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EFFECTS OF DOCOSAHEXAENOIC ACID CONSUMPTION ON ENTERIC REOVIRUS INFECTION: IMMUNOGLOBULIN RESPONSES AND VIRUS CLEARANCE

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in FOOD SCIENCE

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5-1-2008
Date

EFFECTS OF DOCOSAHEXAENOIC ACID CONSUMPTION ON ENTERIC REOVIRUS INFECTION: IMMUNOGLOBULIN RESPONSES AND VIRUS CLEARANCE

By

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A THESIS

submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science

2008

ABSTRACT

EFFECTS OF DOCOSAHEXAENOIC ACID CONSUMPTION ON ENTERIC REOVIRUS INFECTION: IMMUNOGLOBULIN RESPONSES AND VIRUS CLEARANCE

By

Eleni Beli

The possibility that dietary factors can modulate the immune system is of great interest to health professionals and the public. The n-3 polyunsaturated fatty acids, PUFA, found in fish oil, have well recognized anti-inflammatory effects. However, there is concern that these might impair resistance to infectious microbes. Herein, we tested the hypothesis that consumption of the n-3 PUFA, docosahexaenoic acid, DHA, interferes with the *in vivo* immune response to enteric reovirus infection. Mice fed DHA-enriched diets for 4 wks had increased incorporation of DHA and other n-3 PUFA in their membrane phospholipids at the expense of arachidonic acid. Mice fed with DHA-enriched and high-oleic safflower diets were orally infected with reovirus to access antibody production and viral clearance. DHA consumption only transiently interfered with reovirus clearance, but did not affect the humoral responses in the gastrointestinal and systemic compartments or the overall resolution of the intestinal infection. Accordingly, these data are not indicative of a profound impairment of the immune response to reovirus infection.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr Pestka for all his guidance, patience and support for the completion of this project. Also I would like to give special thanks to my committee members, Dr Amalfitano and Dr Hord for the useful comments, specifications and suggestions on the project. Last but not least, I cannot ignore the complete support from Dr Maoxing Li, an experienced virologist researcher in our lab and the technical and moral support from my friend and lab mate Chidozie Amuzie.

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CHAPTER 1

INTRODUCTION

The possibility that food constituents can modify the immune system and attenuate disease is of great interest. Among the nutrients that have become popular as supplements are the n-3 polyunsaturated fatty acids (PUFA) that are found in high concentrations in fish oil. Millions of people in United States take n-3 PUFA supplements in the form of commercially available fish oil capsules and other fortified products.

Nutritionists have long endorsed fish as part of a heart-healthy diet, and the Food and Drug Administration (FDA) in 2004 gave "qualified health claim" status to the n-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) especially against coronary heart diseases (1). Besides heart-related diseases (2-4), there is cumulative experimental and clinical evidence that other inflammatory diseases are benefited by n-3 PUFA consumption. These diseases include rheumatoid arthritis (5), inflammatory bowel disease (IBD) and colitis (6, 7). Additionally, n-3 PUFA are claimed to be a promising preventive therapy for the progression of renal diseases (8-15). There is also some indication that n-3 PUFA contribute to cancer prevention (16). Moreover, studies have shown that DHA promotes the normal brain development of the fetus and the newborn (17-19). The role of n-3 PUFA on neurological and psychological diseases has also been another recent research focus (20-23).

Studies in our laboratory have used an experimental mouse model of human IgA nephropathy (IgAN) to examine the mechanisms of the beneficial effects of n-3 PUFA in

retarding renal disease progression. In this model, a mycotoxin, deoxynivalenol (DON), specifically modulates mucosal immune responses of the mouse, targeting the development, differentiation, and homing of IgA-producing plasma cells in mucosal sites (24). DON dramatically increases IgA in the serum and IgA deposition in kidney. Overall, DON induces in the mouse clinical signs that resemble human IgA nephropathy (25). Dietary supplementation with fish oil retards the progression of DON-induced IgAN- like disease in the mouse by reducing IgA immune complexes in blood and mesangial IgA deposition in kidney (14). The mechanisms by which n-3 PUFA mediate these effects are likely to involve altered balance of n-6 and n-3 derived eicosanoids and reduced inflammatory responses. Among the inflammatory responses generated with DON, interleukin 6, (IL-6), a critical cytokine in promoting differentiation and proliferation of plasma cell precursors, is reduced significantly in n-3 PUFA-fed mice (26). However, it is not known whether fish oil reduces IgA production during normal responses to mucosal pathogens.

Interest in the health benefits of fish oil began with epidemiological studies on Greenland Eskimos (27). It was found that consumption of fats high in n-3 PUFA correlated with low incidence of cardiovascular and inflammatory/autoimmune diseases among the indigenous population. On the other hand, additional studies indicated a higher than normal incidence of tuberculosis infection among this population (28). Many people believed that Eskimo nutritional habits could have reduced their resistance to infections. However, a link between the Eskimo diet and high tuberculosis rates has never been proven epidemiologically, rather, the poor and crowded living conditions among the endogenous population was considered main factor for the prevalence of the epidemic

(29). Human studies addressing exposure to n-3 PUFA and infection did not give clear evidence that a diet rich in n-3 PUFA increases susceptibility to infections (30). Nevertheless, these concerns raised questions about the net health benefits of n-3 PUFA in the view of potentially reduced resistance to infections and a number of animal studies with fish oil gave evidence for possible immunosuppressive effects (31).

Several studies measuring specific cell functions *ex vivo* have shown immunomodulatory effects of n-3 PUFA. However, it is not clear whether modifying the immune function with n-3 PUFA supplementation would have any effect on host resistance to infections. Furthermore, it is not yet determined whether the incidence and severity of infections is increased in populations that are exposed to rich in n-3 PUFA diets during naturally occurring episodes. Thus, measuring the *in vivo* immune responses to an infectious challenge is a reliable method to address potential modulation of the immune system with nutritional interventions. Since, host resistance to infectious diseases is the cumulative result of cellular incorporation, production and activation of various molecules, whole animal infectious models can give strong evidence on the effect of dietary supplementations in infections. Typical endpoints used in similar studies are, general well-being, duration and magnitude of symptoms, clearance of the infectious agent, assessment of antigen-specific antibodies, and markers of innate immunity and anti-inflammatory mediators (32).

To evaluate immunosuppressive effects of fish oil and potential interference with the clearance of infectious microorganisms, numerous mouse models have been used involving both bacterial and viral infectious agents (31). The type of n-3 PUFA, the dose and time period of supplementation, the nature of the pathogen, the route of

administration, and genetic variation of the host can affect determining whether n-3 PUFA impair, enhance, or have no effect on host resistance. To our knowledge, there are no n-3 PUFA studies that address these questions using enteric viral infection as a model for common gastroenteritis (33).

Enteric infection with respiratory enteric orphan virus (reovirus) that is a well-characterized viral host resistance model, has been used with success to evaluate intestinal immunotoxicity (34-37). Reovirus belongs to the *Reoviridae* family, which is the largest of the six families of viruses including some human pathogens that cause common enteric diseases. Enteric reovirus infection has recently been used to study the antiviral effects of 3,3-diindolylmethane, a derivative from indole-3 carbinol found in *Brassica* vegetables that is considered therapeutic for numerous forms of cancer (38). The same model was employed in this study to assess possible suppression of the normal activation of the gut immune system by long-term supplementation with n-3 PUFA.

The objective of this study was to test potential immunosuppressive effects of n-3 PUFA in a mouse model that mimics human gastrointestinal viral infections (39). We had two hypotheses: (1) n-3 PUFA consumption would increase incorporation of these fatty acids into major immune cell populations involved in mucosal and systemic immunity; and (2) n-3 PUFA consumption will result to reduced immunoglobulin responses to reovirus infection and will interfere with viral clearance.

CHAPTER 2

LITERATURE REVIEW

2.1 EFFECTS OF N-3 PUFA ON HOST RESISTANCE

Host resistance models represent the most reliable approach to study the influence of different agents, (toxicants or nutrients) on the functional integrity of the immune system and its ability to eliminate invading pathogens (40). An intact immune system integrates complex signals at the local and systemic level with innate and adaptive mechanisms that initiate both humoral and cell-mediated mechanisms. A complete, functional immune system eliminates most pathogens, while an immunocompromised one encounters difficulties (40). In all these models, a comparison between groups that receive a treatment (such as a drug, a nutrient, or a toxicant) and a placebo is made to estimate the ability of the subjects to deal with the infectious agent. Whether the treatment enhances or suppresses an immune response can be determined by accessing several functions of the immunocompetent cell populations, and the overall clearance of the pathogen from the host. Finding a significant difference in one of the immunological parameters between treatment and control groups will not always yield a definite answer about potential immune stimulation or suppression. This is because it is not always known what percent change in each of the immunological parameters correlates with increased susceptibility and what alternative compensatory mechanisms might be evoked (40). However, clearance of the infectious agent is the outcome of an integrated immune response and, therefore, a strong indicator of possible immune modulation.

Most host resistance models used to test possible adverse immunomodulatory properties of n-3 PUFA, to date, have employed bacteria as the infectious agents (31). Although influenza, cytomegalovirus and murine retrovirus have also been used, there are no studies to our knowledge addressing susceptibility to enteric viruses (31). Relative to foodborne diseases, enteric viruses are among the leader causes of gastroenteritis cases correlated with consumption of contaminated fresh produce or water. Enteric infections induce complex immune responses in the gut, including both non-specific innate responses and adaptive immunity mediated by cytotoxic T-cells and B-cells. Since the majority of diarrheal diseases are attributed to enteric viral infections, a model studying susceptibility towards an enteric viral infection is of great importance.

Effects of n-3 PUFA on the immune responses

There is an extensive literature about the effects of n-3 PUFA on different immunological parameters that ostensible serve as indicators for immune modulation. Since adaptive immunity is absolutely necessary for clearance of most pathogens, the effect of n-3 PUFA on antibody production and cytotoxic T-cell expansion is of interest. In the mucosal compartment, secretion of mucosal IgA is very important protective mechanism and therefore could determine susceptibility of the host. n-3 PUFA down-regulate DON-induced IgA production in serum of the mouse (13, 14), indicating that their consumption might also affect regulation of IgA production in the mucosa. In an influenza host resistance model, it was shown that virus-specific serum IgG and lung IgA production is significantly lower by 7 d post infection (PI) in fish oil-fed mice compared to control-fed mice (41). However, human patients suffering from distal proctocolitis,

administered with n-3 PUFA had the same percentage of IgA and IgG containing cells in the rectal mucosa but significantly reduced IgM cells (7). Finally, significant positive correlations were found between fish oil supplementation and the concentration of IgA in breast milk (42).

Production of antibodies and cytotoxic responses is regulated by T lymphocytes and therefore, the effect of fish oil on this cell population has been studied extensively *in vitro* and *in vivo* (13, 43-48). Some studies report that administration of n-3 PUFA reduces T-cell proliferation and proliferation *ex vivo* in an agonist specific fashion (49-51). Other studies report no effect of n-3 PUFA on antigen-specific CD4+ and CD8+ T lymphocyte proliferation *in vivo* (45, 52). When Byleveld et al, (2000) examined the effect of fish oil on the proliferative and cytotoxic capacities of viral-specific T-cells, they found that spleen T-cell proliferation *ex vivo* was higher, whereas cytotoxicity of lung virus-specific T-cells was significantly lower with fish oil supplementation (46).

Lymphocyte proliferation takes place after antigen-priming of antigen presenting cells (APC). It has been suggested that fish oil might affect antigen presentation process (53-55). APC express major histocompatibility complexes (MHC) on their surfaces that display both self and non-self antigens. When non-self antigens are presented, an immune response is mounted. The nature of the peptide-MHC complex determines which set of lymphocytes will be activated with MHC class I activating CD8+ T cytotoxic cells and MHC class II activating CD4+ T helper cells. *In vitro* studies showed that treatment with n-3 PUFA reduced the expression of MHC-II on IFN-γ activated monocytes and their ability to present antigens to autologous lymphocytes (53, 54). Similarly, treatment *in vitro* with PUFA resulted in B lymphoblasts with lowered susceptibility to lysis by

cytotoxic $CD8^+$ T-cells, suggesting an effect of PUFA on the HLA class I pathway, as the human MHC is named (55). n-3 PUFA have also been shown to interfere with LPS-induced dendritic cell maturation evidenced by the expression of cell surface markers, critical intracellular signaling pathways and cytokine production (56). Thus, n-3 PUFA supplementation might reduce MHC molecule expression and subsequent antigen presentation that drives the adaptive T-cell responses.

The most desirable outcome of the immune responses generated during infection is clearance of the infectious agent. While several n-3 PUFA studies have shown a delay in pathogen clearance (41, 52, 57), the specific part of the immune system that is targeted by n-3 PUFA is unknown. Apart from the adaptive responses the role of innate immune system in mounting a strong immune response is important. EPA, more than DHA, has been shown to reduce innate cellular responses as evidenced by decreased natural killer activity against target cells (58). EPA and DHA were shown to inhibit pathogen killing by macrophages, which are also important in innate immunity (59). One suggested mechanism is that n-3 PUFA interfere with actin nucleation in phagosomes and subsequent with phagocytosis and fusion of the phagosomes with late endocytic organelles, an essential step to kill pathogens. Thus, n-3 PUFA supplementation affects not only adaptive but also innate responses. The magnitude of their impact to immune responses is determined by the specific pathogen encountered, since different pathogens provoke different immune responses.

There also has been extensive research on the effects of n-3 PUFA on the production of cytokines, which are central mediators of immune responses. n-3 PUFA consumption was shown to reduce expression of Th1 cytokines, such as IL-1, IL-2, TNF-

α, and IFN-γ ex vivo (41, 44, 60-62). Mice fed with fish oil and infected with influenza virus, have impaired IFN-γ production, whereas the IFNα/β response remains unchanged (41). Mice fed with fish oil and infected with murine retrovirus, produce less TNF-α, IL-1β, IL-2 and IFN-γ in spleen cultures (63). Reduced IFN-γ production is also observed in studies with mice fed fish oil and infected with *Listeria monocytogenes* (64, 65), sometimes even prior to infection (65). However, DHA has also been shown to impact Th-2 cytokines, like IL-10. A study with piglet neonates immunized with an inactivated influenza vaccine and fed a PUFA-enriched formula, showed significant up-regulation of IL-10 in peripheral blood monocytes accompanied by reduced *ex vivo* virus specific CD4+ and CD8+ T-cells responses (66).

Mechanisms of n-3 PUFA immunomodulatory effects

The most widely accepted mechanism of n-3 PUFA anti-inflammatory properties is their ability to change the lipid mediators of inflammation. Supplementation with n-3 PUFA changes the fatty acid profile of cell membranes and therefore products that are generated by their oxidation. Upon stimulation, PUFA are released from cell membranes and they are converted enzymatically to eicosanoids. Eicosanoids are the most important class of lipid mediators of inflammation and are mainly derived from free C20 fatty acids (67). Since ARA is the dominant C20 fatty acid found in cell membranes of mammals fed with a typical meat-based diet, eicosanoids derived from ARA are important lipid mediators. Metabolism of ARA by cyclooxygenase enzymes (COX) gives rise to the 2-series prostaglandins (PGs) and thromboxanes (TXs), while metabolism by the 5-lipoxygenase (5-LOX) gives rise to hydroxyl- and hydroperoxy- derivatives (5-HETE

and 5-HPETE) and the 4-series leukotrienes (LTs), LTA_4, B_4, C_4, D_4 and E_4 (68). Both PGE_2 and LTB_4 have strong proinflammatory properties such as the induction of fever, increase of vascular permeability, induction of chemokinesis and release of lysosomal enzymes, and enhancing generation of reactive oxygen species and inflammatory cytokines like TNF-α, IL-1 and IL-6 (30, 68, 69).

Increased consumption of n-3 PUFA results in high proportions of those fatty acids in cell membrane phospholipids partly at the expense of ARA. The anti-inflammatory action of n-3 PUFA has mainly been attributed to reduction of the proportion of ARA as a substrate for synthesis of the subsequent proinflammatory eicosanoids (67, 68). Furthermore, n-3 PUFA alter the phospholipid composition of cell membranes reducing the activity of phospholipases involving in the release of ARA from cell membranes (70). Additionally, EPA, as another C20 fatty acid, when is incorporated in membrane phospholipids competitively gains access to the same enzymes that produce eicosanoids from ARA and gives rise to alternative classes of eicosanoids, the 3-series PGs and TXs and the 5-series LTs (68) and other novels lipid mediators termed E-series resolvins. Similarly, DHA gives rise to D-series resolvins, docosatrienes and neuroprotectins (71). These alternative series of lipid mediators are less potent inducers of inflammation and in certain cases exert anti-inflammatory properties counteracting the proinflammatory potential ARA-derived eicosanoids exert (67, 71).

Incorporation of n-3 PUFA into plasma membrane not only alters the lipid products derived from membrane lipids, but also the microenvironment in the membrane. Thus, they could modify the conformation, lateral organization and vertical orientation of molecules involved in interactions with other cells or other molecules (72). Additionally,

because n-3 PUFA also incorporate into internal membranes, uptake of n-3 PUFA can affect molecule trafficking on cell surface (55). Lipid modulation of APC membranes and MHC presentation on their surface possibly has biologically significant effects on viral clearance (72, 73). Moreover, DHA has been shown to inhibit trafficking of RAS and other lipidated proteins to plasma membrane through the secretory pathway (55, 75).

The degree to which the aforementioned mechanisms can explain the effect of n-3 PUFA on host responses is not known. Host resistance is the cumulative result of cellular incorporation and different molecule production and activation. Thus, animal infectious models can give the best indications of the role of n-3 PUFA upon infectious disease resistance. Both bacterial and viral infectious agents have been used to give insight in possible immunosuppressive effects (31). These studies include a wide variety of different bacteria, from Gram-negative, such as *Klebsiella* sp (76), *Salmonella* sp (77, 78), *Bacteroides* spp (79), *Pseudomonas aeruginosa* (77, 80) to Gram-positive microorganisms, such as *Staphylococcus aureus* (79), Group B *Streptococcus* sp (81), and *Listeria monocytogenes* (43, 57, 61, 65, 82-84). The usual endpoint in these studies is host survival, and occasionally, clearance of the infectious agent and specific immunological parameters are measured. In these studies, fish oil has been reported to both enhance and suppress host survival. These differential results indicate that failure to survive a specific infection may be related to the degree in which an overwhelming immune response is the cause of death or the pathogen itself. Apparently, the type of pathogen used and the type of disease it elicits is of particular importance to predict the outcome.

Viral models that have been used to estimate immunomodulatory effects of n-3 PUFA on host resistance include cytomegalovirus (CMV), murine retrovirus (MAIDS), and influenza virus. Mice infected with cytomegalovirus and supplemented with fish oil did not show effects on susceptibility to the infection (80). Mice infected with murine retrovirus and fed with fish oil for 8 wk had significant enhanced survival compared control mice (85). However, using same virus Xi et al, (63) demonstrated that serum levels of IgG and IgM were reduced in mice fed fish oil. Additionally, fish oil consumption reduced virus induction of TNF-α, IL1-β, IL-2 and IFN-γ during the infection. In studies with influenza virus, Byleveld et al (41) reported that fish oil feeding interfered with viral clearance from lungs and reduced IgA secretion in lungs. Furthermore, IFN-γ mRNA expression was found to be down regulated by fish oil supplementation. Overall, those studies suggest that fish oil can enhance or impair immune responses to virus depending on the type of virus used.

2.2 REOVIRUS

Enteric reovirus infection, a well-studied viral host resistance model, has been used effectively to estimate immunotoxicity in the gastrointestinal tract (37). Li et al successfully utilized this model to access modulation of the normal immune responses to an enteric viral infection by natural occurring mycotoxins (34-36, 86). We chose to use the same model to predict possible immunosuppression of the normal immune responses in the gut by long-term supplementation with n-3 PUFA. The following section describes

the enteric reovirus model, and reviews both aspects of reovirus molecular biology and immunity induced upon infection

Genome and life cycle

Reoviridae is the biggest of the six families of viruses with double stranded RNA genome that includes many genera: *Orthoreovirus, Aquareovirus, Cypovirus, Fijivirus, Oryzavirus, Orbivirus, Phytoreovirus* and the human pathogens *Rotavirus* and *Coltivirus* (Colorado tick fever virus). *Orthoreovirus* genus is the prototype of the *Reoviridae* family called also mammalian orthoreovirus or reovirus (respiratory enteric orphan virus).

Reoviruses are grouped in three serotypes: 1, 2 or 3 based on hemagglutination-inhibition tests (87). Prototypical viruses are the Type 1 Lang (T1L) which was isolated from a healthy child, and Type 2 Jones (T2J) and Type 3 Dearing (T3D) that were isolated from children with diarrhea (87). From serological studies it has been shown that the reovirus strains differ mostly in a capsid protein, the σ1 protein. T1L S1 gene is more closely with the T2J than the T3D S1 gene. Despite these differences σ1 protein in all strains share at least five conserved domains. The rest capsid or core proteins of all strains are quite conserved, sharing a 90-98% homology (87).

The reovirus genome consists of 10 distinct segments of double stranded RNA that encode all the proteins the virus needs and comprises a genome of around 23,600 kb (88). The 10 RNA segments have different sizes and are categorized in three groups as large (L1, L2, L3), medium (M1, M2, M3) or small (S1, S2, S3, S4) (87). During infection with different viral strains, reassortment might take place, generating new

viruses containing segments from both strains. Each segment is transcribed to plus-strand RNAs, serving as viral mRNAs. Each mRNA has a long open reading frame, ORF. Each ORF encodes one protein. However, there are 10 RNA segments and the virus encodes twelve proteins. Eight segments code for one protein each from the mRNAs and two segments code for two proteins each. The latter segments include S1 and M3 gene. S1 gene encodes the σ1s and σ1 proteins and M3 gene encodes the μNSC and μ3 proteins. Those extra proteins are encoded from AUG frames within the ORF of the main proteins. Among the 12 translated proteins, eight are structural (λ1, λ2, λ3, μ1, μ2, σ1, σ2 and σ3), and the rest four are non-structural (μNS, μNSC, σNS, and σ1s) (87, 89) and facilitate viral survival, infectivity, replication and assembly to new viruses.

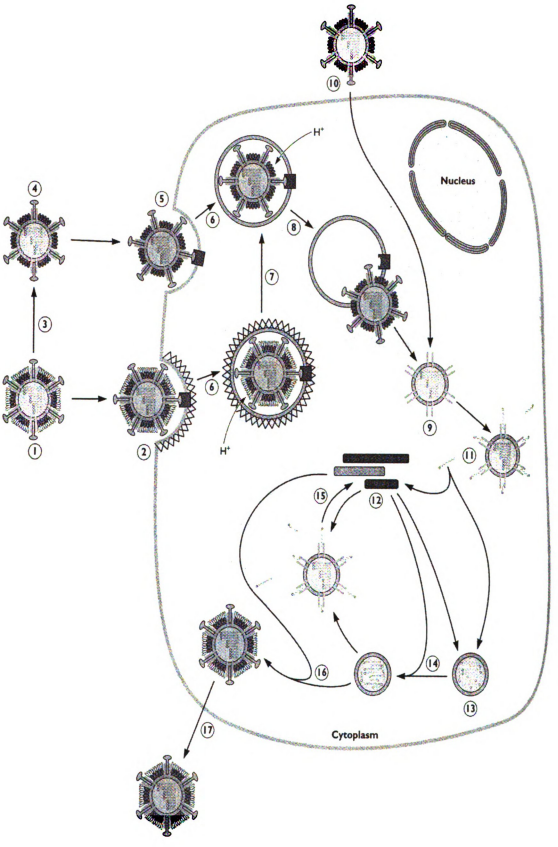
The mature structural form of infectious viruses is the virion released from infected cells. During infection, viral particles undergo a serial of conformational changes that facilitate their entrance in the cytoplasm. The intact virion consists of two capsids composed of structural proteins. The outer capsid is made up largely of σ3 and μ1 protein and is stabilized by interactions with the λ2 protein (87). During infection, virions undergo partial uncoating to yield two types of subvirion particles, infectious subviral particles (ISVPs) and cores.

Viral particles mediate infection by attachment of the virion to cellular receptors. Attachment is mediated by binding of the viral σ1 protein. The σ1 protein contains receptor binding domains (RBD) that determine cell tropism of the variant reovirus strains (90). Until now, two cellular receptors have been recognized, but others remain to be elicited (91). The identified receptors are: α-linked sialic acid and junctional adhesion molecule (JAM) that can be independently bound by reovirus strains (90).

Upon binding, the virion enters the cell by receptor-mediated endocytosis (Fig. 2.1, step 2). In endosomes the virion undergoes acid-dependant proteolytic cleavages and forms the ISVPs (Fig. 2.1, step 7). When ISVPs are formed in the lumen by intestinal proteases, entry of ISVPs into the cell does not depend on endocytosis (Fig. 2.1 step 5) (87, 89). ISVPs are released from the endosome in the cytoplasm as cores (Fig. 2.1, step 8). Replication of reovirus starts within the core particle in the cytoplasm (Fig. 2.1, step 9), with synthesis of 10 capped RNA that serve both as mRNA for translation and as templates for minus-strand synthesis. These RNA are exported from the core and are translated in the cytoplasm (Fig. 2.1, step 10). Transcription of reoviral genomes occurs in two phases: primary transcription occurs within the cores from infecting viral particles the first 6 to 8 hours of infection, while secondary transcription occurs in particles assembled by newly formed proteins reaching maximum rates to 12 hours after infection (Fig. 2.1, step 14-15). Secondary transcription contributes to the majority of RNA transcript produced during infection (87). Shortly after the onset of infection, synthesis of reoviral proteins increases at the expense of cellular proteins (87). Moreover, regulation of the transcription rates occurs with the longer segments to be transcribed at lower rates. Assortment of the viral RNAs occurs when 10 viral mRNAs are packed within a newly formed viral particle (Fig. 2.1, step 13). Generation of the double-strand segments occurs within the nascent particles with synthesis of a minus strand for each plus strand viral RNA that remains associated with each other to form a ds RNA segment (87).

Endosomal and intestinal lumen proteases result to cleavage of the σ3 protein of the intact virion and to generation of ISVPs. Cleavage of the σ3 protein promotes conformational changes of the σ1 structural protein that becomes like an expanded

Figure 2.1. Attachment, endocytosis, replication, assembly and release of reovirus. (1) infectious viral particles; (2) cell attachment via viral protein, σ1 interactions with sialic acid and/or JAM receptors on target cells and receptor-mediated endocytosis; (3) intestinal proteases produce intermediate subviral particles (ISVPs); (4) ISVPs; (5) ISVPs enter target cell via endocytosis; (6) endosomes and lysosomes become acidified; (7) the virion undergoes acid-dependant proteolytic cleavages and forms the ISVPs; (8) release of reovirus ISVPs in the cytoplasm; (9) reoviral cores in the cytoplasm; (10) ISVPs penetrate directly through the cell membrane; (11) transcription occurs within reovirus cores and capped mRNAs are released; (12) primary translation of all 10 mRNA using host translation machinery; (13) viral mRNAs are packed within newly formed viral proteins where new cores assemble; (14) secondary transcription of viral genomes and viral mRNA occurs in particles assembled by newly formed proteins; (15) translation of the secondary viral RNA; (16) assembly of the viral particle with preformed self-assembled complexes of outer-capsid proteins added to subviral particles; (17) mature viral particles are released from the cell by lysis. (Figure reproduced from Flint et al., Principles of Virology, second ed., ASM Press, Washington, 2004 [87]).


Nucleus
Cytoplasm
H+
H+
1
2
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flexible fiber. The σ1 protein is considered as the reovirus cell-attachment protein and its conformational change is believed to mediate interaction with cellular receptors and increase infectivity. The other protein of outer capsid, μ1, facilitates interaction with the membrane bilayers and thus mediates release of ISPVs from endosomes into cytoplasm.

Cores are produced by further release of the σ1 fibers and μ1 subunits and are mostly composed by λ1 and σ2 proteins. In the transition from ISVP to core, parts of the λ2 protein change conformation and rotate to form a turret-like structure, which facilitates entry of nucleotides into the core and exit of newly synthesized viral mRNAs (87, 89).

Assembly of reovirus particles occurs at different stages. In each stage the particles have different functional properties. Before the formation of viral particles, simple complexes are formed by the assembly of RNA with proteins. In more advanced stages, structured particles can be recognized, and transcription of the minus strand occurs. Further, more stable viral particles are formed and finally complete viral particles (87). In the final steps of capsid assembly, preformed self-assembled complexes of outer-capsid proteins are added to subviral particles (Fig. 2.1, step 16) and mature viral particles are released from the cell by lysis (Fig. 2.1, step 17) (87). It is believed that reovirus assembly takes place in specific cytoplasmic inclusions observed during infection, named viral factories (87).

Reovirus effects on host cells

Reovirus infection induces a number of structural and functional changes in the host cell. Part of these changes facilitate viral survival, while others mediate host cell

efforts to alert immune system for the ongoing infection, or to protect the body from systemic spread of the infection. Numerous cell structural changes facilitate viral (89) replication and assembly while others initiate sophisticated signaling cascade to attract immune cells to the focus of infection and evoke responses to eliminate the invading pathogen.

One of the changes that reovirus induces in host cells is the development of cytoplasmic inclusions, called viral factories. Viral factories contain dsRNA, viral proteins and nascent viral particles that are associated with cytoskeleton. These viral factories do not associate with cellular membranes. These inclusions appear as dense granules in the cytoplasm and during infection, increase in size and move towards the nucleus (87). Viral factories do not contain ribosomes, thus, active translation of viral RNA does not occur there. Viral factories are associated with microtubules that play a role in transportation of the viral particles. When cells get infected by reovirus, their cytoskeleton is continuously altered, disrupted and reorganized, changing the shape of the cell constantly (87). Overall, there is agreement that viral factories have a role in viral assembly and maturation of infectious particles (87).

Virus-infected cells undergo further changes that put them into an *antiviral state.* This state is elicited by a complex cellular signal transduction and gene expression initiated with secretion of interferons (IFNs) (89, 92). The various strains have different capacities to induce IFNs, with reovirus T3 to be the most effective. IFNs promote the activation of more than 300 genes participating in the antiviral response that results in cessation of protein synthesis. Inhibition is selective for reovirus proteins and not for cellular proteins (92, 93). Among the key components of this antiviral response is the

RNA-dependent protein kinase, PKR, which inhibits the elF-2 protein synthesis initiation factor by phosphorylation. Reovirus has however evolved mechanisms to override this cellular defense. Reoviral σ3 protein, which has been demonstrated to bind to ds RNA (87, 94), can prevent activation of PKR *in vitro* (87). Activation of PKR has been related to host cell shut off and enhanced viral translation (87). Another important IFN inducible factor, RNase-L, degrades most of cellular and viral RNA (89). PKR together with RNase-L activation contribute to immediate host defense response as evidenced by a general host shutoff. However, reovirus replication is not inhibited neither by PKR nor RNase-L (87). In contrast, reovirus replicates to higher yields in the presence of PKR and RNase-L. Because deletion of these proteins results in impaired reovirus replication, reovirus seems to have evolved effective mechanisms to avoid the actions of these classic IFN-stimulated antiviral responses possibly by benefiting from their expression (94).

Another consequence of reovirus infection is inhibition of cellular DNA synthesis and induction of apoptosis. T3 serotype typically is a more potent inducer of apoptosis than other strains. Reovirus replication is not required for the cell to go to apoptotic death (90, 95). Binding of reovirus outer capsid to cellular receptors is efficient to activate a number of signaling cascades, including c-Jun N terminal kinase (JNK) and extracellular signal related kinase (ERK) that result to apoptotic pathways (90, 95). These signaling cascades activate NF-κB and c-Jun transcription factors, both of which are involved in inducing apoptosis in infected cells, particularly those infected with reovirus T3 (89, 90). Moreover, increased NF-κB activation is linked with increased production of chemokines, cytokines and possibly surface markers signaling the immune cells for the ongoing infection.

Enteric reovirus infection

The various reovirus serotypes differ in their tropism, pathogenesis and potency of the infection. T1L and T3D strains are both able to infect host via the intestine but their capacities to spread and cause disease are different (96). After infection by the oral route, both types bind to M-cells that overlay Peyer's Patch (PP) -dome in the small intestine (96) but only reovirus T1L can replicate in intestinal tissue (95). T3D serotype spreads from the intestinal tract into mononuclear cells of PP, is transmitted to the adjacent neurons and reaches the brain through the vagus nerve (95). T1L serotype spreads to mononuclear cells of PP and mesenteric lymph nodes and concludes in the spleen. Differences occur both in the regions of the intestine they infect, with T3D infecting intestinal intraepithelial lymphocytes and T1L epithelial cells, and on the disease induced with T3D reovirus to be the only one that can cause encephalitis (95).

T1L reovirus has been widely used as a model to study interactions between the mucosal immune system and gut infection. The mucosal immune system is mainly comprised of the gut associated lymphoid tissues (GALT). GALT consists of discrete populations of cells located in PP and gut lamina propria. PP contain B-cells in germinal centers surrounded by mantles of T-cells. Enteric reovirus infection specifically targets PP, ensuring high exposure of virus to mucosal immune cells (34-37). Virus-specific T- and B-cells are primed in the patch and migrate via the afferent lymphatics to the mesenteric lymph node and spleen. From the spleen, primed effectors disseminate via the blood stream to other mucosal sites and the intestine, where they home to the lamina propria. Mature plasma cells secrete antibodies that are transported into the lumen, while

viral-specific cytotoxic T-cells are developed among the intraepithelial lymphocyte populations (97). Migration and specific homing of the effector cells in the intestinal tissues and the sites of infection is mediated with tissue-specific adhesion molecules and chemokine interactions (98).

The kinetics of oral enteric reovirus infection have been well characterized (99). The part of the intestine affected the most is the distal small intestine. Immediately after oral administration, reovirus antigens are found at the dome of M cells, and within 2 d they are detected in PP, where reovirus replication occurs while an immune response is initiated. By the 5^{th} d after inoculation, viral antigens are mostly found in enterocytes of the crypts. Productive infection is limited to immature crypt enterocytes, due to a requirement for an activated RAS signaling pathway that promotes translation of reovirus mRNAs and viral replication (94). Virus is released from cells in the crypts and infects adjacent epithelial cells or is shed in the lumen. By the sixth day after infection, viral titer decreases dramatically in all segments of the intestine except the ileum, where the viral titer can be 2 to 3 log higher compared to other tissues. In neonate mice, the infection spreads systemically, but in adult mice the infection is limited to the mucosa, although viral antigens and antigen-activated cells appear later in the mesenteric lymph nodes and the spleen. Twenty days after infection, the intestine seems to have normal morphology and viral antigens are only detected in mononuclear cells residing in lamina propria.

Reovirus infection triggers significant antibody and cytotoxic responses. The antibody responses are characterized by increased secretion of mucosal IgA and elevation of serum IgG (100, 101). Secretory IgA has a major protective role for the mucosal system. It is a polymeric antibody that is secreted on the mucosal surfaces and neutralizes

the invading pathogens (89, 97, 102, 103). The role of IgA in reovirus infection becomes important for protection against secondary infections (102). While an initial infection can be cleared by mice that lack IgA by other antibodies in the mucosa, upon a secondary infection, the same mice become infected again whereas wild mice were protected (102). Reovirus infection increases the polymeric immunoglobulin receptor (pIgR) mRNA and protein in mucosal epithelial cells, facilitating the transport of IgA to the lumen (104). Thus an intact viral-specific IgA response is essential for protection and clearance of the virus in the guts.

Activation of B-cells by CD4+ T-helper cells with the secretion of cytokines, activation of B1 cells by T-cell independent mechanisms and a pleiotropic redundancy among many cytokines in the intestine are among the mechanisms involved in mucosal IgA production (105). Other thymus-independent mechanisms through γδ T-cells are equally important for the induction of IgA (105). Almost up to 40% of IgA production in the murine gastrointestinal tract is derived by B1 cells (105). Induction of IgA is also upregulated by cytokines as IFN-γ, IL-4, TNF-α and IL-1 and the transcription factor IRF-1 (105, 106). Other cytokines such as IL-5, IL-6 and IL-10 also regulate production of IgA in the gut (103, 106). The biological role of all these cytokines on the secretion of IgA is not clearly defined. IL-6 is considered a major regulator of mucosal IgA induction, however genetic deficiency in IL-6 does not abrogate antigen specific IgA in the gut, demonstrating the complexity of the regulatory mechanisms for mucosal IgA generation (107). Retinoic acid potentially synergizes with IL-6 or IL-5 in the mucosal compartment to regulate IgA secretion (105). The organism has developed many complementary ways to maintain intact IgA production indicating its critical role for the protection (105).

In addition to mucosal IgA, specific IgG is induced upon enteric reovirus infection. The majority of reovirus-specific serum IgG is IgG2a and IgG2b, while levels of virus-specific IgG1 are low (97, 106, 108). Reovirus-specific IgG prevents viral infection by inhibiting virus-cell interactions, penetration and uncoating of the virus. In addition to being produced in systemic lymphoid tissue and found in serum, antigen specific IgG is also detected in mucosal tissues (97). In the absence of virus-specific IgA, passive transfer of specific IgG facilitates clearance of reovirus in severe immunodeficient mice (SCID) and immunoglobulin knockouts (101). However, in immunocompetent mice, systemic administration of reovirus-specific IgG cannot inhibit viral replication in the intestine (101). So the role of IgG antibodies seems to be important in preventing viral systemic spread rather than within the gut mucosal surfaces.

After oral reovirus infection, a robust cellular response is induced. CD4+ T helper cells are differentiated mostly towards Th1 cells while a weaker Th2 response is promoted (109). Th1 cells produce cytokines such as IFN-γ, TNF-α, TNF-β and IL-2 that activate cell-mediated immune responses, whereas Th2 cell produce cytokines such as IL-4, IL-5, IL-10 and TGF-β that mediate antibody production. Reovirus infection induces IFN-γ in PP and intraepithelial lymphocytes (IEL), synthesis of IL-5, IL-6 and IFN-γ in PP cultures (109) and synthesis of IL-12 and IFN-γ in mesenteric lymph nodes (110). In IEL, 4 d post infection (PI), the strong inflammatory cytokines IL-6, IL-12 and IFN-γ that lead to T-cell activation are expressed. Additionally, IEL express IL-17 and stem cell factor (SCF), the colony-stimulating factors GSCF and GM-CSF, and the proteins MCP-1 and MCP-5, which contribute to the mobilization and/or cell proliferation of dendritic cells, monocytes and macrophages (111). IEL from reovirus

infected mice have been shown to have elevated levels of IL-10 (111), while PP have less IL-10 expression (110) suggesting differential expression of cytokines in different tissues. IL-10 has been suggested to delay viral clearance (112-114). Other cytokines such as IL-2 and IL-4 were shown to be only modestly increased in reovirus infected mice at 4 d PI compared to non-infected mice (106, 109, 111), suggesting that these two cytokines may be expressed at earlier time points or may be part of an ongoing process of lymphocyte proliferation in non-infected mice to maintain a homeostatic process of T-cell and/or B-cell expansion in the small intestine (111).

Enteric infection with reovirus induces strong cell-mediated immune responses in the gut that are characterized by the appearance of $CD8^+$ virus specific cytotoxic lymphocytes in PP and IEL. Virus-specific precursor cytotoxic T-cells are found in a frequency of 200 every 10^6 $CD8^+$ IEL, and they express α/β TCR (115). Their role is to lyse virus-infected cells in an MHC-restricted manner (98). Reovirus infection induces monoclonal or oligoclonal expansion of TCR subpopulations (115). Additionally, thymus-independent cytotoxic T-cells contribute to reovirus clearance from the gut. Thymus-deficient mice are resistant to reovirus infection, indicating a role for thymus-independent lymphocytes, natural killer cells and macrophages (97, 115). SCID mice, which do not have any T and B-cells but still have natural killer cells, are not able to overcome reovirus infection and die from the induced disease, confirming the role of thymus-independent T-lymphocytes in protection (115).

In general, the complexity of the immune responses in the mucosa is demonstrated by the synergistic action of different concurrent mechanisms and intracellular signaling events. Apart from the adaptive responses, innate mucosal

responses are equally important upon a viral infection. Secretion of antibodies of low specificity, complement activation, expression and activation of Toll-like Receptors (TLR), production of type I IFNs and cell mediated innate responses seem to play a role in dealing with the infection (92). Innate cell response includes the first cells to arrive at the site of infection and involves neutrophils, macrophages, natural killer cells and other granulocytes. Innate responses in the mucosa include also peristalsis of mucosal smooth muscle, mucus and enzyme secretion, and defensins; these mucosal functions are considered to have a contribution to protection against pathogens. However, we know that specifically for reovirus infection, defensins do not play important role since they are only effective against enveloped viruses or bacteria (116).

Among the innate responses responsible for viral clearance, induction of the type I IFNs, IFN-α and IFN-β, has been shown to be critical. These cytokines put the cell into an antiviral state and help evoke further adaptive responses. A recent study on the role of type I IFNs on reovirus infection showed that these cytokines are indispensable for reovirus clearance in the intestine (73). The study suggested that the major source of type I IFNs is PP, the first site of the intestine that encounters the virus (117), were resident dendritic cells take up antigens. In contrast, epithelial cells, natural killer cells or TLR signaling were shown not to be as critical as IFN signaling for reovirus clearance (74). Based on these findings, more extensive studies need to be done focusing on how nutritional modifications such as those of n-3 PUFA supplementation shape the innate immune responses.

Overall, studies on reovirus-induced immunity have been well-characterized, and thus reovirus can be a good tool to study alterations of the immune system in response to

external factors. Thus, it is possible to assess modifications of normal responses to interventions with nutrients, drugs and toxicants. Furthermore, because quantitative judgments about overall immune function are very difficult, a common practice is to measure aspects of immunity (certain cell functions, cytokine production, phagocytosis, etc) to have a possible insight for the status of the immune system. Since reovirus is the subject of ongoing research about the innate and adaptive immune responses, it is feasible to employ the appropriate tests and measurements to study immune modulation with nutrients, xenobiotics and drugs.

In the present study, we contribute to a growing field of research focusing on targeting the immune system with functional foods or nutritional supplements. The possibility that with selective nutritional choices we can enhance the immune system is particular interesting for science and attractive for the food industry. Several nutrients have been shown to be essential for a sufficient immune function, such as essential amino acids, the essential fatty acid linoleic acid, vitamin A, folic acid, vitamin B6, vitamin B12, vitamin C, vitamin E, Zn, Cu, Fe, and Se (118). Their deficiency leads to decreased immune responses to pathogens and increased susceptibility. Increased intakes of some nutrients above habitual and recommended levels can enhance certain aspects of immune function. However, excess amounts of other nutrients might impair immune functions. It is not clear, however, whether a normal immune function can be "improved". Yet, it is possible to measure aspects of the immune function and thus, we can define the status of the immune system. This study describes the effects of a dietary manipulation on the *in vivo* response to an enteric viral challenge, and the antibodies in peripheral blood and

fecal secretions in response to the virus. Also we determined the severity of the infection in the mouse, tracking the eradication of the infectious agent (30).

CHAPTER 3

DHA SUPPLEMENTATION MODULATES THE LIPID PROFILE OF LYMPHOID ORGANS AND PERITONEAL MACROPHAGES

3.1 ABSTRACT

Consumption of n-3 polyunsaturated fatty acid- (PUFA-) enriched diet for an extended period alters the lipid profile of cells within human and animal tissue. Such a change might impact the anti-iflammatory effects n-3 PUFA. The purpose of this study was to test the hypothesis that consumption of fish oil will result in elevation of n-3 PUFA membrane lipid profile of lymphoid organs and peritoneal macrophages. Female B6C3F1 mice were fed AIN-93G semipurified diets containing 1% corn oil and either 6% fish oil enriched in docosahexaenoic acid (DHA diet) or 6% safflower enriched in oleic acid (control diet). To determine if these diets resulted in elevated n-3 PUFA in spleen, PP and the peritoneal macrophage cell population, these lymphoid tissues were isolated after 2, 4, and 6 wk feeding. Membrane phospholipids were extracted, esterified and analyzed with flame ionization gas chromatography. Fatty acid profiles from the experimental samples reflected the compositions of the diets. The DHA diet increased n-3 PUFA concentration in all tissues at the expense of n-6 PUFA but the kinetics of these changes varied among the various tissues. From this study it was determined that 4 wk feeding was sufficient for incorporation of n-3 PUFA and reduction of arachidonic acid in all excised tissues.

3.2 RATIONALE

Consumption of a n-3 PUFA-enriched diet for an extended period alters the lipid profile of cells within human and animal tissue. Since it is assumed that an alteration in the immune function must be preceded by a change in fatty acid composition of tissues of interest, it is necessary to determine the appropriate feeding period required to attain maximal n-3 PUFA incorporation. In this study we fed mice with DHA enriched diet for various time periods to track the extent of n-3 PUFA incorporation into two lymphoid tissues, spleen and Peyer's Patches (PP), and into peritoneal macrophages. The goal of this study was to determine the kinetics of possible changes within cell membrane lipid profiles.

3.3 INTRODUCTION

Lipids, as macronutrients, are rapidly taken up from food and distributed to every cell membrane in the body. Thus, the type of fat consumed with diet can influence the lipid profile of cell membranes. Eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) are among the longest unsaturated n-3 PUFA derived from fish oil. EPA has 20 carbons and 5 double bonds and DHA has 22 carbons and 6 double bonds. These n-3 PUFA have beneficial effects on several inflammatory responses in cells. While the n-6 PUFA content in tissues is typically high, consumption of diets rich in EPA and DHA significantly increase n-3 PUFA (119-124). In most tissues, these fatty acids are mainly

esterified in phosphatidylcholine and phosphatidylethanolamine classes of membrane phospholipids (125, 126). There are great differences among tissues and organs relative to lipid level and type. Some tissues are more resistant than others in changes imposed by the diet fatty acid composition (127).

Among different cell types, neuronal cells are characterized by endogenously high PUFA content. This high level of PUFA is assumed to contribute to the unique properties of neuronal membranes, which include increased ability to fuse or exfoliate membrane vesicles at high speed (127, 128). It is thus suggested that increased incorporation of n-3 PUFA in other cell types can change their membrane properties towards those of neuronal cells. In *in vitro* studies, membranes rich in DHA are thinner, more permeable and less stable, a fact that enhances vesicle exfoliation, fusion and flip-flop (129). Moreover, enrichment of membranes with DHA induces unique packing arrangements of the membrane lipids that can affect protein trafficking to membranes and therefore cell signaling (130). Thus, n-3 PUFA content of cell membranes influences major aspects of cell functionality and may have important implications to immune responses.

A well-accepted mechanism for the anti-inflammatory properties of n-3 PUFA is their ability to incorporate into cell membrane phospholipids. It is known that upon stimulation, arachidonic acid, (ARA) is released from cell membranes and is metabolized enzymatically to eicosanoids, potent proinflammatory factors (67). Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes will convert free arachidonic acid to give rise to the 2-series prostaglandins (PGs) and thromboxanes (TXs), hydroxyl- and hydroperoxy derivatives (5-HETE and 5-HPETE) and the 4-series leukotrienes (LTs) (68). Both PGE_2 and LTB_4 promote production of cytokines like TNF-α, IL-1 and IL-6

(30, 68, 69). Increased consumption of n-3 PUFA results in high incorporation of those fatty acids in cell membrane at the expense of ARA reducing the proportion of ARA as an available substrate for synthesis of proinflammatory eicosanoids (67, 68). Additionally, EPA and DHA incorporated into membrane phospholipids can competitively gain access to the same enzymes that produce eicosanoids from ARA and give rise to alternative classes of eicosanoids. EPA results to the 3-series PGs and TXs, the 5-series LTs and the E series resolvins (68) and DHA to D-series resolvins, docosatrienes and neuroprotectins (71). These alternative series of lipid mediators have less proinflammatory properties and in certain cases exert anti-inflammatory effects (67, 71). Thus, incorporation of n-3 PUFA can differentially impact inflammation.

Immune cells are distributed through the body and can be found in higher numbers concentrated in the lymphoid tissues. One of the secondary lymphoid organs of the immune system is the spleen. Spleen contains a variety of immune cells concentrated in the white pulp region. In the spleen, T- and B - lymphocytes contact antigen presenting cells, macrophages and dendritic cells to survey for possible evidence of infectious agents or other contaminants. It has been reported altered splenic fatty acid composition with diets rich n-3 PUFA characterized by substantial increases of n-3 PUFA and reductions of n-6 PUFA (121, 131). Additionally, functional modifications have been reported such as suppression of lymphocyte proliferation and Il-2 secretion (132).

Peyer's Patches (PP) are lymphoid tissue aggregations associated with the mucosa within the small intestine. They are composed mostly of B-cells that are organized in large, domed follicles. T-cells occupy the areas between follicles. A specialized epithelium made up of M cells covers the surface of the follicle. Antigen presenting cells,

macrophages and lymphocytes are the main immune cells in PP. To our knowledge, there is little information about n-3 PUFA incorporation in this tissue.

While spleen and PP are organized aggregations of lymphocytes, macrophages, are another important immune cell population distributed throughout all the body. Macrophages are generated from circulating monocytes in the blood and upon stimulation they migrate to the tissues. Macrophages play a key role in host responses against pathogens with their ability to phagocytose, release important cytokines and produce cytotoxic mediators. Moreover, macrophages are significant message-producing cells characterized by an intense oxidative and lipid metabolism generating reactive oxygen species and lipid mediators; thus, their functions partly depend on their lipid composition (133). Dietary n-3 PUFA can potentially modulate many of these functions. Feeding n-3 PUFA diets results in macrophages that produce less eicosanoid upon LPS stimulation (134). Macrophages from mice fed with fish oil diet produce significantly lower levels of proinflammatory cytokines such as TNF-α, IL-1β (135) and IL-6 (134). In addition higher levels of IL-10, an anti-inflammatory cytokine, are observed upon fish oil feeding (135).

This study was conducted to test the hypothesis that a DHA-enriched diet alters the fatty acid profile of major lymphoid tissues. Specifically, a diet enriched in DHA was administered to mice for different time periods and the fatty acid profiles of spleen, PP and peritoneal macrophages were determined using flame ionization gas chromatography. We observed that feeding with DHA diet enriched these tissues in n-3 PUFA at the expense of n-6 PUFA, but the extent of the change varied among the three different tissues.

3.4 MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. To identify fatty acid composition among isolated phospholipids, the following standards were used: PUFA-1 marine source (Supelco, PA), PUFA-2 animal source (Supelco, PA), GLC #538 which consisted from 21 fatty acid methyl esters (FAMEs) and GLC#463 which consisted from 52 FAMEs (Nu-CHECK, MN). All included EPA, DHA and ARA. Detailed description of all methyl esters for each standard is provided at the appendix.

Animals. Female B6C3F1 mice (4 wk old) were purchased from Charles River Laboratories (Portage, MI). Housing, handling and sample collection procedures conformed to the policies and recommendations of MSU Laboratory Animal Research Committee and were in accordance with guidelines established by the National Institutes for Health. Mice were housed three per cage in a humidity (45-55%)- and temperature (23-25^{o}C) controlled- university animal care facility room with a 12 hr light and dark cycle. Mice were acclimated for 1 wk prior to experiment initiation and fed experimental diets until they were terminated.

Diets. Experimental diets were based on the AIN-93G formulation (Dyets Inc., Bethlehem, PA) of Reeves (136) with minor modifications. Both diets contained 920 g/kg AIN-93G formulation (except for oil), 10 g/kg AIN-93VX Vitamin Mix, and 10 g/kg corn oil (Dyets Inc., Bethlehem, PA). In control diets, 60 g/kg high oleic acid safflower oil (Hain Celestial Group, Inc) were added, whereas in DHA diets 60 g/kg MEG-3^{TM} DHA-enriched oil (min 55 % DHA and 3 % EPA, Ocean Nutrition Canada Ltd,

Darmouth, Nova Scotia) were added. Fatty acid compositions of the diets, based on supplier data, are summarized in Table 3.1 Diets were prepared every 2 wk, stored in aliquots at -20°C. Fresh diet was fed to mice daily.

Experimental design and sample preparation. Mice were fed control or DHA diets for 2, 4, or 6 wk prior to termination. Four days before the completion of the feeding period, mice were injected intraperitoneally with 1.5 ml of 3% (w/v) aged Brewer's thioglycollate broth to elicit macrophage migration to the peritoneal cavity. Mice were anesthetized and killed by cervical dislocation at intervals. Peritoneal exudates were collected by washing out the peritoneal cavity with 10 ml cold HBBS buffer. Spleen and PP were excised. Peritoneal exudates were concentrated by centrifugation and all experimental samples were stored under N_2 atmosphere in microtubes at -80°C until further analysis.

Lipid extraction, phospholipid isolation and FAME formation. Lipid extraction was performed as described by Hasler et al. (1991) with slight modifications (137). The frozen peritoneal exudate pellet suspended in 0.6 ml PBS. One hundred micrograms (100 μg) of spleen, seven PP and the peritoneal exudate suspension in PBS, were homogenized with 2.4 ml of a 2:1 mixture (v/v) of chloroform-methanol. A second extraction was further performed with 86:14:1 mixture (v/v) of chloroform-methanol-saline. To remove remaining water, anhydrous sodium sulfate was added in the extracts for overnight incubation at -20°C, and filtered with #1 filter paper. Phospholipids were then separated from the lipid extract using solid-phase extraction. First, the extract was applied to Maxi-clean Silica cartridges, 600 mg (Alltech Associates, IL) and neutral lipids and free fatty

Table 3.1 Lipid composition in experimental diets

Experimental Diets	Oil source			Fatty acids[1]						
	Corn oil	High Oleic safflower	DHA-enriched fish oil	Linoleic acid [2,3]	Oleic acid [2,3]	EPA[4]	DHA[4]	Total (n-6)	Total (n-3)[4]	Ratio (n-6)/(n-3) fatty acids
	g/kg diet									
Control	10	60	0	14.6	47.6	0	0	14.6	0.1	146/1
DHA	10	0	60	6.1	2.6	6.0	34.8	6.1	46.8	1/7.5

[1] Refers to major fatty acids as reported by supplier.
[2] Supplied by corn oil which contains 61.2% linoleic acid and 26% oleic acid
[3] Supplied by high oleic safflower oil that contained nearly 75% oleic acid and 14% linoleic acid.
[4] Supplied by concentrated fish oil that contained 58% DHA and 10% EPA.

acids were eluted with 10 ml chloroform followed by 5ml chloroform-methanol 20:1 (v/v). Phospholipids were then eluted with 10 ml methanol and collected to clean, screw-capped, glass tubes. Following evaporation under streaming N_2, fatty acid methyl esters were prepared by incubation with 1.5 ml 14% trifluoride boron in methanol for 1 hour at 90°C. Fatty acid methyl esters (FAME) were isolated with hexane extraction, dried and resuspended to 50 µl of chloroform before the performance of the analysis.

Fatty acid analysis. The fatty acid profile compositions of the experimental samples were analyzed by capillary gas chromatography (GC-2010-FID, Shimanzu Scientific Instruments, Chicago IL). An EC^{TM}- WAX capillary column 30 m length and 0.25 mm inside diameter (Alltech, IL) was used for separation of FAME. Oven parameters included a 10°C/min increase from 40 to 190°C with a 5 min hold and a further 10°C/min increase from 190 to 240°C for a 20 min hold. Peaks were assigned based upon retention times of commercial standards. The results are reported as percentage of total phospholipids and they were calculated by dividing the integrated area of each peak by the total area of all identified peaks x 100. Percentages less than 0.5% were considered minimum level of detection.

Statistics. Data was analyzed using the Sigma Stat Software version 9 (Jandel Scientific, San Rafael, CA). Data was subjected to two-way ANOVA for time and diet factors. Differences were considered significant at $p<0.05$. All pairwise multiple conparisons were done using Holm-Sidak method.

3.5 RESULTS

Fatty acid composition of spleen

The effect of the diets on splenic fatty acid composition is shown in Table 3.2. Among the monounsaturated fatty acids, oleic acid, 18:1n9, (OA), was detected in high levels in spleen. Feeding with DHA diet had no significant impact on the levels of OA in spleens compared to those at the start of the experiment. However, consumption of control diet increased OA incorporation in the splenic tissue by almost 61% by the 6^{th} wk of feeding. OA was incorporated in a time-dependant way with maximum levels reached at 6 wk.

The PUFA profile in spleen was modified significantly for both n-6 and n-3 fatty acids. Of the n-6 PUFA, ARA underwent the greatest changes. DHA diet decreased ARA content by almost 60% within the first 2 wk, a stable reduction that was not further augmented significantly with longer feeding time. In contrast, ARA content in control-fed mice increased in the spleen in a time-dependent way, reaching a final increase of 33% within 6 wk. Other n-6 PUFA affected by the diets include linoleic acid, 18:2n-6, (LA), and gamma-linolenic acid, 18:3n-6, (GLA). Both diets reduced LA levels compared to 0 wk. However, the DHA diet contributed to smaller LA reduction (~41%) than control diet (~54%) at 4 wk feeding. For both diets, the magnitude of LA reduction became smaller with longer feeding time. GLA also was reduced compared to 0 wk, and DHA diet contributed to a greater reduction than the control diet after 4 wk feeding.

Consumption of the DHA-enriched diet, greatly, modified the n-3 PUFA profile in the spleen. It is observed that n-3 PUFA extensively incorporate upon DHA

Table 3.2. Fatty acid composition of spleen phospholipids in mice fed control or DHA enriched diet[1-3]

			0 wk	2 wk		4 wk		6 wk	
			Baseline	Control	DHA	Control	DHA	Control	DHA
	Fatty acid[2]		% of total phospholipids						
14:0	Myristic acid		1.6 ± 0.1	1.9 ± 0.6	1.6 ± 0.3	1.8 ± 0.0	1.3 ± 0.2	1.1 ± 0.2	1.5 ± 0.3
16:0	Palmitic acid		32.1 ± 2.6	32.7 ± 2.4	37.6 ± 1.1	33.4 ± 2.4	35.6 ± 3.4	30.5 ± 1.0	37.2 ± 3.6
16:1	Palmitoleic acid		0.7 ± 0.3	1.2 ± 0.4	1.0 ± 0.0	0.6 ± 0.1	1.6 ± 0.2	0.5 ± 0.0	1.5 ± 0.2
18:0	Stearic acid		23.5 ± 0.3	23.1 ± 3.4	21.4 ± 1.0	22.2 ± 1.0	18.7 ± 1.8	20.3 ± 1.6	18.9 ± 2.2
18:1(n-9)	Oleic acid	OA	12.6 ± 0.1^{a}	17.6 ± 0.4^{b}	13.5 ± 0.3^{a*}	17.9 ± 1.2^{b}	13.3 ± 1.2^{a*}	20.3 ± 1.0^{b}	14.4 ± 0.3^{a*}
18:2(n-6)	Linoleic acid	LA	8.0 ± 1.6^{b}	3.8 ± 0.3^{a}	4.6 ± 0.4^{a}	3.6 ± 0.1^{a}	4.7 ± 0.3^{a}	4.2 ± 0.4^{a}	5.1 ± 0.3^{a}
18:3(n-6)	Gamma-linolenic acid	GLA	2.8 ± 1.0^{b}	1.2 ± 0.6^{b}	1.2 ± 0.3^{b}	2.3 ± 0.2^{b}	0.7 ± 0.2^{a}	1.4 ± 0.1^{b}	0.8 ± 0.4^{a}
20:4(n-6)	Arachidonic acid	ARA	14.4 ± 2.4^{b}	16.3 ± 2.0^{b}	5.7 ± 0.9^{a*}	17.4 ± 1.4^{b}	5.3 ± 0.5^{a*}	19.1 ± 2.0^{b}	4.7 ± 0.4^{a*}
18:3(n-3)	Alpha-linolenic acid	ALA	<0.5	<0.5	0.7 ± 0.4	<0.5	<0.5	<0.5	<0.5
20:5(n-3)	Eicosapentaenoic acid	EPA	<0.5^{a}	<0.5^{a}	3.8 ± 0.4^{b*}	<0.5^{a}	4.9 ± 0.2^{b*}	<0.5^{a}	4.4 ± 0.58^{b*}
22:5(n-3)	Docosapentaenoic acid	DPA	0.6 ± 0.1^{a}	<0.5^{a}	2.1 ± 0.3^{b*}	<0.5^{a}	2.6 ± 0.3^{b*}	<0.5^{a}	2.2 ± 0.4^{b*}
22:6(n-3)	Docosahexaenoic acid	DHA	3.7 ± 1.0^{a}	2.7 ± 0.3^{a}	9.6 ± 1.6^{b*}	2.1 ± 0.4^{a}	11.3 ± 1.4^{b*}	3.0 ± 0.2^{a}	9.5 ± 1.4^{b*}
total (n-6)[4]			25.2 ± 3.2^{b}	21.9 ± 2.2^{b}	11.5 ± 1.1^{a*}	22.5 ± 0.7^{b}	10.7 ± 0.8^{a*}	24.2 ± 2.2^{b}	10.6 ± 0.9^{a*}
total (n-3)			4.3 ± 1.1^{a}	2.7 ± 0.3^{a}	16.3 ± 2.1^{b*}	2.1 ± 0.4^{a}	18.8 ± 1.9^{b*}	3.0 ± 0.2^{a}	16.0 ± 2.2^{b*}

[1]Only the major fatty acids are shown.
[2] Values are means ± SEM, n=3. Within a diet group, means with superscripts without a common letter, differ, $P < 0.05$. *Different from control at that time, $P < 0.05$.
[3] When analyte was non-detectable, the limit of detection (0.5%) was employed for statistical purposes.
[4] Total (n-6) and total (n-3) fatty acids correspond to the sum of the PUFA shown in the table.

supplementation in as little as 2 wk. EPA concentrations were increased to 4-5% in DHA-fed mice, while at all times it was undetectable in control-fed mice. Docosapentaenoic acid, 22:5n-3, (DPA) increased from 0.6 to 2.1- 2.6% within 4 to 6 wk. DHA was endogenously present at 1.5 – 3.0% at 0 wk and reached final levels of almost 9.5% of total phospholipids. Maximum incorporation of total n-3 PUFA and significant reduction of total n-6 PUFA was observed within 4 wk of DHA feeding.

Fatty acid composition of Peyer's patches (PP)

Fatty acid composition of PP is shown in Table 3.3. The DHA diet had no effect on OA concentration comparable to 0 wk. However, the control diet increased OA by 39% after 6 wk feeding reaching 21% of total phospholipids final concentration.

Concerning n-6 PUFA, ARA levels changed significantly upon feeding with the experimental diets. PP from DHA-fed mice had lower concentrations of ARA compared to PP from control-fed mice. Levels of ARA reduced by almost 52% with the DHA diet but they were unchanged with the control diet. Furthermore, LA was reduced in both groups compared to 0 wk, but there were no differences between the two diets relative to the magnitude of reduction. Incorporation of GLA was also not affected by the diets.

Among n-3 PUFA, EPA was detected only in the DHA-fed mice to 3.8 - 4.2% levels. DHA was increased from 2% at 0 wk to almost 8.2% within 2 wk, however, the final concentration was around 5.9% of total phospholipids after 6 wk feeding with the DHA diet. In control-fed mice, DHA was between 1-2%. Additionally, DHA diet increased DPA from undetectable levels to 0.6-1.5% after 4 wk of supplementation.

Table 3.3. Fatty acid composition of PP phospholipids in mice fed control or DHA enriched diet[1-3]

			0 wk	2 wk		4 wk		6 wk	
			Baseline	Control	DHA	Control	DHA	Control	DHA
	Fatty acid[2]		% of total phospholipids						
14:0	Myristic acid		2.1 ± 0.4	2.1 ± 0.2	1.9 ± 0.1	2.6 ± 0.5	2.3 ± 0.3	2.6 ± 0.5	2.2 ± 0.3
16:0	Palmitic acid		30.1 ± 4.9	29.6 ± 3.7	29.4 ± 5.0	30.8 ± 3.5	35.5 ± 3.3	29.1 ± 3.4	32.5 ± 3.6
16:1	Palmitoleic acid		<0.5	<0.5	<0.5	<0.5	0.7 ± 0.7	1.5 ± 0.2	1.1 ± 0.2
18:0	Stearic acid		29.1 ± 5.7	32.4 ± 3.9	30.7 ± 6.4	27.6 ± 3.5	26.0 ± 3.3	25.2 ± 4.3	25.6 ± 4.0
18:1(n-9)	Oleic acid	OA	15.1 ± 1.1	16.7 ± 1.6	13.8 ± 0.1	20.3 ± 1.5	15.2 ± 0.4*	21.1 ± 3.0	15.9 ± 0.7*
18:2(n-6)	Linoleic acid	LA	9.4 ± 3.0[a]	5.5 ± 1.8[b]	4.3 ± 1.5[b]	5.6 ± 1.1[b]	4.4 ± 0.5[b]	6.9 ± 0.5[b]	4.6 ± 0.8[b]
18:3(n-6)	Gamma-linolenic acid	GLA	2.5 ± 1.3	2.0 ± 0.2	1.3 ± 0.2	2.5 ± 0.7	2.9 ± 1.1	2.8 ± 1.1	2.5 ± 0.7
20:4(n-6)	Arachidonic acid	ARA	9.4 ± 1.5[b]	9.1 ± 1.8[b]	5.8 ± 1.5[b]	10.4 ± 0.6[b]	4.6 ± 0.5[a*]	9.6 ± 0.8[b]	4.5 ± 0.6[a*]
18:3(n-3)	Alpha-linolenic acid	ALA	<0.5	0.7 ± 0.3	<0.5	<0.5	<0.5	<0.5	0.5 ± 0.4
20:5(n-3)	Eicosapentaenoic acid	EPA	<0.5[a]	<0.5[a]	4.0 ± 1.8[b*]	<0.5[a]	3.8 ± 0.2[b*]	<0.5[a]	4.2 ± 0.7[b*]
22:5(n-3)	Docosapentaenoic acid	DPA	<0.5	<0.5	0.6 ± 0.0	<0.5	1.5 ± 0.4*	<0.5	0.6 ± 0.2
22:6(n-3)	Docosahexaenoic acid	DHA	1.9 ± 0.8[a]	1.8 ± 0.6[a]	8.2 ± 3.3[b*]	1.0 ± 0.6[a]	5.2 ± 0.4[b]	1.7 ± 0.1[a]	5.9 ± 0.3[b*]
total (n-6)[4]			21.3 ± 5.7[a]	16.7 ± 3.8[a]	11.4 ± 2.9[a]	18.5 ± 0.1[a]	11.9 ± 1.5[a]	19.3 ± 0.2[a]	11.6 ± 0.5[a*]
total (n-3)			2.1 ± 0.7[a]	2.5 ± 0.3[a]	12.8 ± 5.6[b*]	1.0 ± 0.6[a]	10.5 ± 1.0[b*]	1.7 ± 0.1[a]	11.2 ± 0.8[b*]

[1]Only the major fatty acids are shown.
[2] Values are means ± SEM, n=3. Within a diet group, means with superscripts without a common letter, differ, $P < 0.05$. *Different from control at that time, $P < 0.05$.
[3] When analyte was non-detectable, the limit of detection (0.5%) was employed for statistical purposes.
[4] Total (n-6) and total (n-3) fatty acids correspond to the sum of the PUFA shown in the table.

Fatty acid composition of peritoneal macrophages

The fatty acid composition of peritoneal macrophage phospholipids is shown in Table 3.4. Feeding with experimental diets altered the composition of cell membranes of the major fatty acids. After 6 wk feeding, OA was unaffected in the DHA- and was increased by almost 43% in the control-fed mice.

Peritoneal macrophages from mice fed DHA had also significantly modified n-6 PUFA profile. DHA diet resulted in lower ARA at all time points compared to control-fed mice. The magnitude of reduction reached almost 40%. Additionally, both diets reduced LA content in peritoneal macrophages compared to 0 wk. DHA diet resulted in a smaller reduction of LA than control diet, similarly to what observed in the spleen.

Incorporation of n-3 PUFA in peritoneal macrophages was slower compared to other tissues. Levels of total n-3 PUFA reached maximum (15%) only after 6 wk feeding. Moreover, EPA and DHA were detected in significantly higher levels in the DHA-fed mice compared to control-fed only after 4 wk feeding. While initially in undetectable or very low levels, EPA and DPA reached 4% at 6 wk with the administration of the DHA diet. DHA increased from 3% to a final concentration of 7.4 %.

3.6 DISCUSSION

Gas chromatography was used for fatty acid analysis. This method of fatty acid analysis is robust, precise, linear over a wide range of fatty acid concentrations, and is considered the gold standard for measuring fatty acids (138). Compounds are identified mainly by comparison of retention time with those of purchased standards. Four different standards

Table 3.4. Fatty acid composition of peritoneal macrophage phospholipids in mice fed control or DHA enriched diet[1-3]

			0 wk	2 wk		4 wk		6 wk	
			Baseline	Control	DHA	Control	DHA	Control	DHA
	Fatty acid[2]		% of total phospholipids						
14:0	Myristic acid		3.4 ± 0.2	2.8 ± 0.2	2.6 ± 0.2	4.2 ± 0.5	2.4 ± 0.2	2.6 ± 0.2	2.3 ± 0.5*
16:0	Palmitic acid		29.1 ± 5.0	31.4 ± 5.3	33.8 ± 9.5	41.7 ± 5.7	31.4 ± 9.2	30.1 ± 4.3	31.9 ± 4.7
16:1	Palmitoleic acid		<0.5	0.6 ± 0.6	<0.5	<0.5	1.1 ± 0.6	<0.5	0.8 ± 0.3
18:0	Stearic acid		25.5 ± 2.5^{a}	21.8 ± 1.1^{a}	23.3 ± 3.5^{a}	35.3 ± 2.2^{b}	20.9 ± 3.5^{a}*	21.5 ± 1.7^{a}	19.1 ± 0.9^{a}
18:1(n-9)	Oleic acid	OA	17.7 ± 1.6	24.4 ± 2.3	16.6 ± 2.1	29.1 ± 5.9	17.7 ± 3.2*	25.3 ± 2.4	16.3 ± 1.6*
18:2(n-6)	Linoleic acid	LA	6.1 ± 1.4	5.8 ± 0.5	4.1 ± 2.4	2.6 ± 1.3	4.4 ± 1.7	3.8 ± 0.4	5.7 ± 1.2
18:3(n-6)	Gamma-linolenic acid	GLA	3.9 ± 0.7	3.2 ± 0.5	5.6 ± 1.0	5.9 ± 1.6	4.6 ± 0.4	4.1 ± 0.5	2.6 ± 0.1
20:4(n-6)	Arachidonic acid	ARA	10.2 ± 1.9	17.2 ± 1.3	7.3 ± 0.9*	13.3 ± 1.3	6.1 ± 0.7*	10.7 ± 3.8	6.0 ± 0.1
18:3(n-3)	Alpha-linolenic acid	ALA	0.8 ± 0.8	<0.5	<0.5	<0.5	0.7 ± 0.7	<0.5	<0.5
20:5(n-3)	Eicosapentaenoic acid	EPA	<0.5^{a}	<0.5^{a}	<0.5^{a}	<0.5^{a}	1.4 ± 0.7^{b}*	<0.5^{a}	4.0 ± 0.3^{c}*
22:5(n-3)	Docosapentaenoic acid	DPA	0.5 ± 0.2^{a}	<0.5	1.8 ± 0.0^{a}*	<0.5	3.5 ± 1.3^{a}*	<0.5	3.9 ± 0.5^{b}*
22:6(n-3)	Docosahexaenoic acid	DHA	3.0 ± 0.7	2.1 ± 0.2	4.8 ± 2.9	2.9 ± 2.5	5.8 ± 2.5	1.4 ± 0.2	7.4 ± 1.0*
total (n-6)[4]			20.1 ± 3.8	26.2 ± 2.1	16.9 ± 4.2	21.8 ± 4.0	15.2 ± 2.0	18.6 ± 3.9	14.2 ± 1.0
total (n-3)			4.2 ± 3.6	2.4 ± 0.6	6.6 ± 4.4	2.9 ± 2.5	11.4 ± 3.8*	1.4 ± 0.2	15.3 ± 1.9*

[1]Only the major fatty acids are shown.
[2] Values are means ± SEM, n=3. Within a diet group, means with superscripts without a common letter, differ, $P < 0.05$. *Different from control at that time, $P < 0.05$.
[3] When analyte was non-detectable, the limit of detection (0.5%) was employed for statistical purposes.
[4] Total (n-6) and total (n-3) fatty acids correspond to the sum of the PUFA shown in the table.

containing a diverge variety of fatty acid methyl-esters (FAME) were used to identify peaks within the samples (Appendix).

The results of the present study suggest that by modifying the lipid components of a diet, we can alter the fatty acid composition of those immune tissues. First of all, we observed that DHA diet increased n-3 PUFA concentrations in all tissues at the expense of n-6 PUFA. Additionally, we observed differences in the extent and rate of these changes among the various tissues. Finally, administration of the diets affected cell membrane levels of only those fatty acids that were present in higher amounts in the diets.

Lipids are among the main components of cell membranes, and affect cells' fluidity, architecture, and possibly cell signaling. Translocation of long chain fatty acids across the cell membranes is achieved by passive diffusion, or by certain membrane proteins. In general, membrane transport involves the following steps: absorption, membrane translocation and desorption of fatty acids in the cytoplasm. Translocation within the membrane might occur by diffusion or might involve a membrane associated protein (139). Free fatty acids in the cytoplasm are coupled with fatty acid binding proteins (FABP) that solubilize the fatty acids from membranes. Fatty acids are then esterified by long chain fatty acyl CoA synthetases. Fatty acid binding proteins differentially target fatty acids to phospholipids, triacylglycerols or cholesterol esters, according to cell type, and its function (139).

In the present study we observed that experimental supplementation with DHA enriched n-3 PUFA content in tissue membrane phospholipids. DHA diet increased total n-3 PUFA incorporation in all tissues examined and decreased ARA levels, and

subsequently total n-6 PUFA. Displacement of ARA from the tissue and replacement by EPA or DHA suggests a competition for incorporation of PUFA in membrane phospholipids. The displacement of ARA from membranes has important implications for the anti-inflammatory properties of fish oil by reducing the production of ARA-derived eicosanoids (67). Thus, incorporation of n-3 PUFA in membranes impacts inflammation and supports the contention that a change in the lipid profile of immune cells has a role in induction of inflammation.

Our results further suggest that the type of fat in the diet was reflected in the changes of the fatty acid profile of the tissues. DHA diet contained 6% fish oil, which consisted of 58% of DHA and 10% EPA. Not surprisingly, this diet increased mainly incorporation of DHA in membrane phospholipids and in a smaller degree incorporation of EPA. Control diet contained no n-3 PUFA and its consumption resulted in tissues with unchanged DHA levels. Additionally, control diet contained 6% high oleic acid safflower and resulted in significant increases of OA in all tissues. Although control diet contained higher amounts of LA than DHA diet, only in PP fatty acid profile was this differential incorporation observed.

Our study suggests that not all tissues exchange their fatty acids to the same extent. Spleen and PP were more responsive to changes in their fatty acid profile than peritoneal macrophages. Although peritoneal macrophages reached a maximum incorporation of n-3 PUFA comparable to spleen, this was apparent after 6 wk feeding, while it was apparent at 2 wk for spleen and PP. Moreover, ARA was reduced in all tissues studied, with PP and spleen being more responsive to changes than peritoneal macrophages. One possible explanation might be that these experimental samples are

consisted of different cell populations. Spleen contains lymphocytes and a great amount of erythrocytes that are not present in PP or in peritoneal macrophages. Each of these populations has different turnover rate. Thus, different cells might be more or less resistant to changes in their fatty acid profiles accordingly to their turnover rate (140).

In summary, our results suggest that the type of fat in a diet affects membrane fatty acid composition. PP and spleen had rapid changes of their n-3 PUFA within 2 wk, while peritoneal macrophages incorporated long chain fatty acids in a slower rate. Based on these results, we could say that 4 wk feeding period is sufficient for a good incorporation of n-3 PUFA into membrane phospholipids of major secondary lymphoid organs. Future studies on the impact of n-3 PUFA on the structural and functional characteristics of cell membranes are likely to increase our understanding of n-3 PUFA immunomodulatory effects.

CHAPTER 4. EFFECTS OF DOCOSAHEXAENOIC ACID CONSUMPTION ON ENTERIC REOVIRUS INFECTION: IMMUNOGLOBULIN RESPONSES AND VIRUS CLEARANCE

4.1 ABSTRACT

In addition to their well-known anti-inflammatory properties, n-3 polyunsaturated fatty acids (PUFA) have been reported to impair host resistance to infectious agents. We hypothesized that docosahexaenoic acid (DHA) consumption may reduce immunoglobulin responses to reovirus infection and therefore may reduce viral clearance. Mice were fed with either control AIN-93G diets or AIN-93G diets containing 3% DHA for 4 wk. They were then, orally gavaged with reovirus serotype 1, strain Lang (T1/L). Reovirus induced robust IgA and IgG2a responses within 7 to 14 days (d). Reovirus-specific IgA in fecal pellets increased similarly in control and DHA-fed infected mice from 2 to 8 d post infection (PI); however, at 10 d PI, DHA-fed mice secreted significantly more reovirus-specific IgA. Furthermore, specific IgA and IgG production did not differ in Peyer's patch (PP) and spleen cultures derived from the two groups at 14 d post-infection (PI). Reovirus-specific IgA and IgG2a titers were also not significantly different in sera collected from mice at 7 and 14 d PI. When plaque-forming units (PFU) were assessed in PP-depleted intestinal tissues, no significant differences were detected between the two groups. However, real-time PCR indicated that, reovirus (L2 gene) RNA/mg feces in infected DHA-fed mice was higher at day 2, 4 and 6 d PI than in infected control-fed mice but by 8 d PI, the virus was completely cleared in both groups.

Additionally, more viral RNA was detected in PP throughout the time course of the infection in DHA-fed mice. Taken together, these data suggest that in this murine model, DHA consumption transiently interfered with enteric reovirus clearance, but did not affect the humoral response to the virus or overall resolution of the intestinal infection.

4.2 RATIONALE

Dietary exposure to the mycotoxin deoxynivalenol (DON) induces upregulation of IgA production in the mouse and causes clinical signs analogous to early stages of human IgA nephropathy (IgAN) (25, 141, 142). Consumption of n-3 polyunsaturated fatty acids (PUFA)-enriched fish oil attenuates DON induced murine IgA nephropathy (IgAN) by suppressing the induction of proinflammatory cytokines involved in IgA dysregulation (13, 14, 143). In this study we investigated whether n-3 PUFA attenuate IgA production only in pathological situations, such as defined in the DON – IgAN model, or whether they also impair IgA and other mucosal responses to common pathogens. We used enteric reovirus infection model to examine the effects of a n-3 PUFA, docosahexaenoic acid (DHA), on immunoglobulin responses and viral clearance.

4.3 INTRODUCTION

Among the dietary lipids, n-3 PUFA derived from fish oil are considered to be particularly beneficial for human health. Nutritionists have long endorsed fish as part of a heart-healthy diet. In 2004 the Food and Drug Administration (FDA) gave "qualified

health claim" status to two n-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Specifically they stated that "supportive but not conclusive research shows that consumption of EPA and DHA omega-3 fatty acids may reduce the risk of coronary heart diseases" (1). Besides heart-related diseases (2-4), there is experimental and clinical evidence that other inflammatory diseases can be prevented by or treated with n-3 PUFA administration. These include rheumatoid arthritis (5), inflammatory bowel disease (IBD), colitis (6, 7) and immunoglobulin A nephropathy (IgAN) (8-15). There is also some indication that n-3 PUFA contribute to colon cancer prevention by increasing the cytotoxicity of several anticancer drugs and by reducing angiogenesis and induction of inflammation and metastasis (16, 144). Moreover, studies have shown that DHA promotes the normal brain development of the fetus and the newborn (17-19). The role of n-3 PUFA in neurological and psychological diseases has been another recent research focus (20-23).

Studies in our laboratory have used an experimental mouse model of human IgA nephropathy (IgAN) to examine the mechanisms of the beneficial effects of n-3 PUFA in retarding renal disease progression. In this model, a mycotoxin, deoxynivalenol (DON), specifically modulates mucosal immune responses of the mouse, targeting the development, differentiation, and homing of IgA-producing plasma cells in mucosal sites (24). DON dramatically increases IgA in the systemic compartment, where IgA is normally found in small amounts. Dietary supplementation with fish oil retards the progression of DON-induced IgAN- like disease in the mouse by reducing IgA immune complexes in blood and mesangial IgA deposition in kidney (14). The mechanisms by which n-3 PUFA mediate these effects are likely to involve altered balance of n-6 and n-3

derived eicosanoids and reduced inflammatory responses. Among the inflammatory responses generated by DON, interleukin 6 (IL-6), a critical cytokine in promoting differentiation and proliferation of plasma cell precursors, is reduced significantly in n-3 PUFA-fed mice (26). Therefore n-3 PUFA reduce the progression of the disease. However, it is not known whether fish oil reduces IgA production during normal responses to mucosal pathogens.

Numerous models have been used to evaluate immunosuppressive effects of fish oil and whether n-3 PUFA interfere with the clearance of infectious microorganisms (31). The type of n-3 PUFA, the dose and time period of supplementation, the nature of the pathogen, the route of administration, and genetic variation of the host can be factors determining whether n-3 PUFA impair, enhance, or have no effect on host resistance. Only a few studies with viral agents have been done to assess possible immunomodulatory effects of n-3 PUFA and to our knowledge, no studies have employed an enteric viral agent.

The viral models used to estimate possible modulatory effects of n-3 PUFA on host resistance include cytomegalovirus (CMV), murine retrovirus (MAIDS), and influenza virus. Mice supplemented with fish oil and then infected with CMV were just as susceptible to the infection as control mice (80). Mice fed with fish oil for 8 wk and then infected with MAIDS had significant enhanced survival compared to control mice (85). Using the same model Xi et al. (63) demonstrated that serum levels of IgG and IgM were reduced in mice fed fish oil. Additionally, fish oil consumption reduced virus induction of TNF-α, IL1-β, IL-2 and IFN-γ during the infection. In studies with influenza virus, Byleveld et al. (41) reported that fish oil feeding interfered with viral clearance

from lungs and reduced IgA secretion in the respiratory mucosal surfaces. Furthermore, they reported that IFN-γ mRNA expression was down regulated by fish oil supplementation. These studies suggest that fish oil can enhance or impair immune responses to viral infections depending on the virus used.

Respiratory enteric orphan virus (reovirus) belongs to the *Reoviridae* family, the largest of the six families of viruses with double stranded RNA genome. Type 1 L reovirus (T1/L) has been widely used as a model for an enteric infection (34-37, 86). Among other genera included in the same family is rotavirus, a human pathogen, which is leading cause of gastroenteritis among children and infants (108). Reovirus infection often occurs in humans, but most cases are mild or subclinical.

Intestinal infection by reovirus in the mouse is well-studied. Briefly, reovirus infects the small intestine with preference for the ileum (99). Reovirus entry is mediated through attachment to receptors on M cells residing on the dome of Peyer's patches (PP) (96). M cells conduct vesicular transport of the antigen from the mucosal surface into PP, a site where antigen presentation takes place and immune responses are initiated (90, 145). Reovirus infection induces robust antibody reponses, as evidenced by mucosal IgA production, as well as serum IgA and IgG production (100, 108, 115). Furthermore, reovirus induces strong cell-mediated responses in the gut as evidenced by the appearance of reovirus-specific CD4+ and CD8+ cytotoxic lymphocytes (CTLs) in PP and intraepithelial lymphocytes (IEL) (97, 100, 115). Oral exposure to reovirus induces a robust Th1 response mixed with some Th2 cytokines that is characterized by increased IFN-γ, IL-5 and IL-6 production in the PP (109, 111). Intestinal infection of wild type mice is a self-limited infection that is cleared within 7 to 10 days.

Reovirus has been successfully used to evaluate intestinal immunotoxicity (34-37). By using as infection model this well-characterized enteric virus in the mouse it should be possible to investigate nutritional modulation of normal immune responses. The objective of this study was to test the hypothesis that n-3 PUFA consumption will result to reduced immunoglobulin responses to reovirus infection and will interfere with viral clearance. The results suggest that n-3 PUFA did not markedly alter mucosal or systemic immunoglobulin production to reovirus and only transiently delayed clearance of the virus from the intestine.

4.4 MATERIALS AND METHODS

Chemicals and virus. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Reovirus serotype 1, strain Lang (T1/L) was used for all the experiments. The virus was grown in L929 fibroblast cells at 34°C in DMEM medium with 5% (v/v) fetal bovine serum (FBS; Atlanta Biologics, Atlanta, GA), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Third-passage plaque-purified virions were used for mouse infection and prepared by extraction with 1,1,2-trichloro-1,2,2-trifluoroethane followed by discontinuous CsCl gradient centrifugation (36, 102). Titers of the purified virus were determined by plaque assay as described by Cuff et al. (146).

Animals. Female B6C3F1 mice (4 wk old) were purchased from Charles River Laboratories (Portage, MI, USA). Housing, handling and sample collection procedures conformed to the policies and recommendations of MSU's Laboratory Animal Research Committee and were in accordance with guidelines established by the National Institutes

for Health. Mice were housed three per cage in a humidity (45-55%) and temperature (23-25°C) controlled university animal care facility room with a 12 hr light and dark cycle. Mice were acclimated for 1 wk prior to experiment initiation and fed experimental diets (detailed description provided later) until they were terminated. Reovirus-exposed mice were held in microisolator cages under negative pressure laminar flow in a Biosafety Level 2 room at the Michigan State University Research Containment Facility.

Diets. Experimental diets were based on the AIN-93G formulation (without oil) (Dyets Inc., Bethlehem, PA) of Reeves (136) with minor modifications. Both diets contained 920 g/kg AIN-93G, 10 g/kg AIN-93VX Vitamin Mix, and 10 g/kg corn oil (Dyets Inc., Bethlehem, PA). In control diets, 60 g/kg high oleic acid safflower oil (The Hain Celestial Group, Inc) were added, whereas in DHA diets 60 g/kg MEG-3^{TM} DHA-enriched oil (min 55 % DHA and 3 % EPA, Ocean Nutrition Canada Ltd.) were added. Diet composition of the main fatty acids as provided by the suppliers, is summarized in Table 3.1. Diets were prepared every 2 wk, stored in aliquots at -20°C. Fresh diet was fed to mice daily.

Experimental design and sample preparation. Mice were fed control and DHA diets for 4 wk prior to reovirus infection. They were then infected by oral gavage with $3x10^7$ plaque-forming units (PFU) in a total volume of 100 µl borate-buffered saline (pH 7.4) containing 2% (w/v) of gelatin (100). Mice were fed control or DHA diets unitl experimental termination. At specific time intervals, mice were bled from the lateral saphenous vein into heparinized tubes and fecal pellets were collected. Plasma was separated from blood samples and stored at 4°C for later analysis. Fecal pellets were stored at -20°C until analysis. Pellets were weighed and suspended in PBS to a 10%

(w/v) final concentration, held on ice for 2 h, and sonicated for 15 sec. Suspencions were centrifuged at 1,600 x *g* for 10 min at 4°C and 1.4 ml of the supernatant was cleared by centrifugation at 18,000 x *g* for 10 min at 4°C. Supernatants fractions were used directly for specific antibody detection by ELISA or for total RNA isolation.

Upon experiment termination, mice were euthanized with isoflurane, and exsanguinated via the caudal vena cava. PP, spleen and intestines were collected from the euthanized mice and were processed as described below.

Lymphoid fragment cultures. To assess *ex vivo* immunoglobulin secretion, cultures of PP, spleen and lamina propria were used (36). Briefly, 7 PP per mouse were removed from the intestine, pooled and washed three times in sterile HBSS (Gibco, Grand Island, NY) with 5 μg/ml of gentamicin and twice with tissue culture medium consisting of RPMI 1640 medium supplemented with 10% (v/v) FBS (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 0.5 μM 2-mercaptoethanol, 5 μg/ml of gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Individual patches were cut in half and incubated in 6-well plates with 2 ml of culture media for 5 days at 37°C under 5% CO_2 without additional stimulation.

Spleen cultures were washed with tissue culture media, cut into small pieces (2 x 2 mm) and incubated in 6-well plates with 2 ml of culture media for 5 days at 37°C under 5% CO_2 without additional stimulation. Culture supernatants were harvested and stored at 4°C for antibody detection by ELISA without further dilution.

Lamina propria cultures were established after removing PP, by cutting the intestine longitudinally into 2–3 cm long pieces. Fragments were washed three times with HBSS with 0.2% $NaHCO_3$, 0.1M HEPES and 5 μg/ml gentamicin. Half of the fragments

were stored in 1 ml of sterile PBS containing 0.5% gelatin (w/v) (gel saline) at –80°C to be used for viral titer detection. The other half of the fragments was incubated for 30 min twice in 5 mM EDTA in HBSS to remove the epithelial cells. Fragments were washed two more times with culture media and finally incubated in 6-well plates with 2 ml of culture media for 5 d at 37°C under 5% CO_2. Culture supernatants were harvested and stored at 4°C for antibody detection by ELISA without further dilution.

Virus plaque assay. Viral titers were measured by standard viral plaque assay (147). Briefly, tissues were placed in 1 ml of sterile gel saline, freeze-thawed 3 times, homogenized, and then sonicated. Serial dilutions (100 µl) in gel saline were incubated on monolayers of L929 fibroblasts in 12-well tissue culture plates for 45 min at 34°C and thereafter overlaid with 3 ml of 1% (w/v) agar in Medium 199 with 5% FBS (v/v) and cultured at 34°C. Plaques were visualized with neutral red and counted after 7 d incubation.

Real-time PCR. For viral RNA detection in fecal pellets, total RNA was extracted from 200 µl supernatant of 10% (w/v) fecal suspension using Trizol reagent (Invitrogen, Carlsbad, CA). PCR primers were selected from published sequence of λ2 core spike (L2 gene) of reovirus T1/L (88) as follows: forward, 5' CTG ACG TCG ATC AGG TCG TTG 3' and reverse, 5' GAT GTG GCA TGC ATG CAT GAG 3'. The size of the expected amplicon was 97 bp. To denature dsRNA, 1 µg of the template was incubated for 5 min at 95°C with 250 pM of random primers (Promega, Madison, WI) in a total volume of 12 µl and then cooled on ice. Reverse transcriptase reaction was performed by adding 4 µl of RT reaction buffer, 2 µl of 0.1M DTT, 1 µl of 10 mM dNTP, and 1 µl of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and incubating at 42°C for

60 min. Purified reoviruses were added into 10% (w/w) of fecal pellet suspension from vehicle mice at concentrations of 0, 10^1, 10^2, 10^3, 10^4,10^5 10^6, 10^7 PFU/ml for standard curve and sensitivity determination (36).

IFN-γ mRNA was detected in total RNA isolated from PP with Trizol reagent and treated with the Turbo DNA-free kit (Ambion, Austin, TX) according to the manufacturer's protocol. Probe and primers for mouse IFN-γ mRNA and 18S rRNA (endogenous control) were purchased from Taqman gene expression assays (Applied Biosystems, Foster City, CA). PCR reactions for IFN-γ mRNA and 18S rRNA quantification were performed on the ABI PRISM 7700 Sequence Detector System using the One-Step RT-PCR Master Mix Reagents kit according to manufacturer's protocol. Ct values were related to RNA concentrations using standard curves derived from serial dilutions of total RNA and normalized by dividing the IFN-γ mRNA Ct values by 18S rRNA Ct for each well. All the results were expressed relative to vehicle, control fed group, which was mock-infected group with PBS and sacrificed at the end of the experiment.

ELISA. Serum, culture fluids and supernatant of fecal suspensions were assayed for virus-specific antibodies by ELISA using a modification of the procedure of Major and Cuff (147). Briefly, 96-well plates were coated overnight at 4°C with 50 µl /well of purified reovirus T1/L, diluted to $1x10^8$ particles/ml in 0.1 M $NaHCO_3$ coating buffer (pH 9.6). Plates were washed four times with PBS with 0.1% Tween 20 (PBS-T) and blocked with 100 µl of blocking buffer (3% w/v bovine serum albumin, BSA in PBS-T) for 2 h at room temperature. Serial dilutions (100 µl) of serum, culture fluids, or fecal supernatants were added to each well in duplicates and incubated at 4°C for 16 h. The

plates were washed seven times with PBS-T, and 100 µl of goat anti-mouse IgG2a-, or IgA-HRP conjugates (1:3000) in blocking buffer was added to each well. Plates were incubated at room temperature for 2 h. Following seven washes with PBS-T, 100 µl of K-Blue Max TMB substrate (Neogen, Lansing, MI) was added to each well, and plates were incubated at room temperature for 5 min. Reactions were terminated by adding 50 µl of 2N H_2SO_4 and the absorbance was read at 450 nm on Vmax Microplate Reader (Molecular Devices Corp. Sunnyvale, CA). Absorbance at 450 nm was used as endpoint for fecal, PP, spleen and intestinal fragments cultures. Plasma antibody titers were defined as the last dilution yielded absorbance of 0.2 or higher at 450nm; a log of the dilutions was used to calculate the geometric means.

Statistics. We used 2-way ANOVA to determine the effects of time and diet. Multiple pairwise comparisons were performed using the Bonferroni corrected t test. Data were reported as means ± SEM. The critical level for normality and equal variance test was a = 0.01 and the critical level for the rest of the comparisons was a = 0.05. When used for statistical analysis, nondetectable (ND) data were assigned the limit of detection value. Data are representative of 2 separate experiments. Statistical analysis performed with the Sigma Stat Software version 9.0 (Jandel Scientific, San Rafael, CA).

4.5 RESULTS

DHA consumption does not affect intestinal response to oral reovirus challenge

Following oral reovirus infection, reovirus-specific IgA is produced in the lamina propria and is secreted into the lumen, contributing to early clearance of the virus from

the gut (100, 146) as well as protection against reinfection (102, 146). The reovirus-specific IgA responses in control and DHA-fed mice were compared over 10 d. In infected control and DHA-fed mice, reovirus-specific IgA antibody was detectable beginning 6 d post infection (PI) and was highly elevated at 8 d PI and 10 d PI (Fig. 4.1). Both infected control and DHA groups exhibited robust and similar induction of specific IgA. At 10 d PI, DHA-fed mice, secreted statistically significant levels of specific IgA compared to mice fed control diet.

Ex vivo antibody secretion by isolated PP was compared in the two groups after reovirus infection 14 d PI. Similar levels of reovirus-specific IgA (Fig. 4.2A) and IgG2a (Fig. 4.2B) were induced in both PP cultures from infected control- and DHA-fed mice. *Ex vivo* IgA secretion was also compared in lamina propria cultures. Both control and DHA-fed mice showed a similar robust induction of specific IgA at 5 and 10 d PI (Fig. 4.3). Secretion of reovirus-specific IgA from lamina propria cultures resembled the secretion of reovirus-IgA into lumen as evidenced by ELISA of the feces. In feces, IgA and IgG2a were also similar in control- and DHA- fed mice with the only difference being observed at 10 d PI, where the response was higher in DHA-fed mice. Overall, these data suggest that the induction of mucosal IgA was not significantly affected by DHA supplementation in the diet.

DHA consumption does not affect the systemic humoral responses to reovirus

Reovirus infection by the oral route results not only in production of mucosal antibodies, but also in circulating specific IgA, IgG and IgM in serum (108). These antibodies enhance protection against systemic spread of the infection and also contribute

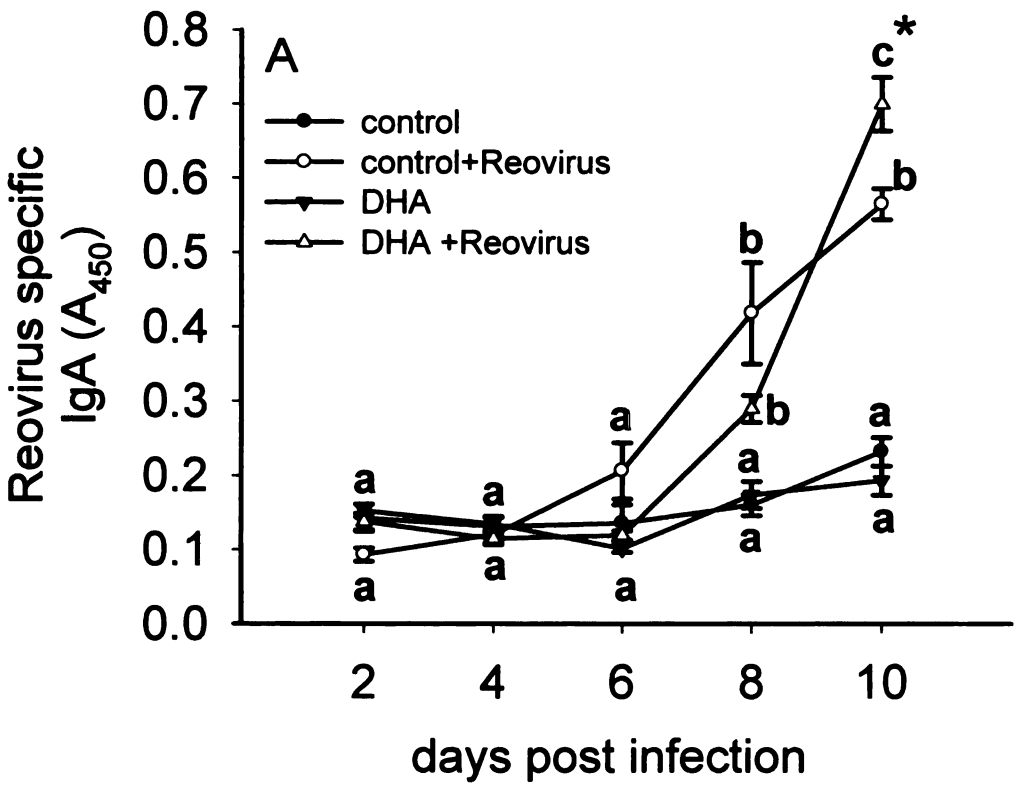

Figure 4.1. Effects of DHA on fecal IgA levels in response to reovirus. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. ELISA absorbances for reovirus-specific IgA in feces collected at different time points PI. Values are means ± SEM, n = 4. Within a group, means without a common letter differ, $P < 0.05$. * Different from infected mice fed a control diet at that time, $P < 0.05$.

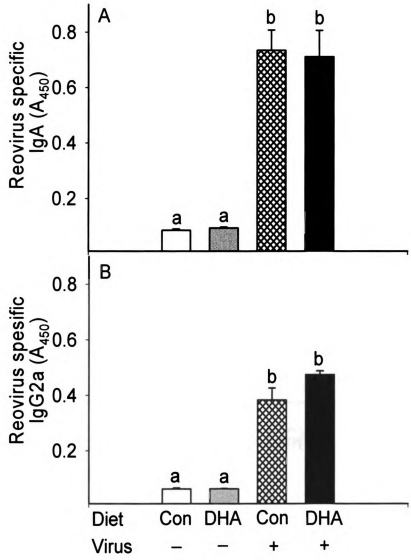

Figure 4.2. Effects of DHA on the production of virus-specific antibodies in ex vivo culture of PP. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. PP were collected at 14 d PI and production of reovirus-specific IgA (A) and IgG2a (B) were determined in ex vivo cultures. Values are means ± SEM, n = 6. Within a group, means without a common letter differ, $P < 0.05$.

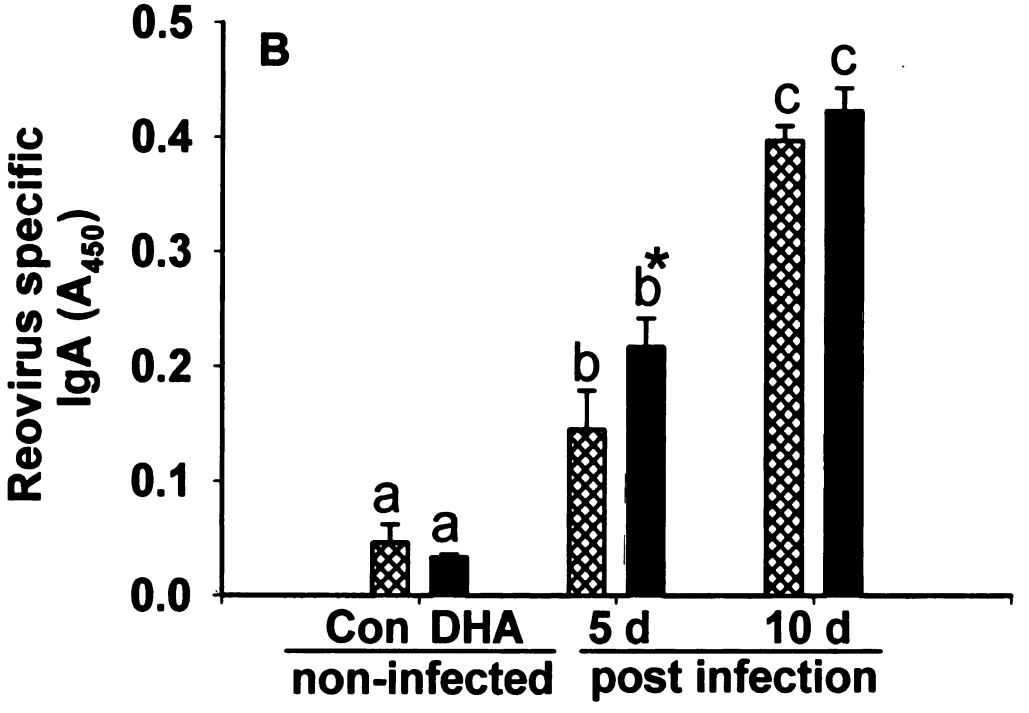

Figure 4.3. Effects of DHA on the production of reovirus-specific IgA in lamina propria cultures. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. Intestines were removed at 0, 5 and 10 d PI and processed to *ex vivo* cultures that were incubated at 37°C under 5% CO_2 for 5 d with no additional stimulation. Values are means ± SEM, n = 6. Within a group, means without a common letter differ, $P < 0.05$. * Different from infected mice fed a control diet at that time, $P < 0.05$.

to immunological memory (101, 102, 108). When reovirus-specific responses were assessed at 7 and 14 d PI, both serum IgA and IgG2a antibodies were induced after infection but were not affected by DHA supplementation (Fig. 4.4). Similar results were observed for reovirus-specific IgA and IgG2a production in spleen cultures obtained from control and DHA- fed mice at 14 d PI (Fig. 4.5). Thus, as observed for mucosal Ig responses, antibody induction in the systemic compartment was not significantly affected by DHA supplementation.

Effects of DHA on viral presence and clearance from intestinal tissues

Reovirus infection causes a self-limited infection that immunocompetent, adult mice are able to overcome within 10 d (87). Reovirus is shed into the intestinal lumen during infection and it is not carried asymptomatically after the course of the infection. It thus, can be continuously monitored in fecal pellets by real time PCR during the course of the disease. Mice fed DHA diet had a significantly higher fecal viral burden than control-fed mice as evidenced by nearly one log more viral RNA in their feces at 2, 4, and 6 d PI (Fig. 4.6). However, by 8 d PI viral RNA was undetectable in both groups suggesting that the ability to ultimately clear the infection was not affected.

To determine if DHA interfered with viral replication in PP, PP were collected at 1, 3, and 7 d PI and total RNA assessed for reovirus RNA. At 1 d PI, both groups had same load of viral RNA in their PP, indicating no differences in the entry and uptake of reovirus. However, at 3 d PI, significantly more viral RNA was detected in the DHA-fed group. While there was no detectable viral RNA in the infected control group at 7 d PI, the infected DHA group had nearly $6x10^4$ copies of viral RNA/μg total RNA (Fig. 4.7).

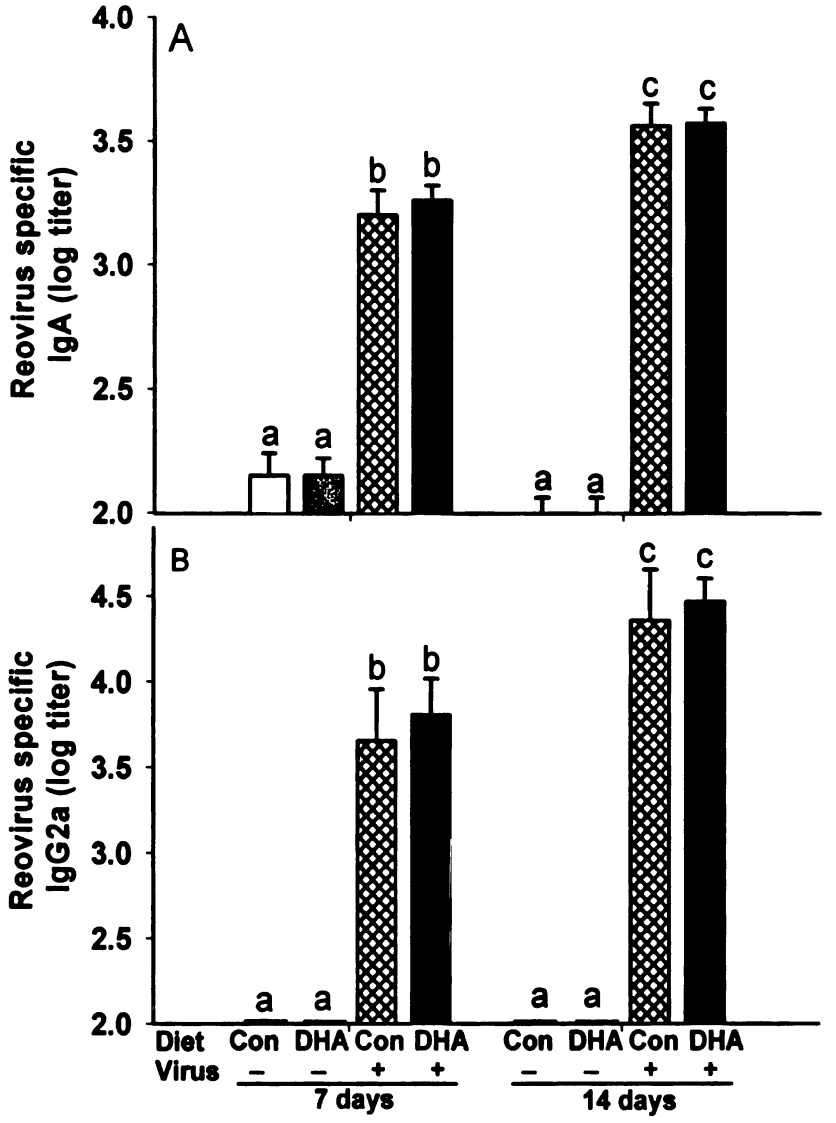

Figure 4.4. Effects of DHA on generation of plasma virus-specific antibody responses. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. Blood was collected at 7 and 14 d PI and specific IgA (A) and IgG2a (B) titers determined in plasma by ELISA, n = 6. Values are means ± SEM, n = 6. Within a group, means without a common letter differ, $P < 0.05$.

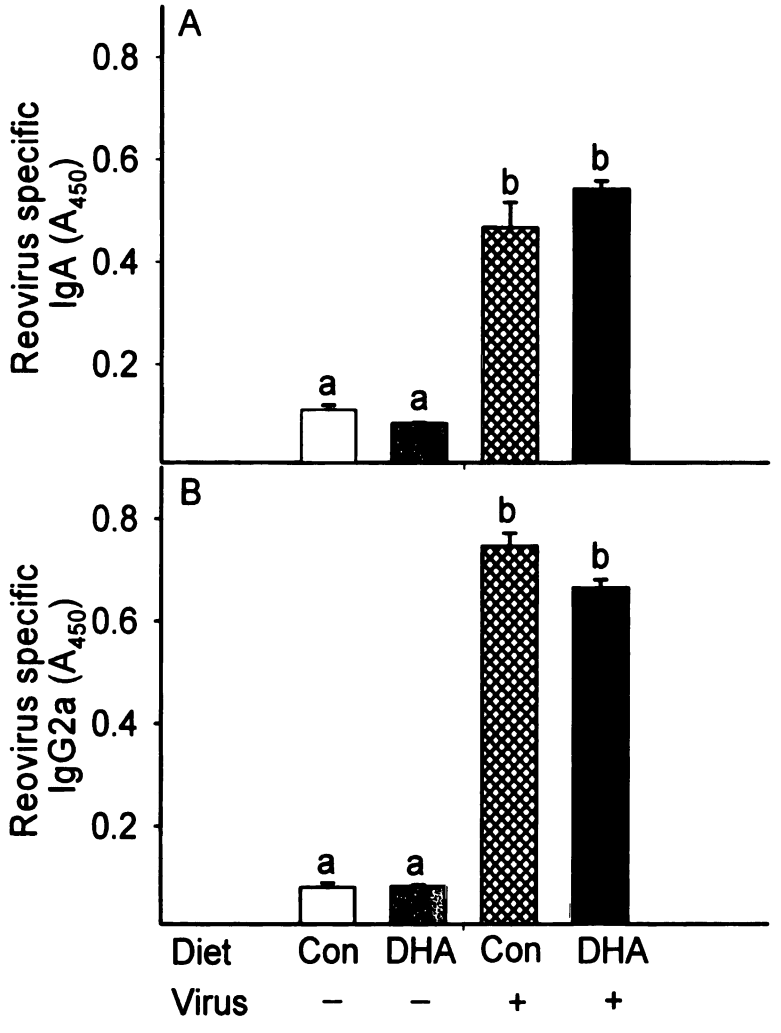

Figure 4.5. Effects of DHA on the production of reovirus-specific IgA and IgG2a in *ex vivo* spleen cultures. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. Spleens were collected at 14 d PI and production reovirus-specific IgA (A) and IgG2a (B) were determined in ex vivo cultures. Values are means ± SEM, n =6. Within a group, means without a common letter differ, $P < 0.05$.

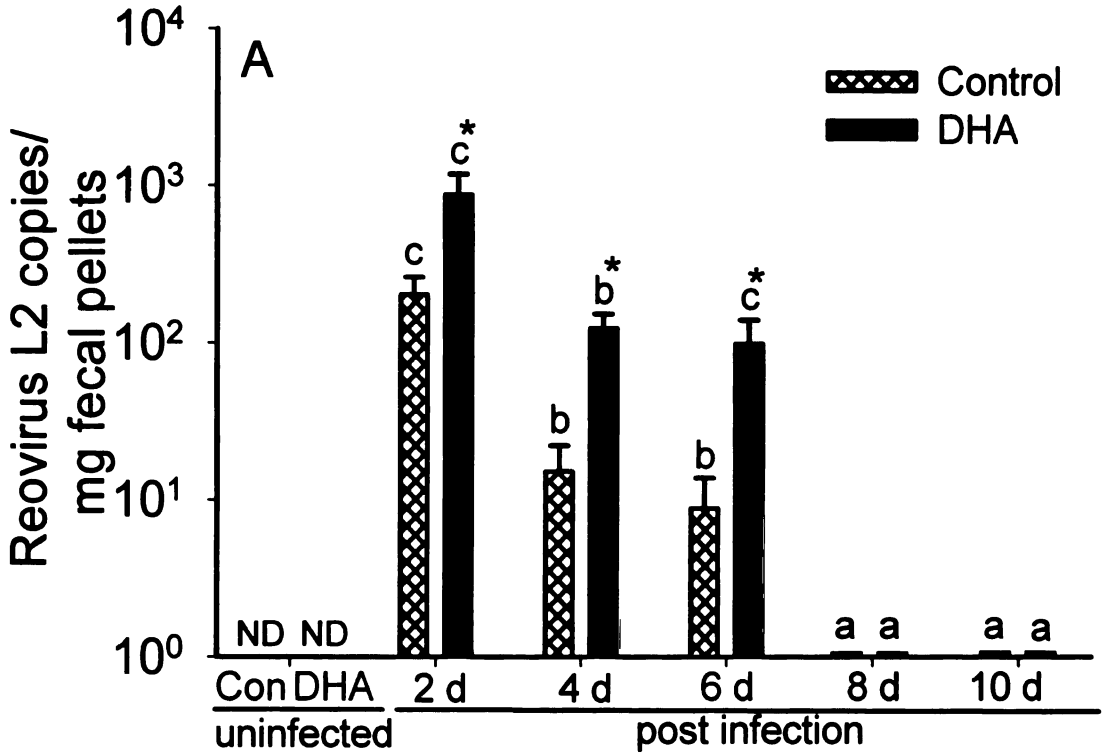

Figure 4.6. Effect of DHA diet on reovirus L2 RNA in feces. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. . Fecal pellets were collected at intervals and total RNA analyzed by PCR, n = 4. Values are means ± SEM, n = 6. ND, not detectable. Within a group, means without a common letter differ, $P < 0.05$. *Different from infected mice fed a control diet at that time, $P < 0.05$.

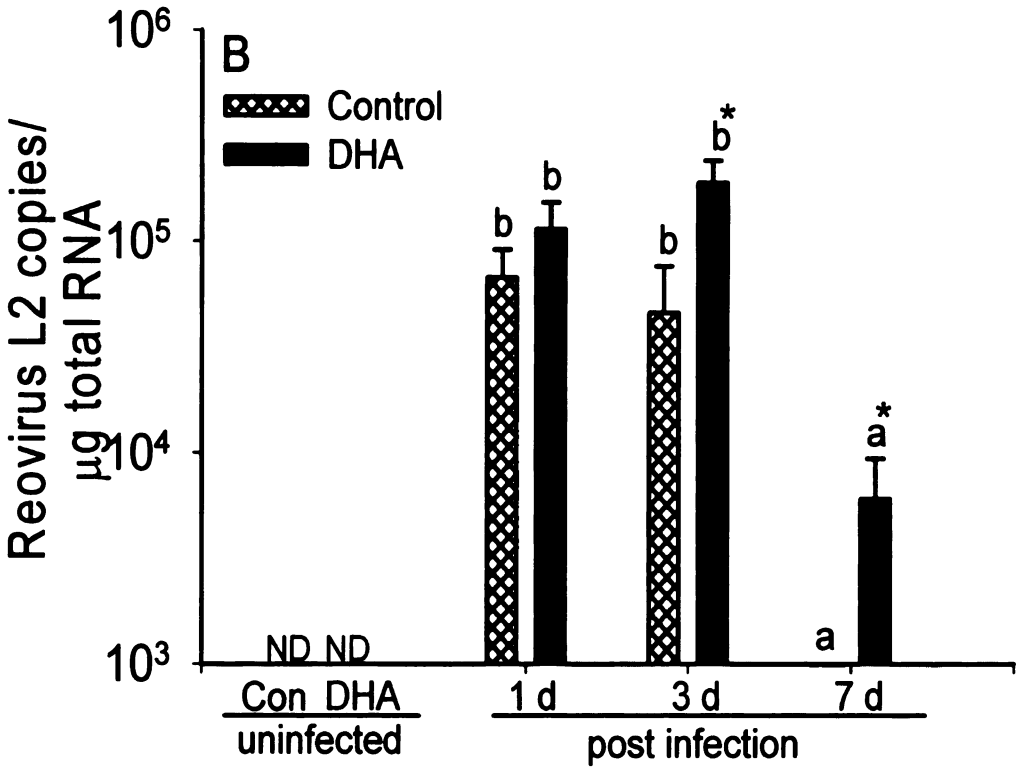

Figure 4.7. Effect of DHA diet on reovirus L2 RNA in PP. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. PP were collected at 0, 1, 3, and 7 d PI and total RNA analyzed by PCR. Values are means ± SEM, n = 6. ND, not detectable. Within a group, means without a common letter differ, $P < 0.05$. *Different from infected mice fed a control diet at that time, $P < 0.05$.

A plaque assay was used to determine if viral RNA levels in feces corresponded with higher numbers of viral particles in intestinal tissues after the removal of PP. Both infected control and DHA groups carried a virus load of almost 10^5 PFU/intestine at 5 d PI, which was reduced by one log at 10 d PI (Fig. 4.8). No statistical differences were detected between control and DHA-fed mice. These data suggest that while more viral RNA was detected into the intestinal lumen of DHA-fed mice, the high DHA diet did not increase the number of reovirus infectious units in the intestine.

To determine if the expression of IFN-γ must have a role on the differences observed for viral RNA in PP, we assessed IFN-γ mRNA expression in PP at 1, 3, and 7 d PI. No significant differences on the IFN-γ relative expression were detected although there was a trend for the DHA fed mice to have lowered IFN-γ expression at 3d PI (p = 0.178) and at 7d PI (p= 0.193) (Fig. 4.9).

4.6 DISCUSSION

Nutritional interventions can potentially shape the immune responses, increasing or decreasing susceptibility to infections. In this study we tested the hypothesis that n-3 PUFA consumption will result to reduced immunoglobulin responses to reovirus infection and will interfere with viral clearance. The gut mucosal lymphoid tissue responds to reovirus infection with both secretion of antibodies and activation of cytotoxic T-cells. In the gut, PP have key role in the induction of immune responses.

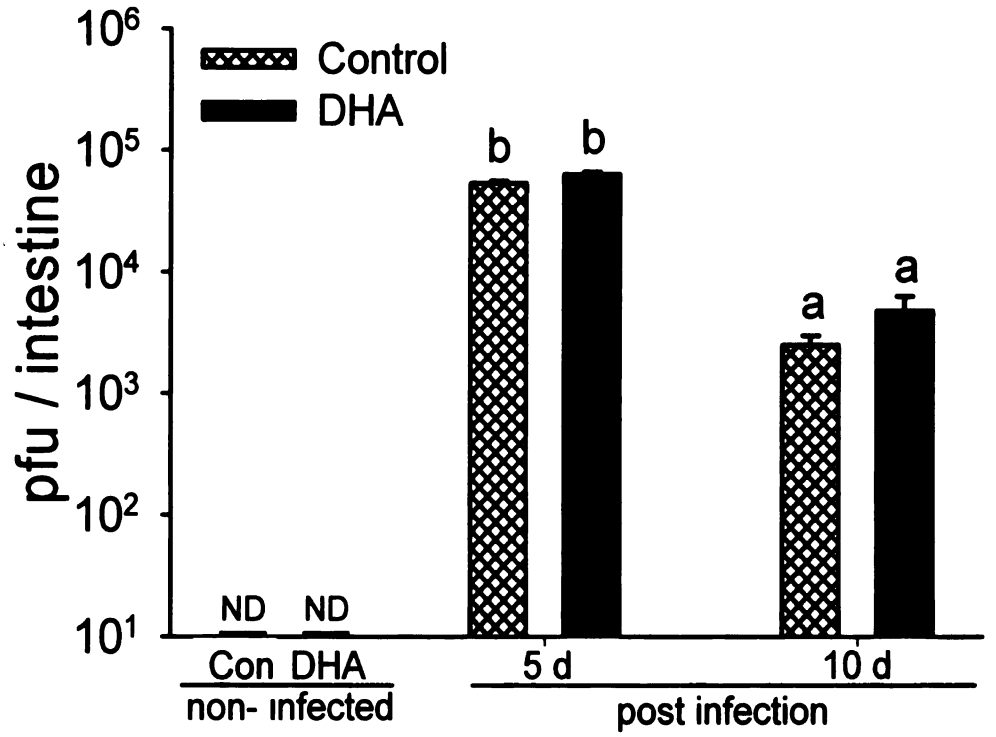

Figure 4.8. Effect of DHA diet on infectious reovirus in intestinal tissues. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. Intestines were removed at 0, 5 and 10 d PI and processed to determine virus titers. Values are means ± SEM, n = 6. ND, not detectable. Within a group, means without a common letter differ, $P < 0.05$.

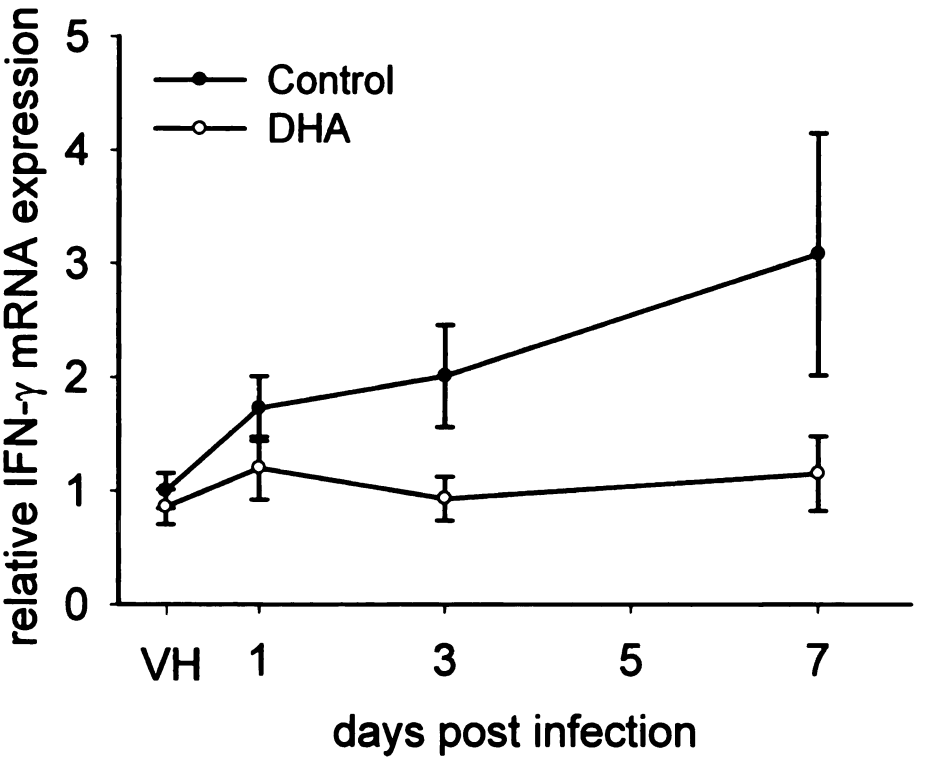

Figure 4.9. Effect of DHA diet on IFN-γ mRNA expression in PP. Mice were fed with DHA and control diet for 4 wk before infection with $3x10^7$ PFU of reovirus. PP were collected at 0, 1, 3 and 7 d PI. Total RNA isolated and IFNγ mRNA was detected by RT-PCR. Data are means ± SEM, n=6 of IFN-γ mRNA copies per μg RNA. The data were normalized to the uninfected control diet group.

They are the first site of reovirus infection, contain aggregated lymphocytes, elicit important innate immune responses and alert the immune system for reovirus invasion. Reovirus antigens are taken up in this site and transfer to spleen where activation of B and T-cells takes place. Thus, in PP both humoral and cytotoxic responses are initiated. During reovirus infection, IgA producing cells are increased and robust specific-IgA responses are elicited in PP (100). Reovirus-specific IgA contributes to clearance of the virus from the gut (100, 146) and protects against reinfection (102). Additionally, production of circulating specific IgA, IgG and IgM, enhance protection against systemic spread of the infection and contribute to immunological memory (101, 102, 108).

The effects of n-3 PUFA on the induction of antibodies at the mucosal sites are not well understood. Supplementation with n-3 PUFA significantly attenuated DON-induced IgA elevation in serum resulted from dysregulation of the mucosal immune responses (13, 14). Furthermore, induction of influenza-specific lung IgA and serum IgG were reduced in mice fed fish oil (41). However, some studies imply that supplementation with fish oil has no effect on antibody secretion. For example, fish oil supplementation had no significant effect on secretion of IgA in breast milk, yet, it was positively correlated with increased IgA (42). Additionally, patients suffering from distal proctocolitis have similar percentages of IgA and IgG containing cells in the rectal mucosa with administration of n-3 PUFA, while a significant reduction in IgM containing cells was observed (7).

The present study demonstrates that fish oil did not deleteriously impact the kinetics of virus-specific antibody secretion in the mucosal compartment. This was consistent with our inability to see differences in antibody production from PP, lamina

propria and spleen cultures. However, DHA-fed mice secreted statistically significant more reovirus-specific IgA at 10 d PI in the intestinal lumen as detected in fecal samples (Fig. 4.1). This extended elevation of antibody secretion for the DHA group could be related to increased shedding of reovirus during the infection among the DHA-fed mice.

It was notably that more viral RNA was detected in feces and PP during the infection in DHA-fed mice. However, we did not observe differences on the infectious particles detected in intestinal tissues by plaque assay. It could be speculated that due to reduced antiviral responses, DHA permitted higher viral RNA replication but there was insufficient assembly of mature infectious viruses in the intestinal epithelial cells. Another possibility could be related to epithelial cells which are not a mere passive barrier but produce many cytokines potentially affecting the balance between crypt cell proliferation and epithelial cell death (148). A Th1 cytokine response induces epithelia cell proliferation while a Th2 response induces apoptosis (149, 150). Since n-3 PUFA have been shown to suppress Th1 responses (151), they might result in enhanced turn-over rates of differentiated epithelial cells shed into the lumen causing more viral RNA to be detected in feces. Both of these hypotheses need further investigation. Overall though, the results suggest only a transient effect of n-3 PUFA on viral clearance and no effect on the overall host's ability to resolve the infection.

In addition to antibody secretion and viral clearance, the profile of cytokines induced upon infection determines the outcome of the disease. Here we only detected IFN-γ mRNA expression in PP to see if a polarized Th1 response is altered by the DHA diet. We observed a trend towards lower IFN-γ expression in DHA-fed mice that was not proven statistically. It is known that supplementation with n-3 PUFA reduces expression

of Th1 cytokines, such as IL-1, IL-2, TNF-α, and IFN-γ *ex vivo* (41, 44, 60-62). More specifically, mice fed with fish oil and infected with influenza virus exhibit impaired IFN-γ production in the lung (41). Furthermore, mice fed with fish oil and infected with MAIDS had reduced TNF-α, IL-1β, IL-2 and IFN-γ in spleen cultures (63). Reduced IFN-γ production was shown also in studies employing fish oil with *Listeria monocytogenes* (64, 65), sometimes even before the infection (65). Overall these studies are consistent with the trend toward reduction in IFN-γ observed here.

Viral clearance in the intestine is facilitated by several immune responses elicited by multiple cells. The classic view of the immune responses focuses on the production of antibodies and cell cytotoxicity. Reovirus-specific IgA production protects against reinfection but its role in the clearance of the initial infection might not be indispensable (102). Thus, n-3 PUFA effects on IgA secretion in the mucosa might not directly affect early clearance of reovirus. In contrast, a diminished cytotoxic T-cell response might be detrimental for the clearance of reovirus infection. Besides, n-3 PUFA has been previously related to reduced cytotoxic responses (46, 48).

Specifically for the mucosal system, cytotoxic responses involve cells that are activated by both thymus-dependent and thymus-independent mechanism. *In vitro* studies show that treatment with n-3 PUFA reduces the expression of major histocompatibility complex (MHC) molecules on antigen presenting cell (APC) surfaces, a key mechanism for thymus-dependant activation of cytotoxic T-cells (53-55, 102). Treatment with DHA results also in B-cells with reduced levels of MHC-I on their surface, possible interfering with their ability to present antigens (55). Furthermore, n-3 PUFA can alter critical intracellular signaling pathways and cytokine production patterns and thus direct essential

cell-differentiation steps involving in dendritic cell maturation (56). Such interference with antigen presentation could impair the cellular immune responses to an infection and possibly delay viral clearance.

Byleveld et al, (2000) showed that with fish oil supplementation, the cytotoxicity of lung influenza-specific T-cells significantly reduced (46). However, in the mucosal system thymus-independent cytotoxic cells contribute equally if not in a higher degree in reovirus clearance (115). These cells are activated in a MHC-I independent way, and lowered expression of those molecules on APC might not be reflected as impairment of the cellular cytotoxic responses. Thus, fish oil might not be as potent suppressor of viral clearance in the intestine as is considered for bacteria in agreement with our study, where only a transient delay was observed for reovirus clearance. Moreover, reovirus infection in mice with non-functional MHC-I molecules generates a robust reovirus-specific antibody production (147), further suggesting that possible reduction of MHC-I molecule expression by fish oil might not be affect the antibody responses, in agreement again with our results. However, the effect of fish oil on antigen presentation is not clearly understood and *in vivo* experiments need to be done.

In addition to the classical adaptive immune responses described earlier, focus lately has been given to the contribution of the innate immune responses to host resistance to infection. Among the innate responses type I IFNs seem to be indispensable for reovirus clearance in the intestine in contrast to natural killer cell activation or TLR signaling {Johansson, 2007 355 /id}. IFN-α and IFN-β are produced within the first hours of the infection, drive the infected cell into an antiviral state and direct an effective adaptive response to clear the pathogen. Upon reovirus infection, type I IFNs are mainly

produced in PP, where reovirus is encountered for the first time (117). Fish oil supplementation and infection with influenza virus had no adverse effects on IFNα/β expression in the lung (41), but the impact of n-3 PUFA on IFN type I cytokines in the gut mucosal is not well-known. The effect of fish oil on this type of innate responses in the intestine is another possible mechanism that needs further investigation to understand the observed higher viral load for the DHA-fed mice.

The amount of n-3 PUFA consumed by mice in this study exceeded that normally consumed by humans. Typical n-3 PUFA intake recommendations for healthy people are 0.3 - 0.5 g/d for DHA and EPA along with 0.8 - 1.1 g/d for α- linolenic acid, while 3 g/d is recommended for disease prophylaxis and therapy (15). The dose used in the study corresponds almost five times the maximum recommended consumption levels of n-3 PUFA for humans and it would be similar to a 16 g/d consumption of DHA and EPA combined (15). Nevertheless, the observation that this high dose did not affect antibody production and overall viral clearance suggests that immunosuppression of the mucosal immune responses would not be a major safety concern.

Overall our studies suggest that while DHA consumption transiently delayed viral clearance, robust reovirus-specific antibody responses were generated and thus, virus shedding ultimately cleared in both the groups. Future studies with enteric viral models are essential to understand how nutritional interventions exert immunomodulatory effects on the mucosal system and how the immune system compensates for any alteration among its normal functions.

APPENDIX

Table 1. The following reference standard was purchased from Supelco, PA. The information presented here is what the company provided us.

GLC REFERENCE STANDARD PUFA-1, marine source Cat #47033		
CHAIN		**ITEM**
C14:0	METHYL	MYRISTATE
C16:1	METHYL	PALMITOLEATE
C18:0	METHYL	STEARATE
C18:1n9	METHYL	OLEATE
C18:1n7	METHYL	VACCENATE
C18:2n6	METHYL	LINOLEATE
C18:4n3	METHYL	STEARIDONATE
C20:1n9	METHYL	11-EICOSENOATE
C20:5n3	METHYL	EICOSAPENTAENOATE
C22:1n11	METHYL	13-DOCOSENOATE
C22:1n9	METHYL	11-DOCOSENOATE
C22:5n3	METHYL	DOCOSAPENTAENOATE
C22:6n3	METHYL	DOCOSAHEXANEONATE
C24:1n9	METHYL	NERVONATE

Table 2. The following reference standard was purchased from Supelco, PA. The information presented here is what the company provided us.

GLC REFERENCE STANDARD PUFA-2, animal source Cat #47015-U		
CHAIN		**ITEM**
C14:0	METHYL	MYRISTATE
C16:0	METHYL	PALMITATE
C16:1n7	METHYL	PALMITOLEONATE
C18:0	METHYL	STEARATE
C18:1n9	METHYL	OLEATE
C18:1n7	METHYL	VACCENATE
C18:2n6	METHYL	LINOLEATE
C18:3n6	METHYL	G-LINOLENATE
C18:3n3	METHYL	LINOLENATE
C20:1n9	METHYL	11-EICOSENOATE
C20:4n6	METHYL	ARACHIDONATE
C20:5n3	METHYL	EICOSAPENTAENOATE
C22:4n6	METHYL	ADRENATE
C22:6n3	METHYL	DOCOSAHEXANEONATE

Table 3. The following reference standard was purchased from Nu-CHECK, MN. The information presented here is what the company provided us.

GLC REFERENCE STANDARD SPECIAL PREPARATION Cat # 538		
CHAIN		**ITEM**
C14:0	METHYL	MYRISTATE
C14:1	METHYL	MYRISTOLEATE
C16:0	METHYL	PALMITATE
C16:1	METHYL	PALMITOLEATE
C18:0	METHYL	STEARATE
C18:1	METHYL	OLEATE
C18:1	METHYL	VACCENATE
C18:2	METHYL	LINOLEATE
C18:3	METHYL	LINOLENATE
C20:0	METHYL	ARACHIDATE
C20:1	METHYL	11-EICOSENOATE
C20:2	METHYL	11-14 EICOSADIENOATE
C20:3	METHYL	11-14-17 EICOSATRIENOATE
C20:4	METHYL	ARACHIDONATE
C20:5	METHYL	EICOSAPENTAENOATE
C22:0	METHYL	BEHENATE
C22:1	METHYL	ERUCATE
C22:6	METHYL	DOCOSAHEXAENOATE
C23:0	METHYL	TRICOSANOATE
C24:0	METHYL	LIGNOCERATE
C24:1	METHYL	NERVONATE

Table 4. The following reference standard was purchased from Nu-CHECK, MN. The information presented here is what the company provided us.

GLC REFERENCE STANDARD

SPECIAL PREPARATION
Cat # 463

CHAIN	ITEM	
C4:0	METHYL	BUTYRATE
C5:0	METHYL	PENTANOATE
C6:0	METHYL	CAPRONATE
C7:0	METHYL	HEPTANOATE
C8:0	METHYL	CAPRYLATE
C9:0	METHYL	NONANOATE
C10:0	METHYL	CAPRATE
C11:0	METHYL	UNDECANOATE
C11:1	METHYL	UNDECENOATE
C12:0	METHYL	LAURATE
C12:1	METHYL	DODECENOATE
C13:0	METHYL	TRIDECANOATE
C13:1	METHYL	TRIDECENOATE
C14:0	METHYL	MYRISTATE
C14:1	METHYL	MYRISTOLEATE
C15:0	METHYL	PENTADECANOATE
C15:1	METHYL	PENTADECENOATE
C16:0	METHYL	PALMITATE
C16:1	METHYL	PALMITOLEATE
C16:1T	METHYL	PALMITELAIDATE
C17:0	METHYL	HEPTADECANOATE
C17:1	METHYL	10-HEPTADECENOATE
C18:0	METHYL	STEARATE
C18:1	METHYL	OLEATE
C18:1T	METHYL	EALIDATE
C18:1	METHYL	PETROSELINATE
C18:1	METHYL	VACCENATE
C18:1T	METHYL	TRANSVACCENATE
C18:2	METHYL	LINOLEATE
C18:2T	METHYL	LINOELAIDATE
C18:3	METHYL	GAMMA-LINOLENATE
C19:0	METHYL	NONADECANOATE
C19:1	METHYL	7-NONADECENOATE

Table 4. Continued

C18:3	METHYL	LINOLENATE
C20:0	METHYL	ARACHIDATE
C20:1	METHYL	5-EICOSENOATE
C20:1	METHYL	8-EICOSENOATE
C20:1	METHYL	11-EICOSENOATE
C20:2	METHYL	11-14 EICOSADIENOATE
C20:3	METHYL	HOMOGAMMA LINOLENATE
C20:4	METHYL	ARACHIDONATE
C20:3	METHYL	EICOSATRIENOATE
C22:0	METHYL	BEHENATE
C22:1	METHYL	ERUCATE
C20:5	METHYL	EICOSAPENTAENOATE
C22:2	METHYL	DOCOSADIENOATE
C22:3	METHYL	DOCOSATRIENOATE
C22:4	METHYL	DOCOSATETRAENOATE
C24:0	METHYL	LIGNOCERATE
C22:5	METHYL	DOCOSAPENTAENOATE
C22:6	METHYL	DOCOSAHEXAENOATE
C24:1	METHYL	NERVONATE

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