EFFECTS OF CALORIE RESTRICTION AND ω -3 DIETARY FAT ON AGING IN SHORT- AND LONG-LIVED RODENTS

Dean A. Troyer¹, Jaya T. Venkatraman²,
and Gabriel Fernandes³

Departments of ¹Pathology and ³Medicine
The University of Texas Health Science Center
at San Antonio,

7703 Floyd Curl Drive
San Antonio, TX 7828

and

²Department of Physical Therapy, Exercise
and Nutrition Sciences,

State University of New York at Buffalo
301 Parker Hall, Buffalo, NY 14214

ABSTRACT

Aging is accompanied by a steady increase in the incidence of spontaneous tumors and a decline in immune function. Calorie restriction (CR) or supplementation with ω -3 fats prolongs life span, suppresses tumorigenesis, and ameliorates immune function in a variety of experimental models. We suggest that decreased oxidant stress and upregulation of apoptosis mediate the effects of calorie restriction on immunity and longevity. CR prolongs life span in several animal models and our studies have examined the effects of CR on the immune system and on tumorigenesis. CR maintains naive T cells, prevents the rise in "double-negative" T cells, maintains lymphocyte responsiveness to mitogens, and preserves Dexamethasone induced apoptosis in spleen cells of MRL/lpr mice. CR also modulates the expression of inflammatory mediators and cytokines. CR decreases the Sjögren's syndrome-like chronic inflammation of salivary glands of B/W animals while increasing expression of the immunosuppressive cytokine TGF β 1 and decreasing expression of the pro-inflammatory cytokines IL-6 and TNF α . The autoimmune disease in the B/W mouse also affects the kidneys, and we find that renal expression of platelet derived growth factor-A, (PDGF-A) and thrombin receptor are decreased in CR animals. Similarly, CR decreases the expression and localization of plasminogen activator inhibitor type 1 in glomeruli of B/W animals. CR also modulates expression and function of androgen receptors and the binding of insulin to liver nuclei. Finally, CR suppresses the development of breast tumors in the Ras oncomouse. These effects of calorie restriction are paralleled in short-lived B/W animals fed diets supplemented with ω -3 fatty acids. Omega-3 fatty acids induce the expression of hepatic antioxidant enzymes, and enhance apoptosis in lymphocytes of B/W animals.

To whom all correspondence should be addressed:
Gabriel Fernandes, Ph.D.; Department of Medicine; Clinical Immunology; The University of Texas Health Science Center at San Antonio; 770 Floyd Curl Drive; San Antonio, TX 78284-7874. Telephone: (210) 567-4663; Fax: (210) 567-4592

CALORIE RESTRICTION

*Effects of CR on the NZBxNZWF1 (B/W)
and MRL/lpr Mice*

Restriction of calories (without nutritional deficiency or malnutrition) remarkably retards age related decrements in immune function (1), delays tumorigenesis (2) and prolongs life span (3). The initial observations in rats by McCay (4) have been confirmed and extended to several other species (3). In our studies, the defined standard diet contains 5% fat and is pair fed to restricted and ad libitum animals beginning at 4 weeks of age (5). Caloric restriction is achieved by reduction of caloric intake to 40% of ad libitum over a four week period. The only dietary constituent that is altered in restricted animals is the vitamin content, which is increased proportionately to compensate for reduced total food intake. Both the relative amounts and sources of protein, carbohydrate, and fats can influence longevity (6), however, repeated studies in both short and long-lived rodent strains have demonstrated the efficacy of reduced energy intake (7), indicating the wide applicability of calorie restriction in animal studies. The effects of calorie restriction are not thought to be due to malnutrition or starvation. The body weights of calorie restricted animals plateau or stabilize (but do not decline) whereas ad libitum fed animals continue to gain weight into, indicating that the animals are not undernourished from a caloric standpoint (8). In fact, caloric intake per kilogram body weight is virtually the same in calorie restricted and ad libitum fed animals (5). Although the mechanisms involved in amelioration of the aging process and leading to prolongation of life span by decreased energy intake remain obscure, experimental evidence indicates that the immune system and the process of tumorigenesis are affected by CR.

Decreased energy intake ameliorates the progressive glomerular and vascular disease in the autoimmune-prone B/W mouse which develops immunological and renal disease that resembles human lupus erythematosus (9). Although the causes of lupus and autoimmunity remain poorly understood, it is clear that the immune system plays an important role in this disease. Our

previous studies have indicated that CR prevents the loss of helper T-cell and cytotoxic T-cell function in B/W mice (9). Further, memory (Th-2) cells, more likely to be autoreactive, are increased in *ad libitum* fed B/W animals while CR fed mice have more naive T (Th-1) cells. Splenocytes from B/W mice fed a CR diet (vs. *ad libitum* feeding) also maintain greater responsiveness to mitogens, including Con A (1,9). The autoimmune disease of the B/W mice also manifests itself in the salivary glands, and we find that in older mice, CR increases the expression of TGF β 1 in salivary glands (10). This was paralleled by a decrease in the expression of the proinflammatory cytokines IL-6 and TNF α . We note here that while the role of TGF β as a critical modulator of the immune system is undisputed, increased TGF β may affect different organs differently. Overproduction of TGF β in the kidneys may promote inflammation and fibrosis while TGF β may be a beneficial immunomodulator of autoimmune disease in lymphocytes, particularly in the context of autoimmune diseases (11). Thus, TGF β is secreted by many cell types, including activated T and B lymphocytes and mediates a negative feedback loop to inhibit T cell activation in response to IL-1 (12). TGF β also inhibits proliferation and antibody secretion from B cells (9). On the other hand, TGF β may play a central role in the complex pathology of the kidney observed in B/W mice as a promoter of inflammation and glomerular pathology (11). In summary, CR is a powerful modulator of disease in the B/W mouse which is a close model of the human autoimmune diseases, lupus erythematosus and Sjogren's syndrome. CR also has important effects on inflammatory mediators in the kidneys of B/W mice including decreased expression of plasminogen activator inhibitor type 1, platelet-derived growth factor-A, and thrombin receptor (5,14). The effects of CR on autoimmune disease in the short-lived B/W mouse range from preservation of greater numbers of naive T cells to modulation of T and B cell responsiveness by cytokines.

CR also modulates immune system function and prolongs life span in MRL/lpr mice. The Fas gene is defective in these mice which develop enlargement of lymphoid organs and auto-immune disease resembling human lupus (15). The Fas defect is linked to accumulation of CD4⁺ CD8⁻ (double-negative) self-reactive T lymphocytes. Defective apoptosis due to the Fas defect may account for the autoimmune disease. In MRL/lpr mice fed a CR (vs. AL) diet, CR decreases the accumulation of CD4⁺ CD8⁻ cells, enhances apoptosis in response to dexamethasone, and increases IL-2 expression (16).

Amelioration of Immune Function in Long-Lived Strains of Animals

In longer lived strains of animals, CR also extends life span and ameliorates age-related changes in the immune system. Splenocytes from long-lived (B6xDBA/2)F1 mice maintained from 16 weeks onward on a 40% CR diet, show higher proliferative responses to PHA,

Con A, and staphylococcal enterotoxin B (superantigen, SEB) at 30 months of age (17). Superantigens or bacterial toxins are implicated in the stimulation of T-cell subsets in rodents and humans (18,19). The development of self-superantigens may also be a mechanism to eliminate autoreactive T cells through clonal deletion (20). There may be very different activation requirements for naive vs. memory T cells, and it is possible that the progressive accumulation of memory T cells with age accounts for the decreased responsiveness of splenocytes from *ad libitum* fed animals to Con A and SEB. The decrease in memory T cells and higher levels of naive T cells observed in CR animals may account for the more youthful immune profile and enhanced responsiveness of splenocytes to mitogens as compared to *ad libitum* fed animals.

We and others have also studied the ability of CR to suppress progressive renal disease, delay the onset of spontaneous malignancies, and lower elevated glucose and cholesterol levels in long-lived Fischer 344 rats (21,22). When fed a diet whose content of calories is reduced by approximately 40%, mean life span of the Fischer 344 rat increases from approximately 23 months to 35 months (23). On a number of occasions, it has been noted that this effect of calorie restriction is not due to malnutrition, since the animals fed a CR diet actually ingest the same or slightly more calories per kilogram body weight as the *ad libitum* fed animals (23). This is due in part to the fact that body weights of the CR fed animals are proportionately smaller. We have extended these studies to an analysis of the effects of CR on fatty acid composition of membranes, immunological parameters, membrane fluidity and calcium fluxes in splenocytes. Spleen cells from CR fed animals showed enhanced proliferative responses in response to Con-A. In line with the increased proliferative response, splenocytes from CR fed animals showed the following: increased IL-2 production in response to Con-A, increased high-affinity IL-2 receptor expression, and increased calcium fluxes in response to Con A. In addition, fatty acid composition and membrane fluidity were altered by CR. In older CR animals, membrane fluidity was decreased along with a decrease in the arachidonic acid content of splenocyte lipids (24).

In addition to changes in the immune system, aging is associated with increased blood glucose levels and the development of insulin resistance (25). Hepatocyte insulin receptors drop by 50% at 24 months of age while receptor numbers in the CR animals were maintained at about twice the number in the *ad libitum* animals (26). We have also studied the effects of CR on the age-dependent decreased responsiveness of hepatocytes to androgens (27). Although CR doesn't prevent declines in circulating testosterone concentrations in the Fischer-344 rat, preservation of androgen responsiveness is a feature of hepatocytes isolated from CR animals. Aging in *ad libitum* fed animals is accompanied by loss of cytosolic androgen binding protein, decreased expression of senescence marker protein (SMP-2), but

increased expression of the androgen-inducible α_{2u} globulin. Taken together, these findings concerning androgen receptivity and insulin receptor number indicate that CR maintains target organ responsiveness to hormonal agonists in spite of decreasing hormone levels in the case of testosterone. In additional studies of the Fischer 344 rats, we found an increased number of binding sites for insulin and IL-2 on splenocytes of CR animals vs. AL. Analysis of phospholipids for fatty acid composition showed that CR prevented the rise in arachidonic acid levels seen in older animals (28). Thus, direct modulation of fatty acid composition of membranes may be an important mechanism through which CR modulates fatty acyl composition and ligand binding sites.

Finally, the effects of 60% CR were examined in a long-lived hybrid, the Fischer-344X Brown Norway F1 (BNF1) rat (29). This hybrid rat has a median life span of 31 months, and does not manifest the same kidney disease as does the Fischer-344 rat. The median life span of *ad libitum*-fed rats was 138 ± 3.4 weeks vs. 185 ± 3.4 weeks for the calorie restricted animals (29). The *ad libitum* fed BNF1 rats demonstrated an age-associated decreased lymphoproliferation in response to PHA and Con A and an increase in memory T cells. CR ameliorated the shift of naive to memory T cells, and maintained splenocyte responsiveness to mitogens. CR also maintained higher levels of the anti inflammatory interleukin-2 (IL-2) and lower levels of pro-inflammatory cytokines IL-6 and tumor necrosis factor- α . These studies indicate that an important effect of CR is to prevent a rise in memory T cells and decrease the expression of proinflammatory cytokines.

In summary, CR consistently maintains responsiveness to mitogens, increases the number of naive T cells while lowering the number of memory T cells. These findings in various strains of rodents stand in some contrast to the less consistent declines in immune function observed in humans (30,31, 32, 33). These findings are therefore difficult to apply directly to humans, however, the diet-induced alterations in such physical properties as membrane fluidity or fatty acid composition are intriguing and merit further study.

CR Suppresses Tumorigenesis

The bulk of human colon, breast, and particularly prostate tumors that develop later in adult life can be viewed as age related processes (e.g. oxidant stress and DNA repair mechanisms) that have a relationship to nutritional factors. CR delays the spontaneous appearance of breast tumors in C3H/Umc mice (34), and similarly, in the transgenic MMTV/v-Ha-ras "Oncomouse," CR delays the onset of tumors and markedly prolongs life span (2). In another mouse model, the p53^{-/-} homozygous knockout, there is rapid evolution of a variety of spontaneous malignancies including lymphoma. CR increased mean survival to 25 weeks vs. 16 weeks for *ad libitum* feed animals (35). Even as dysregulated apoptosis may influence the deterioration of the immune system with aging, neoplasia is increasingly seen as involving both

disordered apoptosis and cellular proliferation. It is likely that CR modulates apoptosis, and this may account for some of the effects of CR to suppress tumorigenesis (36). A recently published hypothesis links upregulation of apoptosis with reduction of oxidant stress and prolongation of life span by CR (37). CR decreases oxidant stress (38) and increases the expression of free radical scavenging enzymes (39). Senescence and resistance to apoptosis are characteristics of aging cells, and it is suggested that if cells damaged by free radical mechanisms cannot be eliminated by apoptosis, the probability of a malignancy correspondingly increases.

Dietary Fats: Vegetable, Fish, or Both?

Increased dietary intake of fish oil lipids, possibly combined with modest CR, may have benefits similar to those observed with CR alone. The content of ω -3 polyunsaturated fatty acids (PUFA) in cellular membranes declines with age, and the content of arachidonic acid in cellular phospholipids increases in AL (vs. CR) fed animals (40,41). Over the past several decades, the association of high levels of dietary saturated fats with increased heart and cardiovascular disease has prompted a shift to consumption of vegetable-derived PUFA typically rich in ω -6 unsaturated fats. Today, 90% of daily fat consumption (average 170 grams) per person in the United States is of plant origin (42). This substitution of PUFA for saturated fats has paralleled a decrease in the incidence of cardiovascular disease. However, vegetable oils (corn oil, soybean oil, etc.) are rich in ω -6 fatty acids. These are precursors for arachidonic acid, the substrate for synthesis of potent inflammatory prostaglandins (PGE₂) and the pro-inflammatory agents thromboxane A₂ (TXA₂) and leukotriene B₄ (LTB₄) (43). In parallel with this increase in consumption of vegetable derived oils, our knowledge of the potential benefits of a diet enriched in fish oil has increased. Diets enriched in either ω -6 or ω -3 fatty acids reduce the incidence of heart attack and atherosclerotic cardiovascular disease (44,45). However, ω -3 lipids have, in addition, favorable effects on immune function (46) and suppression of tumorigenesis (47). It is therefore appropriate to consider decreasing the ratio of ω -6/ ω -3 fats closer to a 5:1 (vs. the current 20:1) ratio in the interest of providing these added health benefits (43). A recent recommendation is that dietary fat intake should be 20% of calories with only 6% of this being saturated fat and the balance from ω -3 and ω -6 oils (48). The optimal composition of the fatty acid portion of the diet will be the topic of continuing study and evaluation as we learn more about the benefits (or detrimental effects) of various fats in relationship to tumorigenesis, cardiovascular disease, and the immune system.

Linolenic acid can serve as a precursor for ω -3 fatty acids, and while the reactions are slow in humans, feeding alpha linolenic acid can also increase the content of ω -3 fats. An additional consideration with regard to distribution and availability of oils is that linoleic acid rich oils resist spoilage and rancidity better than do ω -3

and linolenic acid rich oils. Thus, to make increased fish oils available to the public, improved packaging and preservation methods will be needed. This is relevant to both clinical and experimental uses of FO. When beneficial effects of marine oil supplementation are observed experimentally, the ω -3 fats are substituted for vegetable oils or saturated fats (rather than added to the existing diet), and the oils are stored and fed with adequate antioxidant (usually Vitamin E) supplementation to prevent oxidation and rancidity (49).

Fish Oil Fats and Autoimmune Disease in the Short-lived B/W Mouse

The most widely studied and best understood mechanisms for the effects of FO are its anti-inflammatory properties. These are attributed to two ω -3 fatty acids, EPA (20:5) and DHA (22:6). Like arachidonic acid, EPA and DHA are spared from oxidation and become directly acylated into membrane phospholipids. A relative increase in availability of ω -3 fatty acids inhibits the synthesis of arachidonic acid from its precursor, linoleic acid; leads to competition with arachidonic acid both for acylation at the 2-position of membrane phospholipids and competes as substrate for cyclooxygenase (50,51). Taken together, these effects of EPA and DHA to replace or compete with arachidonic acid lead to increased production of 3-series prostaglandins (PG) and thromboxanes (TX). PGE₃ derived from EPA is less inflammatory than PGE₂ derived from arachidonic acid, and the less active TXA₃ replaces the potent vasoconstrictor and platelet aggregating TXA₂ (51).

The precise relationship of these effects of fish oil fats to the amelioration of autoimmune disease remains unclear. We propose that fish oil fats increase the ratio of (naive) Th-1/(memory) Th-2 cells, and that there is a parallel decrease in the expression of pro-inflammatory cytokines such as IL-1 β , IL-6, TNF α , and IL-2 in target organs. Overabundance and increased activity of Th-1 cells, and pro-inflammatory cytokines, are likely to play a central role in the pathogenesis of lupus erythematosus and other autoimmune diseases. B/W mice spontaneously develop a disease similar to human systemic lupus erythematosus which has been used as an animal model to elucidate the pathogenesis of the disease (53). Immune system abnormalities in these mice include increased expression of proto-oncogenes *c-myc* and *c-ras* in splenocytes and thymocytes, production of autoantibodies against a variety of antigens including DNA, and development of immune complexes which localize in the kidneys. Experimentally, dietary supplementation with fish oil lipids suppresses renal disease and prolongs life span in the B/W mouse (53). Our studies showed mean life span of 402 $\pm$ 26 days for FO vs. 267 $\pm$ 13 days for corn oil fed animals (54). In a cross-sectional study of these animals, the FO group showed increased proliferative response to T cell mitogens (bacterial superantigens) and decreased serum anti-dsDNA antibody titers. Stimulation of splenocytes of 6.5 month old mice with Con A *in vitro* showed higher levels of IL-

2, IL-4, and TGF- β 1, characteristic of younger mice. Although impressive in comparison to fish oil, the results in these studies raise the question of how fish and corn oil compare to other sources of dietary fats.

In additional studies, we compared lard, corn oil, and fish oil at the 20% level on survival of B/W mice, and found that corn oil was intermediate between lard and fish oil (55). Thus, corn oil as a source of dietary lipid in these studies represents an intermediately effective lipid when life span is the measure of effectiveness. The lipid constituents of the diet assume special significance because dietary lipids provide both a high density source of calories and a source of molecules that are directly integrated into cell membranes. The integration of differing fatty acids into cell membranes can affect both membrane fluidity and metabolic pathways which generate inflammatory mediators. This is due, in part to the fact that ω -3 and ω -6 fatty acids are not inter convertible, and thus, raising the dietary content of fish oil relative to that of animal or vegetable derived fats can alter the physical properties of plasma membranes. Although CR exerts its effects in both low and high fat diets, a higher total fat content diminishes the effect of calorie restriction (6). While the substitution of vegetable oils for saturated animal fats in the diet is based on sound data, our understanding of the influence of chemically distinct classes or molecular families of lipids is in its infancy. The potential detrimental effects of trans fatty acids have been noted (56). Additionally, the isomerized forms of linoleic acid, found in red meat and dairy products are referred to as conjugated linoleic acid and have been found to have anti-proliferative (57) and anti-tumor activity (58). Finally, constituents of olive oil, present in the Mediterranean Diet, and including higher levels of oleic acid, are associated with decreased atherosclerosis and a lower incidence of breast cancer (59). Thus, the beneficial effects of fish oil are apparent when compared to corn oil and lard, but other lipid constituents may have additional positive or negative effects. Although the benefits of a reduction in saturated fat and total fat intake are widely accepted, the "ideal" fat constituents of the diet have not been determined. Future studies will be needed to refine our knowledge of the most beneficial dietary fats.

The renal disease accounts for much of the morbidity and accelerated mortality in the B/W mouse, and diets containing 10% fish oil fats or purified EPA markedly reduce proteinuria and histological evidence of glomerular disease in the B/W mouse (60). Our own studies of renal disease in the B/W mouse indicated that proteinuria was reduced and delayed by fish oil supplementation, and at 11 months of age approximately 50% of the fish oil fed mice were alive while all of the corn oil fed mice were dead (61). Fish oil suppressed the expression of the pro-inflammatory cytokines IL-1 β and IL-6, and also suppressed TNF α production. These cytokines stimulate production of PGs, and it is interesting to speculate that feeding fish oil fats ameliorates renal disease in short-lived B/W mice by both suppressing

pro-inflammatory cytokine production and altering the profile of prostaglandins produced upon stimulation by way of substituting EPA and DHA for arachidonic acid in membrane phospholipids. In additional studies we have shown that at 6.5 months of age, kidney tissue from fish oil fed B/W mice also had reduced TGF β 1 mRNA and protein levels compared to corn oil fed animals (62). The alteration of cytokine production by fish oil supplementation is organ specific. Spleen cells from fish oil fed animals produce higher levels of TGF- β 1 (63). In contrast to the kidney where TGF β is pro-inflammatory, in lymphoid cells, TGF β is immunosuppressive and decreases the production of pro-inflammatory cytokines. In this study, we also determined by flow cytometry that fish oil increased the number of naive (Th-1) T cells and decreased the number of memory (Th-2) T cells in splenocytes along with decreased message for *c-myc* and *c-H-ras*. These findings point to the modulation of gene expression by ω -3 fatty acids.

In confirmation of these findings suggesting that gene expression is altered by ω -3 fatty acids, we have found that fish oil feeding in B/W mice also induces the expression of the antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase. In both liver and spleen, we found that fish oil feeding suppressed expression of message for these enzymes (61,64). In the liver, antioxidant enzyme activity was determined, and also found to be higher in fish oil fed animals. For these studies, both CO and FO fed animals received antioxidant supplementation, vitamin E, approximately 215 mg/kg diet. Thus, contrary to earlier concerns raised about the tendency of ω -3 fatty acids to peroxidize during storage, when fed with adequate antioxidant supplementation, fish oils actually enhance the antioxidant defense system. In summary, the pathogenesis of the renal disease in B/W mice is closely related to the localization of immune complexes within the kidney. Increased oxidant stress and formation of reactive oxygen intermediates are also likely to play a role in the pathogenesis of autoimmune renal disease (65), and fish oil supplementation enhances the expression of the antioxidant enzymes may thereby protect against oxidant stress.

Finally, we have studied the effects of fish oil supplementation on some aspects of apoptosis. We found that dexamethasone induced apoptosis of splenocytes was higher in fish oil fed mice than corn oil fed animals (66). In collaborative studies, we also found that splenocytes from fish oil supplemented mice showed increased expression of Fas, a positive modulator of apoptosis (67). While the causes of human lupus remain unknown, dysregulation of apoptosis is likely to be involved (68). Modulation of programmed cell death by diet would be particularly relevant to the programming events involved in production of T cells. Ninety-five percent of all marrow stem cells transported to the thymus are deleted by apoptosis, and only 5% survive. Dysregulated apoptosis could lead to an increased number of autoreactive T

cells and increased autoimmunity. A possible connection between dietary restriction of calories and the maintenance of lower numbers of double negative (CD4⁺/CD8⁻) T cells may reside in the interaction of the endocrine and immune systems in the thymus. The thymic hypertrophy observed in neonatally adrenalectomized animals suggests that a baseline level of circulating glucocorticoids is necessary for maintenance of normal thymic function (69). CR fed mice demonstrate increased glucocorticoid levels during night time activity and peaking just prior to food intake (70). The increase in TCR receptor mediated antigen-specific depletion of immature thymocytes observed with the glucocorticoid-receptor blocker, RU-486 supports a role for glucocorticoids in modulating thymic function (71). Our previous studies have indicated that delayed thymic involution and autoimmune disease is observed in animals fed a low protein or calorie restricted diet (72). Both the locally generated (73) and systematically generated glucocorticoids (74) may modulate the maturation of immune cells in the thymus.

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