains about 50% PUFA and may be a good dietary source of PUFA. The purpose of this study was to investigate the effect of diets supplemented with algae on blood plasma lipids.

Methods: Rats were fed with four different semisynthetic diets: 1) standard, with 5% soybean oil; 2) PUFA-free with 5% coconut oil; 3) PUFA-free with 10% algae; 4) PUFA-free with 15% algae. After 32 days the levels of plasma fatty acids, triglycerides, and cholesterol were studied.

Results: Rats fed the PUFA-free diet demonstrated an absence of linolenic acid (LNA) in plasma; however, supplementation with algae resulted in the same level of LNA as controls, increased levels of eicosapentaenoic acid and docosahexaenoic acid, and a decreased level of arachidonic acid. Dietary supplementation with 10% and 15% algae decreased the plasma cholesterol to 54% and 25% of the control level, respectively ($p<0.0005$). Plasma triglyceride levels decreased significantly ($p<0.005$) after diet supplementation with 15% algae.

Conclusion: *Algae Aphanizomenon flos-aquae* is a good source of PUFA and because of potential hypocholesterolemic properties should be a valuable nutritional resource.

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INTRODUCTION

Previous research identified the important role of dietary polyunsaturated fatty acids (PUFA) in human health. A deficiency in n-3 PUFA has been linked to immunosuppression,[1] arthritis,[2] cardiovascular diseases,[3-6] mental[7,8] and dermatological[9] problems. Human and animal models containing n-3 PUFAs have anti-inflammatory activity[2,10,11] that may be mediated by decreasing the arachidonic acid level and thereby suppressing the production of specific cytokines.[12] Furthermore, n-3 fatty acids have been shown to decrease certain cancer risks,[13,14] prevent platelet aggregation,[6,15] and to lower blood cholesterol, possibly by stimulating its excretion into bile.[3,16]

The North American diet is believed to be deficient in PUFA, especially in n-3 fatty acids.[17] Dietary supplementation with fish oil rich in n-3 eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) has been recommended as a potential treatment for hypercholesterolemia.[15,18] Much empirical evidence over the past decade suggests that *Aphanizomenon flos-aquae (Aph. flos-aquae)*, a blue-green alga growing naturally in Upper Klamath Lake, Oregon, may be a good dietary source of PUFA. Nearly 50% of the lipid content of dried *Aph. flos-aquae* (5% to 9% of total dry weight) is composed of PUFA, mostly n-3 α-linolenic acid.

In our experiments using rats as the animal model, *Aph. flos-aquae* not only served a source of dietary PUFA but also significantly lowered blood cholesterol and triglyceride levels.

METHODS

Animals: Thirty-two adult male Sprague-Dawley rats were randomly distributed into 4 groups. Animals were placed into individual wire cages, and maintained at 22° C with a 12-hour light-dark cycle. Food and water were supplied *ad libitum.* For 32 days the animals were fed with the following semipurified test diets based on the American

Institute of Nutrition (AIN-76) standard:

1. Standard diet containing 5% soybean oil (SBO);
2. PUFA-deficient diet containing 5% coconut oil (PUFA-D);
3. PUFA-deficient diet containing 10% algae (Alg10);
4. PUFA-deficient diet containing 15% algae (Alg15).

The algal material used in this study was supplied by Cell Tech (Klamath Falls, OR) and contained 6.3% lipids. Feed was provided by Purina Test Diets (Richmond, IN).

After the feeding trial, the animals were fasted overnight and euthanised by carbon dioxide inhalation. Plasma was collected by heart puncture in a tube containing 100 µl 0.5 M EDTA (pH 8.0), centrifuged at 3,000 g for 15 minutes, and stored at -80°C.

Lipid Analysis: Blood fatty acid analysis was performed using a direct transesterification method[19] as modified by Mosers.[20] In brief, 250 µl of plasma was vortexed with 1 ml methanol:methylene chloride (3:1). 50 nmol of 17:0 free fatty acid (internal standard) in 50 µl of hexane was added to this mixture. Under continuous vortexing 200 µl of acetyl chloride was added and the mixture was incubated in the oven at 75°C for one hour. After cooling for 15 min at room temperature 4 ml of 7% potassium carbonate was added, vortexed, and then 2 ml of hexane was added. The mixture was vortexed for 60 sec and then centrifuged at 1750 g for 10 min at 4°C. The hexane layer was removed, 2 ml of acetonitrile was added and the mixture was centrifuged at 1120 g for 5 min at 4° C. The hexane layer was removed, dried under nitrogen to a final volume of approximately 100 µl, and 1 µl of the sample was used for analysis. Fatty acid identification was performed on a Hewlett-Packard 5890 series II model gas chromatograph-mass spectrometer GC-MC with a Hewlett-Packard 5971 mass spectrometer (Hewlett-Packard, Wilmington DE). Soybean and coconut oils were methylated by acid methanolysis before fatty acid analysis. The algae material was soaked in methanol, extracted and then methylated by acid methanolysis prior to fatty acid analysis.

Plasma triglycerides and cholesterol were measured on the automated clinical chemistry analyzer Roche BHO/H917 using corresponding Boehringer Mannheim kits.

Statistics: Statistical difference between groups was determined using unpaired Student's t-test. Difference in fatty acid profiles was evaluated using repeated measures analysis and contrast tests[21]. For all analysis, differences of $p<0.05$ were considered statistically significant.

RESULTS

Dietary Fatty Acids: Fatty acid composition of *Aph. flos-aquae,* soybean oil and coconut oil used in this study is represented in Table 1. The composition of soybean and coconut oil in the present study is close to that found in the

TABLE 1
Fatty acid composition (% of total fatty acids) of soybean oil, coconut oil, and algae

Fatty Acid	Source of Fatty Acids		
	Soybean oil	Coconut oil	Algae
Caprylic (8:0)	-	9.70	-
Capryc (10:0)	-	7.50	-
Lauric (12:0)	-	42.10	-
Myristic (14:0)	-	22.40	9.10
Palmitic (16:0)	14.69	18.20	36.60
Palmitoleic (16:1)	-	-	11.90
Margaric (17:0)	-	-	0.89
Stearic (18:0)	5.40	-	2.70
Oleic (18:1)	26.80	-	6.70
Linoleic (18:2n-6)	44.40	-	7.40
Linolenic (18:3n-3)	8.00	-	22.30
Arachidic (20:0)	0.35	0.14	-
Arachidonic (20:4n-6)	-	-	0.65
Eicosapentaenoic (20:5n-3)	-	-	0.08
Behenic (22:0)	0.33	-	-
Total polyunsaturated	52.40	-	30.43
Total saturated	20.77	100.04	49.29

Table 2
Lipid composition (%) of experimental diets

Indices	Diets			
	SBO	PUFA-D	Alg10	Alg15
Oil Source				
Soybean oil	5.00	0.00	0.00	0.00
Coconut oil	0.00	5.00	4.50	4.250
Algae	0.00	0.00	10.00	15.00
Total fat	5.00	5.00	5.13	5.20
Fatty Acid Content				
Linoleic acid (18:2n-6)	2.22	0.00	0.05	0.07
Linolenic acid (18:3n-3)	0.40	0.00	0.14	0.21
Total polyunsaturated (PUFA)	2.62	0.00	0.19	0.28
Lauric acid (12:0)	0.00	2.11	1.89	1.79
Myristic acid (14:0)	0.00	1.12	1.07	1.04
Palmitic acid (16:0)	0.73	0.91	1.05	1.12
Stearic acid (18:0)	0.27	0.00	0.02	0.03
Oleic acid (18:1)	1.34	0.00	0.04	0.06
Total saturated (SFA)	1.00	4.14	4.03	3.95
PUFA/SFA	2.62	0.00	0.05	0.07
n-6/n-3	5.55	-	0.36	0.36

literature.[22] Soybean oil is rich in linoleic acid (LA, 18:2n-6; 44.4% of total lipids) and contains a substantial amount of α-linolenic acid (LNA, 18:3n-3; 8%). *Aph. flos-aquae* is richer in LNA (22.3%) and contains less LA (7.4%) than soybean oil. *Aph. flos-aquae* has also small amount (0.65%) of arachidonic acid (AA, 20:4n-6) and traces (0.08%) of EPA (20:5n-3). Coconut oil is free of both n-3 and n-6 fatty acids.

Fatty acid composition of the various diets is represented in Table 2. SBO and PUFA-D diets had a total of 5% lipids provided by soybean and coconut oils. Because of a slightly higher amount of lipids in algae (6.29%) than expected (5%), Alg10 and Alg15 diets contained correspondingly 5.13% and 5.20% of lipids. Ratios of PUFA to saturated fatty acids (SFA) and n-6 to n-3 varied considerably between the diets.

Calculations showed that *Aph. flos-aquae* contains 1.40% LNA and 0.46% LA of total algal dry weight. Diets containing 10% and 15% of algae (corresponds to 0.63% and 0.94% of algal lipids) provided a total dietary intake of 0.14% LNA and 0.047% LA for the Alg10 diet, and 0.21% LNA and 0.07% LA for the Alg15 diet (Table 2). Therefore, amounts of n-3 and n-6 PUFA in algae-supplemented diets were significantly lower than in the positive SBO control diet. The SBO diet contained 2.9 times more LNA and 44 times more LA than the Alg10 diet, and 1.9 times more LNA and 32 times more LA than the Alg15 diet. Furthermore, the n-6/n-3 ratio varied significantly between the algal (0.36) and the SBO (5.55) diets.

Because of the high SFA content in coconut oil, algae supplemented diets contained more SFA than the SBO diet (Table 2). Alg10 and Alg15 diets contained four times more SFA than the SBO diet. Therefore, the PUFA/SFA ratio was significantly lower in the Alg10 (0.05) and Alg15 (0.07) diets compared to the SBO (2.62) diet. The main SFA in the Alg10 and Alg15 diets were lauric acid (12:0, ~1.84% of total diet), myristic acid (14:0, ~1.06%) and palmitic acid (16:0, ~1.08%).

Plasma Fatty Acids: Figure 1 shows a full rat plasma fatty acids profile. Plasma palmitate levels increased with palmitate dietary intake (r=0.60), reaching the highest level in the Alg15 group ($p<0.01$). Plasma LA increased with dietary LA intake (r=0.67), being highest in the SBO group ($p<0.001$), which correlates with the high amount of LA in this diet. In rats fed coconut oil deficient in LA, the plasma LA level was 36% ($p<0.0005$) of the SBO control level.

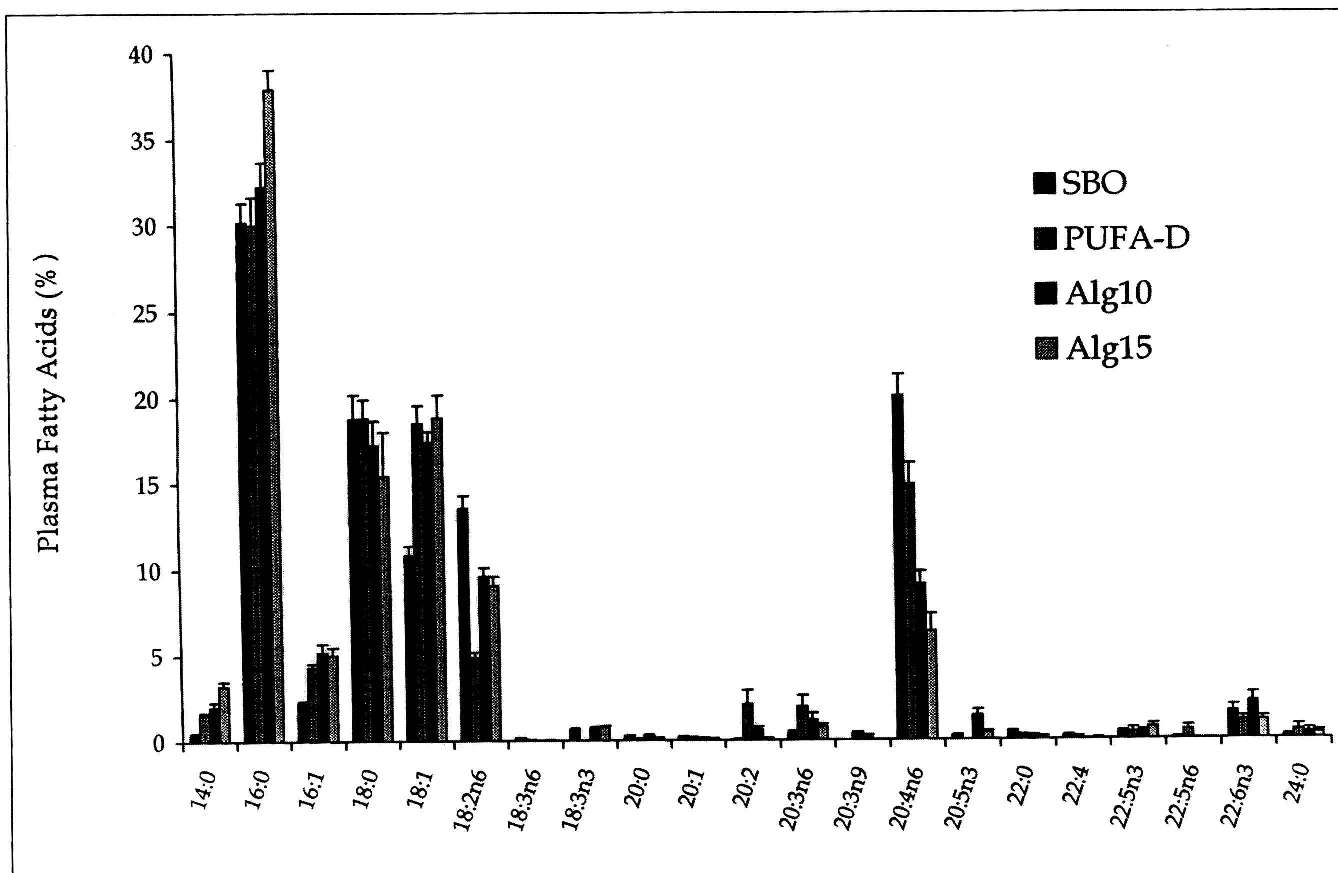

FIGURE 1. Plasma fatty acids profiles in animals fed different diets (Mean±SEM).
In PUFA-D animals diet supplementation with algae increased plasma oleic acid and LA levels but decreased AA level.

When the PUFA-D diet was supplemented with *Aph. flos-aquae,* plasma LA level was restored to 71% (Alg10) and 67% (Alg15) of the SBO level, in spite of the fact that algae supplemented diets contained less than 3% the amount of LA present in the control SBO diet.

Plasma arachidonic acid (AA, 20:4n-6) also decreased with increasing AA dietary intake (r=-0.88) and was highest in the plasma of the SBO group (p<0.001). However, the plasma AA level correlated positively with the dietary level of the AA precursor LA (r=0.64), which was the highest in the SBO diet. Rats fed the PUFA-D diet, which contains no LA, had a plasma AA level significantly lower than controls fed the SBO diet (p<0.01). However, supplementing PUFA-D diet with *Aph. flos-aquae* further decreased plasma AA levels in a dose-dependent manner, in spite of the low LA and AA content in Alg10 and Alg15 diets.

In order to better appreciate the variation in plasma PUFA levels, Figure 2 shows the plasma lipid profile for some PUFAs on a different scale than Figure 1. Rats fed the PUFA-D diet had no plasma LNA, which is consistent with the absence of LNA in coconut oil. However, algae supplementation of the PUFA-D diet restored plasma LNA to the SBO (control) level, in spite of the fact that the algal diets contained only 35% (Alg10) and 52% (Alg15) of the LNA present in the SBO diet.

Feeding rats the PUFA-D diet increased plasma dihomo-α-linolenic acid (DGLA, 20:3n-6) 5 times above the SBO control level (p< 0.05). Algae supplementation of PUFA-D diet decreased plasma DGLA level in a dose-dependent manner. Levels found in the algae-treated animals were still higher than SBO controls, though this difference was not statistically significant (p<0.09).

EPA was absent in the plasma of rats fed the PUFA-D diet. However, when the diet was supplemented with 10% and 15% algae, plasma EPA increased 6 times (p<0.005) and 1.7 times (p>0.1) above the SBO control level, respectively. The DHA (22:6n-3) concentration in the plasma of rats fed the PUFA-D diet was 35% lower than in controls, although this difference did not reach statistical significance. Supplementation with 10% algae increased the plasma DHA level by a factor of 2 (p<0.05), but supplementation with 15% algae did not affect the plasma DHA level. The effect of algae on EPA and DHA levels in rat blood plasma may not be dose-dependent.

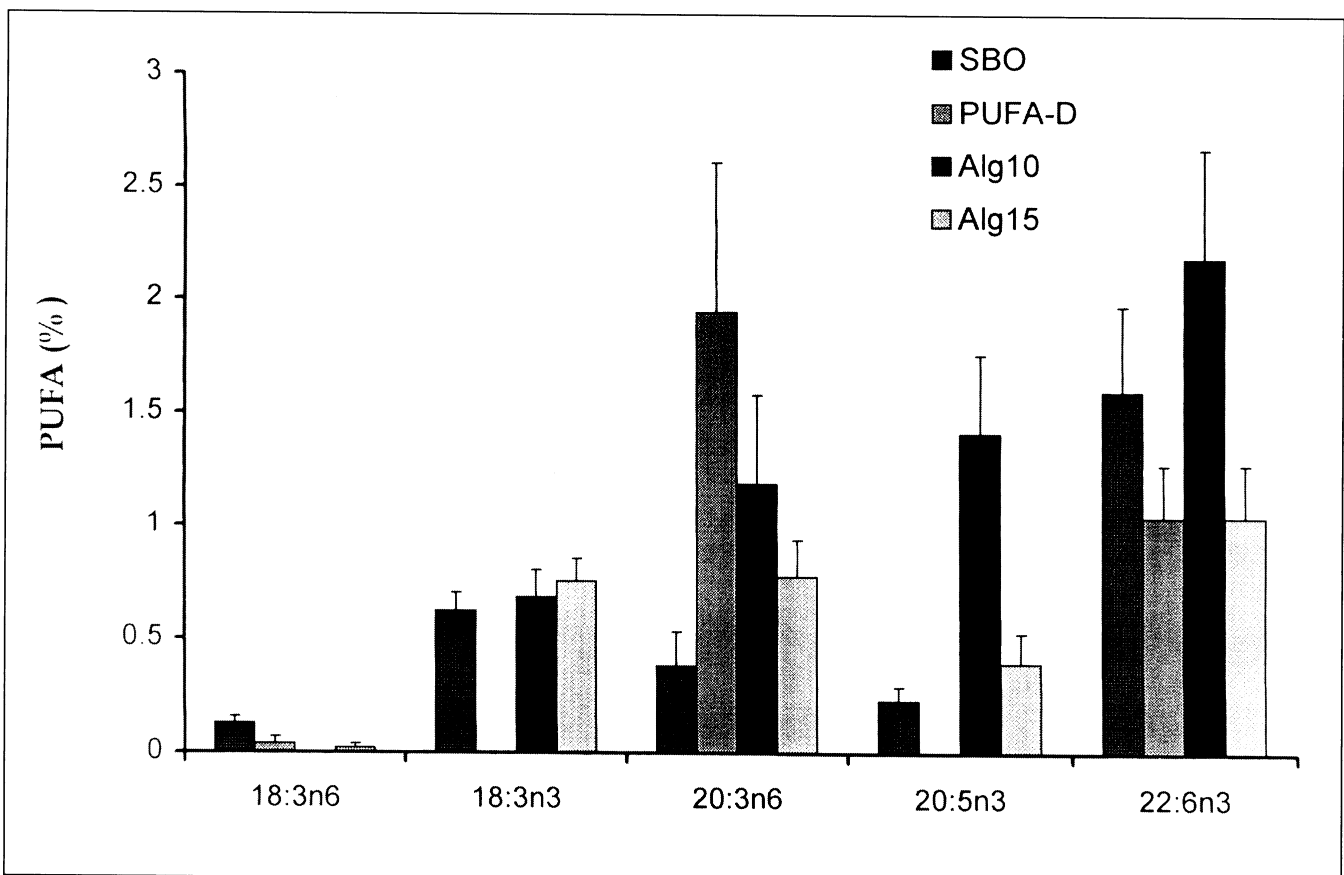

FIGURE 2. Specific PUFA profile in rats plasma (Mean±SEM).

Rats fed PUFA-D diet demonstrated an absence of LNA in plasma, however, animals fed Alg10 and Alg15 diets had the same level of LNA as controls, and increased EPA and DHA levels.

Thus, supplementation of the PUFA-D diet with algae normalizes fatty acid levels in plasma of PUFA deficient animals and makes their PUFA profile similar to controls.

Plasma Triglycerides and Cholesterol: Algae affected not only free fatty acids but also other lipids in the blood. The PUFA-D diet did not significantly decrease plasma triglycerides level relative to SBO controls (Figure 3). However, supplementation of the PUFA-D diet with 15% algae decreased plasma triglycerides to 24% of the SBO control level ($p<0.005$). The PUFA-D diet supplemented with 10% algae did not affect significantly the plasma triglycerides level. Levels of triglycerides in blood plasma positively correlated with PUFA/SFA ratio ($r=0.87$).

Cholesterol concentration in plasma was very sensitive to diet supplementation with algae (Figure 3). Rats fed the PUFA-D diet had a lower cholesterol level ($p<0.05$) than the SBO controls. The PUFA-D diet supplemented with algae caused a further dose-dependent decrease in the plasma cholesterol level. Supplementation with 10% and 15% algae decreased the plasma cholesterol level to 54% and 25% of the SBO control level ($p<0.0005$), respectively. Cholesterol and triglyceride levels were positively correlated ($r=0.91$).

Cholesterol levels also positively correlated to plasma PUFA/SFA ratio ($r=0.81$) and to plasma stearic acid ($r=0.86$). On the other hand, blood cholesterol was strongly negatively correlated with plasma myristic acid ($r = -0.99$). From a dietary standpoint, blood cholesterol was related only to dietary palmitic acid ($r = -0.95$).

DISCUSSION

The results reported here demonstrate that, in the rat model, *Aph. flos-aquae* appears to be a good source of PUFA. Calculations showed a good correlation between dietary and serum levels of LA. However, the correlation between dietary and serum LNA was poor. Rats fed the PUFA-deficient diet supplemented with *Aph. flos-aquae* had blood levels of LNA comparable to levels found in rats fed soybean oil diet containing nearly three times the amount of LNA. This suggests a higher bioavailability of LNA in *Aph. flos-aquae* compared to soybean oil. Furthermore, in spite of the fact that blood levels of LNA

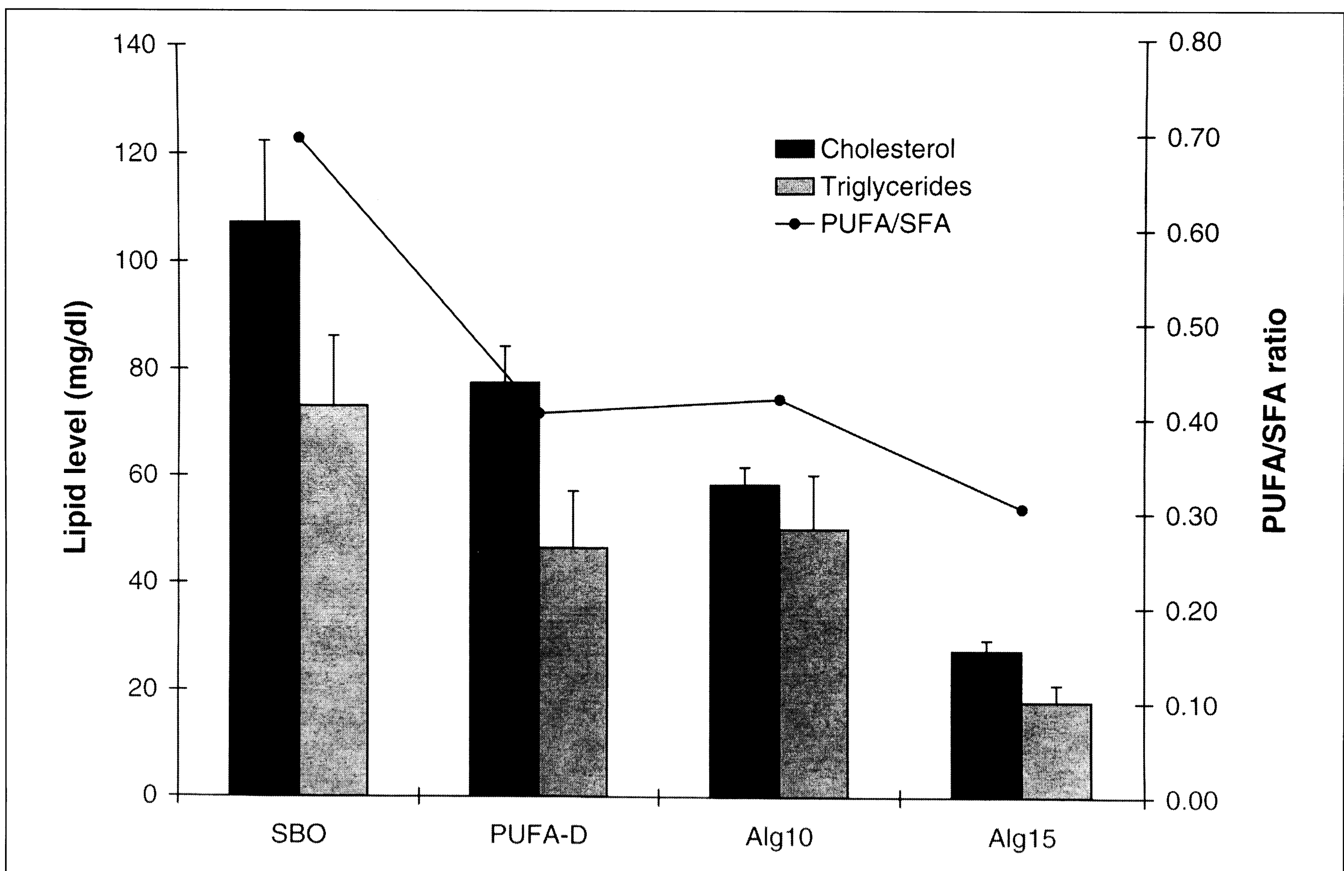

FIGURE 3. Lipid levels and PUFA/SFA ratio in rats fed different diets (Mean±SEM). * P<0.01.
PUFA-D diet supplemented with algae decreased triglycerides and cholesterol levels in rat blood plasma.

were similar in rats fed SBO and algae supplemented diets, there were significantly higher blood levels of EPA in the rats fed the *Aph. flos-aquae* diet. It has been previously suggested that increased dietary SFA increased the rate of conversion of LNA to EPA, whereas increased dietary n-6 PUFA decreased this conversion by 40-50%.[23] This dual effect could explain the fact that rats fed algae supplemented diets, which contained significantly more SFA, had higher blood levels of EPA than rats fed the SBO diet, which contained significantly more LA.

When the two main plasma n-6 PUFA (LA and AA) were analysed as profile, there was a very good positive correlation between LA dietary intake and the total level of n-6 PUFA. However, the n-6 PUFA profiles in rat plasma were different between the various groups. Supplementing diets with algae led to a dose-dependent decrease in plasma AA and concomitant accumalation of of LA. This could be due to *Aph.flos-aquae's* content of phycocyanin. Phycocyanin, the blue pigment in blue-green algae, was recently shown to have significant anti-inflammatory properties[24,25] which seemed to be mediated by an inhibition AA metabolism.[26] The presence of phycocyanin in the algae supplemented diets may have inhibited AA synthesis and consequently promoted the accumulation of LA.

This study suggests that *Aph. flos-aquae* has significant hypocholesterolemic properties when compared to soybean oil. Many studies have demonstrated the hypocholesterolemic properties of n-3 PUFAs[16,27,28] and the negative correlation between PUFA/SFA ratio and blood cholesterol levels.[29,30] In this study, cholesterol levels were positively correlated with the PUFA/SFA ratio. The main SFA present in the diet of the algae-treated groups were lauric, myristic and palmitic acids, which were all demonstrated to promote hypercholesterolemia to some degree.[31-33] This suggests that the hypocholesterolemic effect of *Aph. flos-aquae* is likely to be mediated by factors other than its fatty acid content. Specifically *Aph. flos-aquae* contains a significant amount of chlorophyll (1-2% dry weight) which was shown to stimulate liver function, and increase bile secretion[34]. A synthetic derivative of chlorophyll was shown to reduce blood cholesterol.[35] Therefore, it is possible that *Aph. flos-aquae* chlorophyll is responsible for the increased liver function and secretion of cholesterol into bile. *Spirulina*, another blue-green algae, was also shown to affect cholesterol metabolism by increasing HDL levels. [36] According to other sources[37], hypocholesterolemic effect of blue-green algae *(Nostoc commune)* is related to their fibers.

In conclusion, this study demonstrated that *Aph. flos-aquae* is a good source of PUFA with strong hypocholesterolemic properties. *Aph. flos-aquae's* ability to increase serum level of LNA, EPA, DHA, and lower level of AA in rats makes it a good candidate for future nutritional research in humans.

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