Fish Oil-Fed Mice Have Impaired Resistance to Influenza Infection\(^1,2\)

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Abstract

Dietary fish oils, rich in (n-3) PUFA, including eicosapentaenoic acid and docosahexaenoic acid, have been shown to have antiinflammatory properties. Although the antiinflammatory properties of fish oil may be beneficial during a chronic inflammatory illness, the same antiinflammatory properties can suppress the inflammatory responses necessary to combat acute viral infection. Given that (n-3) fatty acid-rich fish oil supplementation is on the rise and with the increasing threat of an influenza pandemic, we tested the effect of fish oil feeding for 2 wk on the immune response to influenza virus infection. Male C57BL/6 mice fed either a menhaden fish oil/corn oil diet (4 g fish oil:1 g corn oil, wt:wt at 5 g/100 g diet) or a control corn oil diet were infected with influenza A/PuertoRico/8/34 and analyzed for lung pathology and immune function. Although fish oil-fed mice had lower lung inflammation compared with controls, fish oil feeding also resulted in a 40% higher mortality rate, a 70% higher lung viral load at d 7 post infection, and a prolonged recovery period following infection. Although splenic natural killer (NK) cell activity was suppressed in fish oil-fed mice, lung NK activity was not affected. Additionally, lungs of infected fish oil-fed mice had significantly fewer CD8\(^+\) T cells and decreased mRNA expression of macrophage inflammatory protein-1-\(\alpha\), tumor necrosis factor-\(\alpha\), and interleukin-6. These results suggest that the antiinflammatory properties of fish oil feeding can alter the immune response to influenza infection, resulting in increased morbidity and mortality. J. Nutr. 139: 1588–1594, 2009.

Introduction

Dietary long-chain PUFA derived from fish oil have been shown to have beneficial effects on chronic inflammatory and autoimmune disorders (1,2) and long-chain PUFA such as eicosapentaenoic acid [20:5(n-3)] and docosahexaenoic acid [22:6(n-3)] appear to be most beneficial (3,4). A number of studies report that the immunosuppressive effects of PUFA are a result of decreased cytokine production and from reductions in T cell proliferation, activation, and signaling (5–7). Studies of rodents fed fish oil-enriched diets have shown a reduction in natural killer (NK)\(^6\) cell activity (8), decreased lymphocyte proliferation (9,10), and decreased antigen presentation functions (9,11–13). In addition, decreases in ex vivo production of tumor necrosis factor-\(\alpha\) (TNF\(_{\alpha}\)), interleukin (IL)-1, IL-2, IL-6, and interferon (IFN)-\(\gamma\) have also been reported (10,14–18). Whereass the antiinflammatory properties of PUFA may be beneficial for some chronic inflammatory illnesses, these same antiinflammatory properties may be detrimental for response to an infection when an intact immune system is needed to eradicate an invading pathogen. For example, diets supplemented with fish oils have been shown to lower host resistance to Mycobacterium tuberculosis (19), to reduce the survival of mice against infection with Listeria monocytogenes (20,21), and to decrease resistance of mice infected with Salmonella typhimurium (22). Similarly, fish oil feeding can diminish host defense against influenza virus due to delays in viral clearance (23).

Despite the availability of vaccines and antiviral agents, influenza virus continues to be a major cause of morbidity and mortality worldwide (24,25). Influenza virus infects cells of the respiratory system, resulting in an acute and diffuse inflammation of the bronchoalveolar tract (26). Following infection, a coordinated immune response consisting of both innate and adaptive mechanisms results in an accumulation of immune cells and secretion of immunomodulatory proteins designed to limit viral spread. In the mouse model of influenza virus infection, NK cells, neutrophils, and T lymphocytes increase in the lung postinfection (p.i.) and contribute to host protection (27). The secretion of both inflammatory and antiviral cytokines helps to eliminate the virus and reduce further spread. Whereas this inflammatory response is necessary for viral clearance, it also contributes to lung pathology (27). Although there are many studies documenting the antiinflammatory properties of fish oil, there are few studies that have examined the effects of fish oil on viral infection (28).
Because of the known health benefits, fish oil supplementation is on the rise. A survey conducted among health care professionals in the US reported an increase in fish oil supplementation from 24 to 30% in 2006 to 2007 (29). Additionally, in 2007, 37.4% of the U.S. population reported using fish oil or fish oil supplements for health reasons (30). With the anticipated increase of fish consumption and the increase in the usage of fish oil supplements, there is a growing concern that the beneficial antiinflammatory properties of (n-3) fatty acids may have adverse effects when inflammation is necessary to combat infection (1,28,31). This study was undertaken to investigate effects of fish oil feeding on the immune response to influenza virus infection in mice.

Materials and Methods

Animals and diet. Six-week-old male C57BL/6J mice were purchased from Jackson Laboratories. All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Care and Use Committee. All mice were housed under a 12-h-light/dark schedule with free access to food and water. Mice consumed ad libitum a control semipurified diet containing 5% corn oil (D07092502, Research Diets) or a fish oil-corn oil diet at physiological levels (1% corn oil + 4% fish oil, D07092503, Research Diets) for 2 wk (Table 1).

Virus and infection. The mouse-adapted strain of influenza A/Puerto Rico/8/34 (American Type Culture Collection) was propagated in the allantoic fluid of 10-d-old fertilized hen’s eggs and the viral titer was determined by hemagglutination assay (32). Following 2 wk of dietary treatment, mice were anesthetized with an intraperitoneal injection of a ketamine (0.6 mg/kg)/xylazine (0.35 mg/kg) solution and infected intranasally with 0.05 mL of 2 hemagglutinating units of A/Puerto Rico/8/34 virus diluted in PBS. Previous studies from our laboratory determined that this dose of virus is sufficient to effectively elicit an immune response with normal mortality in mice (32). Mice were weighed daily following infection and percent weight loss compared with starting weight at time of infection was calculated.

Pathology. The left lobe of the lung was removed at d 0 (uninfected mice) 3, 7, 10, 15, and 21 p.i. and perfused with 4% paraformaldehyde, paraffin embedded, cut in 6-μm sections, and stained with hematoxylin and eosin. Pathology grading was performed semiquantitatively according to the relative degree of inflammatory infiltration as previously described (33).

Quantification of lung virus titer. As previously described, lung viral titers were determined by a modified tissue culture infectious dose 50 (TCID₀ᵉ₅) using hemagglutination as an endpoint (32). Briefly, supernates from lung homogenates were serially diluted and used to infect Madin–Darby canine kidney cells. Virus titers were determined based on the presence or absence of hemagglutination of human O RBC and TCID₀ᵉ₅ was determined by the method of Reed and Muench (34).

Enumeration of NK cell populations. Lungs and spleens were removed at d 0 and 3 p.i. Lungs were incubated in a collagenase solution (1500 units/lung) for 1 h. Lung and spleen were processed into single-cell suspensions using a stomacher (Seward) and strained through a 40-μm nylon filter. Cell numbers between different samples were equalized to $5 \times 10^6$ cells/sample and stained with fluorescein anti-DX5 isothiocyanate (FITC) and anti-CD3 phycoerythrin (PE) (BD Pharmingen). Fluorescence was measured using a FACS Calibur flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser and a 647-nm diode laser. The lymphocyte population was gated and NK cells were identified as CD3⁻DX5⁺ within the gate.

Quantification of immune cells. To obtain bronchoalveolar lavage fluid whole lungs were lavaged 3 times with PBS at d 3, 7, 10, and 15 p.i. RBC were lysed using ACK lysis buffer (0.15 mol/L NH₄Cl, 1 mmol/L KHCO₃, 0.1 mmol/L Na₂EDTA in double-distilled H₂O, pH 7.4). Samples were washed twice in PBS/2% bovine serum and stained with fluorescent antibody for 30 min on ice, followed by 3 additional washes in PBS/2% bovine serum. At least $5 \times 10^5$ cells were stained with the following anti-mouse monoclonal antibodies: FITC anti-CD3, APC-anti-CD4 and peridinin-chlorophyll-protein anti-CD8, PE anti-CD11b and FITC anti-GR-1 (BD Biosciences). Cell populations were analyzed using a FACS Calibur flow cytometer (Becton Dickinson). T cells were identified using the follow antibodies: FITC-anti-CD3, APC-anti-CD4, and peridinin-chlorophyll-protein anti-CD8 and neutrophils: PE anti-CD11b and FITC anti-GR-1 (35).

Quantiﬁcation of lung mRNA cytokine levels. The right lobe of the lung was removed at d 0 (uninfected mice) 3, 7, 10, and 15 p.i. Total RNA was isolated using the TRizol method and RT was conducted with a Superscript II First Strand Synthesis kit (Invitrogen). Following previously described methods (32), mRNA levels were measured for TNFa, IL-2, IL-6, IL-12, IFNα and IFNβ, regulated upon activation, normal T cell expressed and secreted, macrophage inflammatory protein-1-α (MIP-1α), and glyceraldehyde-3-phosphate dehydrogenase using quantitative real-time PCR. All data were expressed as fold-change from uninfected mice fed the same diet. Control and fish oil-fed mice did not differ in glyceraldehyde-3-phosphate dehydrogenase levels at any time point. All data were expressed as fold-change from uninfected mice fed the same diet.

Statistical analysis. Statistical analyses were performed using JMP 7 Statistical software and SAS 9.1.2 software. Normally distributed data were analyzed by 2-way ANOVA with diet and day p.i. as main effects. Student’s t test was used for post hoc comparison between the dietary groups and Tukey’s honestly significant differences was used for post hoc comparisons among the days p.i. Nonparametric data were analyzed using the Kruskal Wallis test. Counted data were analyzed using logistic regression analysis ($P < 0.05$) when Wald CI for odds ratios did not

Table 1. Composition of experimental diets

<table>
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<tr>
<th>Ingredient</th>
<th>Control1</th>
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</tr>
<tr>
<td>d,l-α-Tocopheryl acetate</td>
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1 Formulated and supplied from Research Diets, Inc. AIN-78A rodent diet with modifications. Control corn oil diet no. D07092502, fish oil diet no. D07092503 containing menhaden oil with 2.1% arachidonic acid, 14.2% eicosapentaenoic acid, and 12.2% docosahexaenoic acid.

2 Vitamin E additionally added to the fish oil diet.
include 1. Survival data were analyzed using the Kaplan-Meier survival estimates. Significance that compares the survival curve estimates of the 2 diet groups was analyzed by the log rank test. Differences were considered significant at \( P < 0.05 \).

**Results**

**Fish oil-fed mice recover more slowly from influenza virus infection.** In mice, weight loss is a marker for illness severity following influenza infection and subsequent weight gain is an indicator for recovery. Both control and fish oil-fed mice lost considerable amounts of weight during the course of infection. Control mice began to regain weight after d 10 p.i.; however, fish oil-fed mice did not begin to regain weight until d 14 p.i. Between d 11 and 18 p.i., the percent body weight change remained significantly lower in the fish oil-fed mice compared with the control mice. By d 20 p.i., the body weights did not differ between the groups (Fig. 1).

**Fish oil feeding results in a higher mortality rate following influenza infection.** Increased severity of infection in the fish oil-fed mice was also reflected in the mortality rates. Beginning at d 11 p.i., fish oil-fed mice had a significantly higher mortality rate compared with control mice. At d 20 p.i., the fish oil-fed mice had a 51% mortality rate compared with a 10% mortality rate in control mice (Fig. 2).

**Fish oil feeding improves lung pathology post-influenza virus infection.** Influenza virus infection results in an infiltration of inflammatory cells in the lung. Lung pathology was examined at various times p.i. Lung pathology was significantly reduced in influenza-infected fish oil-fed mice at d 7, 10, 15, and 21 p.i. compared with infected controls (Fig. 3).

**Fish oil feeding increases virus load in the lung p.i.** Because the fish oil-fed mice had reduced inflammation in the lungs and the inflammatory response is necessary for viral control, lung viral titers were measured in the mice. On d 7 p.i., fish oil-fed mice had a viral load that was 7.1-fold higher than that of controls (Fig. 4).

**Fish oil feeding affects NK cell cellularity and cytotoxicity.** NK cells represent the first line of defense post-influenza infection (36). Previous studies have demonstrated reduced splenic NK activity with fish oil feeding (8). At d 3 p.i., the splenic NK population was significantly reduced in the fish oil-fed mice compared with controls (Fig. 5A) and NK (Fig. 5B). In

**FIGURE 1** Percent body weight change in mice fed control or fish oil diets following influenza infection. Values are means ± SEM, \( n = 61 \) (control) or \( n = 71 \) (fish oil). *Groups differ at each time, \( P < 0.05 \).

**FIGURE 2** Percent survival in mice fed control or fish oil diets following influenza infection. Control, \( n = 61 \) (d 0); fish oil, \( n = 71 \) (d 0). Log rank, \( P < 0.001 \).

the lungs at d 3 p.i., the fish oil-fed mice had less than one-half the total number of NK cells compared with control mice (Fig. 5C); however, lung NK activity did not differ between groups (Fig. 5D).

**Fish oil feeding reduces neutrophils in lung of infected mice.** Recruitment of neutrophils to the site of infection is an essential early component of the immune response to influenza infection (37). In the lungs of fish oil-fed uninfected mice (d 0), the total number of neutrophils was 71% lower than in control mice (Fig. 6). Following infection, neutrophil numbers in the lung increased in both groups; however, the total neutrophil number remained significantly lower in the fish oil-fed mice.

**Fish oil feeding reduces CD8+ T cells in the infected lung.** To determine whether fish oil feeding affects T cell numbers in the lung, total numbers of T lymphocytes (CD3+) as well as CD4+ and CD8+ populations were identified in bronchoalveolar lavage fluid following influenza challenge. Although the CD3+ T cell number peaked at d 10 p.i. in both diet groups, fish oil-fed mice had significantly fewer CD3+ T cells at d 7 and 10 p.i. (Fig. 7A). This reduction in CD3+ T lymphocytes was due to fewer CD8+ T cells (Fig. 7B), as the number of CD4+ T cells did not differ between groups at any time point (data not shown). By d 15 p.i., CD3+ lymphocyte numbers did not differ between groups.

**FIGURE 3** Degree of lung pathology in mice fed control or fish oil diets following influenza infection. Values are means ± SEM, \( n = 3-6 \) per time point except d 10, \( n = 11 \) (control and fish oil). *Different from control, \( P < 0.05 \). Pathology score was as follows: 0, no inflammation; 1+, mild influx of inflammatory cells with cuffing around vessels; 2+, increased inflammation with ~25–50% of the total lung involved; 3+, severe inflammation involving 50–75% of the lung; and 4+, almost all lung tissue contained inflammatory infiltrates.
Fish oil feeding decreases cytokine and chemokine mRNA expression in lungs of influenza-infected mice. A coordinated production of cytokines and chemokines occurs in the lungs during an influenza virus infection (27). To determine whether fish oil feeding could influence production of these inflammatory mediators, mRNA levels were measured for various cytokines and chemokines. In infected mice, fish oil feeding resulted in significantly lower mRNA expression of MIP-1α, TNFα, and IL-6 at d 7 p.i. compared with control mice (Fig. 8). Levels of mRNA for IL-2, IL-12, IFNα, IFNβ, and regulated upon activation, normal T-cell expressed and secreted did not differ between infected diet groups at any time p.i. (data not presented).

**Discussion**

The favorable effects of dietary fish oils stem from the potential of (n-3) fatty acids to reduce excessive inflammation (3,38). Studies have shown beneficial antiinflammatory properties of dietary fish oils on chronic diseases such as rheumatoid and osteoarthritis, inflammatory bowel disease, cardiovascular disease, type 2 diabetes, and Alzheimer’s disease (39). However, suppression of the immune system may be deleterious when inflammation is required to eliminate the invading pathogen, such as during influenza virus infection (28). Following infection with influenza, a controlled and coordinated immune response is essential for resolving the infection. The availability of (n-3) fatty acid-rich fish oil supplements and the increasing inclusion of (n-3) fatty acids in prepared foods (40) add to the importance of understanding how the immunosuppressive properties of fish oils may impact the host’s ability to respond to influenza virus infection.

Mice fed a fish oil-rich diet had decreased lung pathology following influenza infection compared with controls; however, the fish oil-fed mice also had a prolonged recovery time, significantly increased viral titer and importantly, a significantly higher mortality rate. Thus, in the case of influenza virus infection, reduced lung inflammation was associated with a poor outcome p.i. To understand the direct effect of fish oil feeding on the immune response to influenza virus infection, numbers and phenotypes of cells infiltrating the lung tissue were measured. At d 3 p.i., fish oil-fed mice had fewer numbers of NK cells infiltrating their lungs compared with control mice. NK cells provide an early defense against influenza virus infection and are important in reducing viral load prior to activation of the adaptive immune response (36). NK cell trafficking to the site of infection is dependent on a variety of inflammatory mediators, including the expression of a cytokine/chemokine gradient, upregulation of adhesion molecules, and activation of G-protein–coupled receptors (41–43). Fatty acids of the (n-3) group have been shown to decrease surface expression of vascular adhesion molecules and to alter G-protein–coupled membrane receptors (38,44,45), suggesting that fish oil feeding may have interfered with the signaling required for NK cell trafficking into the lungs. However, other possibilities for reduced NK in the lung include a reduction in general numbers, resulting in fewer NK cells trafficking to the lung.

Although the NK cell number was reduced in both the spleen and lungs of fish oil-fed mice, impairment in NK activity was found only in splenic NK cells. The differences between spleen and lung NK activity may be due to exposure to cytokines. There are a number of cytokines that enhance NK cytotoxicity, including IL-12, IL-18, and IFNα and β (36,42); therefore, NK activity in the lungs of fish oil-fed mice may have increased due to exposure to the cytokine milieu at the site of infection. During influenza virus infection, production of inflammatory cytokines occurs at the site of infection and therefore the spleen is not exposed to this localized inflammatory response. Indeed, Yaqoob et al. (8) demonstrated enhanced NK activity in PUFA-treated NK cells following exposure to IFNγ.

Fish oil feeding also affected numbers of neutrophils in the lung following infection. During early virus-induced inflammatory responses, neutrophils rapidly traffic into infected airways, where they play a critical role in limiting virus replication and activating innate immunity (37,46). Neutrophil migration is

**FIGURE 5** Effect of influenza infection and fish oil feeding on spleen NK cell number (A), spleen NK cell cytotoxicity (B), lung NK cell number (C), and lung NK cell cytotoxicity (D) in mice fed control or fish oil diets on d 3 after infection with influenza. Values are means ± SEM, n = 6.

*Different from control, P < 0.05.
that fish oil-fed mice lacked MIP-1α and TNFα compared with infected wild type mice (49). In our study, we showed reduced lung inflammation and delayed viral clearance by neutrophils. Moreover, influenza-infected MIP-1α knockout mice exhibited reduced lung inflammation and delayed viral clearance compared with infected wild type mice (49). In our study, we showed that fish oil-fed mice lacked MIP-1α and TNFα mRNA induction. Together, these data suggest that the failure to upregulate MIP-1α and TNFα mRNA in fish oil-fed mice may have resulted in reduced neutrophil trafficking to the lungs following influenza infection.

On d 7 and 10 p.i., CD8+ T cells were lower than in controls in the lung of influenza-infected fish oil-fed mice. Influenza-specific CD8+ T cells kill infected target cells by direct lysis and also play an essential role in influenza virus clearance and controlling morbidity (50–52). In mice that lack CD8+ T cells, infection with influenza A/PR/8/34 led to increased viral replication and mortality (53). CD4+ T cells, on the other hand, help to resolve inflammation during influenza infection; however, they are not essential for viral clearance (54,55). Our data suggest that reduction in CD8+ T cell numbers p.i. coupled with reduced numbers of neutrophils likely contributed to the increase in lung virus titer in the fish oil-fed mice.

The recruitment of T cells is dependent on cytokine-induced expression of adhesion molecules. For example, TNFα and IL-1β stimulate endothelial cells to increase expression of adhesion molecules, selectins, and integrins (56,57). As expected with an influenza virus infection, cytokine mRNA expression of TNFα peaked at d 7 in control mice; however, this induction did not occur in fish oil-fed mice, suggesting that a lack of TNFα may have contributed to the decrease in CD8+ T cell trafficking to the lung. Alternatives to trafficking include increased apoptosis and/or failure to proliferate in response to antigen stimuli (58,59). However, we examined lung tissue for increased apoptosis by TUNEL staining and found no differences in apoptosis between diet groups (data not shown).

The number of CD4+ T cells in the lung, however, was not affected in fish oil-fed influenza infected mice. Fish oil feeding may have altered pathways required for CD8+ T cell and not CD4+ T cell trafficking. For example, although both CD4+ and CD8+ T cells express CXCR3, administration of anti-CXCR3 antibody reduced CD4+ T cell infiltrate in the brain, whereas CD8+ trafficking was not affected (60). Similarly, CCR5 is also expressed on both CD4+ and CD8+ T cells, although only CD8+ T cell trafficking was affected by lack of CCR5 expression in a mouse model of hepatitis virus (61). Interestingly, mRNA for MIP-1α, the ligand for CCR5, was undetected in the fish oil-fed mice. Our results suggest that chemokines and perhaps their receptor expression may play a key role in the immunomodulatory effects of fish oil during influenza infection.

TNFα is produced by infected lung epithelial cells, activated macrophages, dendritic cells, neutrophils, T cells (CD8+ and CD4+), and NK cells (26,62). During viral infection, TNFα exerts antiviral activity (63), enhances the recruitment of leukocytes to the site of infection, and activates innate immune responses (64,65). IL-6 induction has pleiotropic effects, including the activation of NK cells and macrophages and stimulation of T cell differentiation during influenza infection (64,66). The potential for (n-3) fatty acids to reduce proinflammatory cytokines has been shown previously (67). For example, studies of fish oil-fed mice have demonstrated that injection with lipopolysaccharide decreased the ex vivo production of TNFα, IL-1β, and IL-6 by peritoneal macrophages and decreased TNFα, IL-1β, and IL-6 concentrations in circulation (14,17,68).

Together, these data suggest that the antiinflammatory properties of fish oil that resulted in reduced neutrophils, NK cells, and CD8+ T cells in the lung and decreased expression of mRNA for proinflammatory cytokines likely led to the increased viral titer and subsequent higher mortality rate in the fish oil-fed mice. However, the viral titers began to decrease in both fish oil-fed and control groups at a time when the former were beginning to die. This may be a case of survivor bias and those mice that ultimately died may have had a higher viral titer. We also investigated other possibilities for the high mortality rate of fish oil-fed mice, including impaired liver and/or kidney function, spread of virus to the brain, and increased lung cell apoptosis.
All of these possibilities were negative (data not shown). In the future, other possibilities to explain the increased death rate of fish oil-fed mice will include an investigation to determine whether lung tissue repair mechanisms are impaired (69) or if fish oil-fed influenza-infected mice have increased and/or longer fevers potentially leading to brain damage and ultimately death (70,71).

Further mechanistic studies are needed to determine how PUFA can influence the immune response to influenza infection. For example, studies have shown that lipid raft disruptions by (n-3) fatty acids can affect signaling pathways in T lymphocytes and disrupt immunological synapse formation needed to activate T cells (6,72). However, potential adverse effects of these alterations in vivo during an influenza virus infection have not been investigated.

In this study, we utilized both a physiologically relevant concentration of dietary fish oil supplementation and a natural route of administration of a viral pathogen. Results from our study suggest that fish oil consumption has the potential to increase the severity of an influenza virus infection and perhaps other viral illnesses as well.

Acknowledgment

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Literature Cited


