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DIETARY OXIDATIVE STRESS AND THE POTENTIATION OF VIRAL INFECTION[1]

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ABSTRACT

Oxidative stress is implicated in the pathogenesis of several viral infections, including hepatitis, influenza, and AIDS. Dietary oxidative stress due to either selenium or vitamin E deficiency increases cardiac damage in mice infected with a myocarditic strain of coxsackievirus B3. Such dietary oxidative stress also allows a normally benign (i.e. amyocarditic) coxsackievirus B3 to convert to virulence and cause heart damage. This conversion to virulence is due to a nucleotide sequence change in the genome of the benign virus, which then resembles more closely the nucleotide sequence of virulent strains. Although it has been known for many years that poor nutrition can affect host response to infection, this is the first report of host nutrition affecting the genetic sequence of a pathogen. Further research is needed to determine whether poor host nutrition plays any role in the emergence of new viral diseases via alterations in the genotype of an infectious agent.

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INTRODUCTION

Oxidative stress has been defined as "an imbalance between oxidants and antioxidants in favor of the oxidants" (107). Oxidative stress can occur in vivo if oxygen, instead of following its usual benign reductive pathway down the mitochondrial respiratory chain to form water, undergoes a series of univalent reductive steps to yield reactive oxygen species, which can be highly detrimental to life. This is a consequence of the so-called paradox of aerobic life, the idea that although we cannot exist without oxygen, we are nonetheless still subject to its potentially damaging effects (39). The ability of dietary anti- and pro-oxidants to affect oxidative stress status in vivo has been widely discussed, and the effect of dietary intake of these substances on human health is an area currently undergoing intensive investigation (54).

The role of oxidative stress in viral infection has recently been critically reviewed by Schwarz (101) for RNA viruses, DNA viruses, and retroviruses. Although, where instructive, other viral infections are considered, our review focuses primarily on the influence of dietary-induced oxidative stress on the pathogenesis of coxsackievirus infection. This RNA enterovirus is a member of the Picornavirus family, which also includes the viruses responsible for polio, foot and mouth disease, and the common cold. We have found that dietary oxidative stress has a profound influence on the evolution of the virus in vivo, so that a normally benign strain of the virus converts to virulence.

THE INFLUENCE OF DIETARY OXIDATIVE STRESS ON VIRAL INFECTION

Selenium

Our interest in studying the interaction of dietary oxidative stress and viral infection was stimulated by the Chinese investigations into the role of selenium

(Se) deficiency in Keshan disease. Keshan disease is a cardiomyopathy characterized by necrotic lesions throughout the myocardium, with varying degrees of cellular infiltration and calcification (53). The scientists in China had convincingly shown that the incidence of Keshan disease was closely associated with poor Se status: The disease was found only in those areas of China with Se-poor soils and occurred only in populations with low dietary intakes of Se and that had low blood and hair Se levels (66). Despite their success in linking Keshan disease with Se deficiency, the Chinese nutritionists realized that its etiology was more complex because the disease also exhibited remarkable seasonal and annual fluctuations (118). Such variations are, of course, more typical of an infectious agent than a nutritional deficiency. The Chinese recognized this possibility and were able to isolate a variety of viruses from some Keshan disease patients, including a coxsackievirus B4. Furthermore, this virus caused much more severe heart damage when inoculated into Se-deficient mice than when given to Se-adequate mice (6). These observations were published in 1980 and suggested that the cardiomyopathy due to Keshan disease really had a multifactorial etiology that included both nutritional and infectious aspects.

In 1992, we formed a collaboration to investigate the effect of nutrition on the course of viral infection. Because of the Chinese studies indicating a role for Se nutriture in the pathogenesis of coxsackievirus infection, and because of our own expertise in these areas, we began by looking at the effect of Se deficiency on the heart damage caused in mice by infection with coxsackievirus B3 (CVB3). Our first experiment compared the heart pathology induced by a virulent strain of CVB3, CVB3/20, and showed that Se-deficient mice were indeed more susceptible to the cardiotoxic effect of the virus than were Se-adequate[2] controls (11). The hearts of the infected Se-deficient mice clearly suffered more damage (defined as inflammatory foci associated with myocardial cell necrosis and dystrophic calcification) than the hearts of the infected Se-adequate controls. The deficient infected mice also experienced higher cardiac and hepatic viral titers than the Se-adequate infected controls, indicating that the virus replicated more readily in the deficient animals.

Our next experiment with the virulent CVB3/20 virus examined the effect of passing the virus isolated from the hearts of Se-deficient or -adequate primary mice into a second group of mice that had been fed the adequate diet. Rather

[2]Our Se-adequate diet (11) was supplemented with 0.2 mg of Se/kg as sodium selenite because the protein source (torula yeast) and the added sulfur amino acid (methionine) would not be expected to contribute much additional Se. This amount of Se is 33% higher than that recommended in the AIN-93 diet (94) but is still well within the nutritional range. The level of vitamin E we added to the diets [d-alpha tocopheryl acetate, unless vitamin E deficiency was a variable (see next section)] was 38.4 mg/kg, equivalent to the 50 IU of all-*rac*-alpha-tocopheryl acetate per kg recommended in the AIN-76 diet (2). A nutritionally relevant range of vitamin E intake for rodents is 5–30 mg of alpha-tocopherol per kg (108).

than measure host factors alone (see below), we wanted to determine whether the virus itself had changed phenotypically as a result of its replication in a Se-deficient host. Seven days after infection, none of the hearts of the secondary mice fed the adequate diet and inoculated with virus passed previously through Se-adequate primary mice (identified as CVB3/20Se+) exhibited heart damage. CVB3/20 does not generally cause heart damage in normal mice within 7 days; heart damage generally occurs 10–14 days postinfection. However, the hearts of secondary mice fed the adequate diet and inoculated with virus passed previously through Se-deficient primary mice (identified as CVB3/20Se−) exhibited considerable heart damage by 7 days. By 10 days postinfection, adequate mice infected with CVB3/20Se+ developed mild-to-moderate cardiac damage, whereas the hearts of mice fed the adequate diet and inoculated with CVB3/20Se− had severe heart damage. Moreover, the hearts of mice infected with CVB3/20Se− had viral titers three orders of magnitude higher than those in hearts of mice infected with CVB3/20Se+. In other words, the virus that had previously been passed through a Se-deficient animal had changed phenotypically: It replicated to a greater extent and caused more severe cardiac injury in a secondary Se-adequate mouse than had the virus that had been passed through a Se-adequate animal.

Having confirmed the Chinese report that Se-deficient mice were less resistant than Se-adequate mice to the cardiotoxic effect of a virulent CVB3 (CVB3/20), we turned our attention to the effect of Se nutriture on the cardiotoxic potential of a normally benign CVB3 (CVB3/0). As expected, the amyocarditic strain of coxsackievirus, CVB3/0, caused no heart inflammation or damage when inoculated into normal mice. However, when CVB3/0 was inoculated into Se-deficient mice, significant cardiopathology occurred (9). In accord with these results, viral titers were also much higher in the deficient mice infected with CVB3/0.

Once again we decided to test the effect of prior passage through either a deficient or an adequate host on the virulence of the virus to a subsequent host. When CVB3/0 was first inoculated into a Se-adequate mouse and then isolated from its heart (renamed CVB3/0Se+) and reinoculated into a second Se-adequate mouse, the benign virus remained benign and caused no heart damage. On the other hand, when CVB3/0 was inoculated into a Se-deficient mouse and the virus was isolated from its heart (renamed CVB3/0Se−) and reinoculated into a second mouse that was adequate in Se, significant cardiopathology was observed. The benign amyocarditic CVB3/0 strain had converted to virulence by virtue of its passage through a Se-deficient host. This conversion to virulence was also accompanied by higher cardiac viral titers in the mice infected with CVB3/0Se−.

The phenotypic alteration in the virulence characteristics of both the CVB3/20Se− and CVB3/0Se− suggested that a change in the genome of the

Table 1 Nucleotide composition of coxsackievirus genome at specific positions thought to determine virulence[a]

Viral strain	Strain phenotype	Genomic nucleotide number (5′–3′)						
		234	788	2271	2438	2690	3324	7334
CVB3/20	Virulent	T	A	T	C	A	T	T
CVB3/M	Virulent	T	A	T	C	A	T	T
CVB3/0	Avirulent	C	G	A	G	G	C	C
CVB3/0Se+	Avirulent	C	G	A	G	G	C	C
CVB3/0Se−	Virulent	T	A	T	C	G	T	T

[a]CVB3/20 and CVB3/M are virulent strains that have been sequenced. CVB3/0 was sequenced prior to inoculating either Se-adequate or Se-deficient mice. CVB3/0Se+ was isolated from the heart of a Se-adequate mouse inoculated with CVB3/0. CVB3/0Se− was isolated from the heart of a Se-deficient mouse inoculated with CVB3/0. Three other isolates from Se-deficient mice had the identical sequence. (Adapted from Reference 13.)

viruses might be responsible for these effects. When the genome of the CVB3/0Se− was sequenced, it was found that its base composition was different from that of the wild-type CVB3/0 at six of seven sites thought to determine virulence in CVB3 (Table 1) (12). In addition, the changes all reflected the composition of known myocarditic strains that had been previously sequenced.

Vitamin E and Omega-3 Fatty Acids

Vitamin E deficiency had the same potentiating effects on the infection of mice with coxsackievirus as were seen with Se deficiency (10). The myocarditic CVB3/20 strain caused greater heart damage in vitamin E–deficient compared with vitamin E–adequate mice, and vitamin E deficiency allowed the amyocarditic CVB3/0 to become myocarditic. With both strains of virus, higher virus titers were seen in the vitamin E–deficient animals. However, to obtain the fullest effects of vitamin E deficiency on the virus, it was necessary to add fish oil, long known to be a tocopherol antagonist (38), to the diets fed to the mice. The fact that fish oil had to be added to increase the pathology is perhaps not surprising because of the relatively short feeding period before the mice were infected (4 weeks), which would not allow induction of a severe deficiency of this fat-soluble vitamin. The genomic changes seen in the CVB3/0 strain on passage through vitamin E–deficient mice (13) were identical to those seen after passage through Se-deficient mice, and in both cases the amyocarditic CVB3/0 converted to virulence.

The fact that both Se and vitamin E deficiency in mice resulted in similar exacerbations of viral infection places significant limitations on the mechanistic interpretation of our results. Because both deficiencies led to more or less similar outcomes, it is difficult to propose biochemical mechanisms involving particular selenoproteins or specific membrane effects of vitamin E. Rather,

a broader explanation must be sought, such as a general deleterious impact of oxidative stress on cellular metabolism. The idea that a nonspecific effect of oxidative stress is responsible for the phenomena reported here is also supported by our fish oil studies, in which the consumption of this highly unsaturated fat invariably worsened the response of the vitamin E–deficient mice to viral infection. Moreover, N,N′-diphenyl-*p*-phenylenediamine, a synthetic antioxidant structurally unrelated to vitamin E, which nonetheless substitutes for the vitamin in many models (44), was active in protecting vitamin E–deficient mice against the cardiotoxic effects of the coxsackievirus (MA Beck & OA Levander, unpublished observations). Until a more-comprehensive mechanism can satisfactorily account for all of our observations, we are inclined to accept a generalized increase in cellular oxidative stress as the best explanation for our data.

Iron Overload

Elevated iron levels in the liver due to chronic iron overload (dietary or parenteral) have long been associated with a tendency toward increased hepatic lipid peroxidation. For example, Bacon et al (3) observed conjugated dienes in hepatic subcellular fractions from rats fed carbonyl iron and interpreted that as evidence for iron-induced lipid peroxidation of liver mitochondria and microsomes. Dillard et al (40) found that pentane production (an index of overall in vivo lipid peroxidation) by rats given a course of iron dextran injections could be inhibited 92% by supplementing their antioxidant-deficient basal diet with vitamin E. Dabbagh et al (37) found that a number of endogenous antioxidants, including vitamin E, were decreased in the livers of rats in a dietary iron overload model, whereas hepatic concentrations of F2-isoprostanes, specific by-products of lipid peroxidation, were increased. Likewise, Omara & Blakley (87) showed that hepatic vitamin E concentrations were decreased in mice fed very high levels of iron and suggested that vitamin E might be a useful antidote for iron toxicosis. Immunohistochemical studies revealed the presence of a strong autofluorescence in the livers of iron-loaded rats that was markedly reduced by vitamin E supplementation (89), and vitamin E supplementation also decreased the hepatic levels of thiobarbituric acid–reactive substances in iron-overloaded rats (23).

Given the central role of the liver under conditions of iron overloading, it might be expected that viruses affecting this organ would be prime targets of any effects of increased hepatic lipid peroxidation. Bonkovsky and colleagues reviewed the interrelationship between iron and viral hepatitis and concluded that "... the preponderance of the available evidence suggests to us that iron caused or synergized the hepatotoxicity and perhaps also the hepatocarcinogenicity caused by chronic viral hepatitis" (22). In patients with chronic hepatitis C, phlebotomy normalized serum enzyme levels and improved the response to

interferon therapy, and two large clinical trials are currently under way in the United States to determine what role iron reduction might play in the management of chronic hepatitis C (21). Weinberg (116) presented several examples whereby elevated iron status predisposed one to, whereas iron withholding suppressed, viral infection.

Because of the involvement of the liver in iron overload, one might not necessarily expect that excess iron intake would have any effect on the heart damage caused by the cardiotropic CVB3. However, we found that CVB3/0, the benign (amyocarditic) strain, converts to virulence and causes cardiopathology in vitamin E–deficient mice fed high levels of iron, just as it does in vitamin E–deficient mice fed fish oil (MA Beck & OA Levander, unpublished observations). During the course of infection in vivo, CVB3 first replicates in the gut and liver and then migrates to the heart (see, for example, the viral titer curves presented in Figure 3 of Reference 11), so the virus genome may have been altered while replicating in the highly pro-oxidative milieu of the iron-loaded liver before it ever reached the heart. On the other hand, the antioxidant defense system in the heart has been shown to be relatively weak compared with that in the liver under conditions of copper deficiency, which can lead to cardiac iron accumulation and cardiomyopathy (29). Cardiomyopathy-associated heart failure caused by iron overload has been reported in hereditary hemochomatosis (69), and hereditary hemochomatotic cardiomyopathy has been successfully treated with a combination of phlebotomy and iron chelators (75). Perhaps the cardiomyocyte is more vulnerable than the hepatocyte to the oxidative damage due to iron overload and this is what leads to the cardiotocixity of the coxsackievirus. In any case, the most parsimonious explanation for our results still appears to be an effect of dietary oxidative stress on the virulence of the virus.

Gold Compounds

Gold thioglucose is known to be a powerful in vitro inhibitor of glutathione peroxidase, presumably because of covalent bonding between gold (I) and Se (II) as selenocysteine at the active site of the enzyme (28). Aurothioglucose has also been shown to decrease tissue glutathione peroxidase activity when injected into rats (63) or chicks (68). Gold thioglucose is a stronger in vitro inhibitor of the newly discovered selenoenzyme, thioredoxin reductase (51), than of glutathione peroxidase (59). This compound has been utilized recently in the development of a novel assay for thioredoxin reductase activity in rat liver supernate (60). Substitution of sulfur for Se at the active site of rat liver type I iodothyronine deiodinase resulted in a 100-fold decrease in the sensitivity of 3,3′,5′-triiodothyronine deiodination to competitive inhibition by gold thioglucose (20), and this was taken as evidence for the existence of selenocysteine at the active site of the enzyme. Thus, it appears that inhibition by gold is a

characteristic of many selenoenzymes that have selenocysteine at their active site.

In 1974, Allner et al (1) reported that prior injection of mice with sodium aurothiomalate converted a normally avirulent strain of Semliki Forest virus into a lethal infection. Similarly, Mehta & Webb (80) found that treatment of adult mice with gold sodium thiomalate made normally nonlethal Semliki Forest virus and Sindbis virus infections lethal and increased the virulence of Langat and West Nile viruses. The mechanism by which gold sodium thiomalate achieved these effects is not known, but Mehta et al (79) suggested that stimulation of membrane proliferation by the gold compound might be an important factor affecting virulence. In certain virus infections, biogenesis of smooth membranes is a prerequisite for virus RNA synthesis and maturation.

Kabiri et al (65) showed that pretreatment with a gold salt (sodium aurothiomalate) potentiated coxsackievirus B3 infection in adult mice. No deaths occurred in animals given only the virus, but mice given the gold compound prior to infection suffered a mortality rate of 90%. In general, viral titers in various organs were higher in gold-treated infected mice compared with mice given only the virus. In light of what we now know about the ability of gold compounds to inhibit many selenoenzymes and about the ability of selenium deficiency to increase coxsackievirus virulence, it seems worthwhile to examine the relationship of gold treatment, selenium status, and virulence of several viruses, including coxsackievirus. If gold were indeed shown to increase virulence through its effects on selenium, we would once again be back to oxidative stress as a possible explanation for the results because aurothioglucose was shown to increase iron-induced lipid peroxidation in rat liver microsomes, presumably because of its strong inhibitory action against glutathione peroxidase (14). On the other hand, gold also has other significant biological effects, such as inhibition of the DNA-binding activity of NF-κB and AP-1, cellular transcription factors that regulate a wide variety of cellular and viral genes (55, 121).

[Recent preliminary findings in our laboratory show that administration of gold thioglucose to Se-adequate mice prior to inoculation with CVB3/0 increases the virulence of the virus, resulting in increased viral titers and mortality (AD Smith, CA Guidry, VC Morris, MA Beck, OA Levander, unpublished observations). Further work is needed to clarify the mechanism by which this gold compound potentiates the infection of mice by CVB3/0.]

EFFECT OF OXIDATIVE STRESS ON THE HOST IMMUNE SYSTEM AND THE RESPONSE TO VIRAL INFECTION

The immune system has been shown by many investigators to be affected by the nutritional status of the host (16, 18, 27, 102). In addition, nutritionally deficient

hosts have been shown to be more susceptible to viral infections, and outbreaks of infectious disease often accompany famine. Thus, increased oxidative stress induced by a dietary deficiency in antioxidant nutrients would be expected to affect host immune responses, thus leading to increased susceptibility to viral disease. Although a number of reports document the wide range of effects particular nutrients have on immune functions (for a recent review, see 17), less is known about the effect of antioxidant nutrients on viral disease. Below, we discuss influenza and coxsackievirus, two infections affected by the oxidative stress status of the host, as well as NF-κB, a multisubunit transcription factor (4) that rapidly activates the expression of genes involved in inflammatory responses. In this section, HIV infection and the relationship between oxidative stress and immunity are not discussed, as a recent review on this topic (52) is available.

Influenza virus is a segmented RNA virus that is responsible for a great deal of morbidity and mortality around the world (114). Infection with influenza virus causes damage to both the lungs and airways due to inflammatory responses. Influenza virus directly activates monocytes and polymorphonuclear leukocytes to generate reactive oxygen species (ROS) (92), and ROS can act as a stimulator for T cell proliferation (91). However, the generation of ROS during influenza virus infection will also contribute to the pathogenesis, as treatment of influenza virus–infected mice with superoxide dismutase conjugated to pyran polymer protects from a lethal infection (85). Infection of human airway epithelial cells with influenza virus results in an increase in expression of mRNA for Mn-superoxide dismutase (Mn-SOD), an enzyme important for oxidant defense (64). Interestingly, no effect was seen on mRNA for Cu/Zn SOD, and mRNA levels for catalase were decreased. Using an in vivo model, mice infected with influenza virus were found to have increased mRNA levels for Mn-SOD, heme oxygenase-1, and glutathione peroxidase early postinfection. Cu/Zn SOD and catalase mRNA levels were not affected at any time postinfection (30). Increased oxidative stress in the lungs of influenza-infected mice has also been demonstrated by an increase in superoxide anion radical production by cells found in bronchioalveolar lavage (BAL) fluid, as well as by increased H_2O_2 and decreased ascorbate content of the BAL fluid (24).

The damage that occurs during an influenza virus infection is caused in part by the host cell inflammatory reaction due to recruitment of inflammatory cells to the lungs. The ability of the inflammatory cells to traffic to the lung depends, in part, on local expression of chemokines, low-molecular-weight proteins that act as chemoattractants for immune cells. Previously, we demonstrated that the chemokine macrophage inflammatory protein-1α (MIP-1α) is at least partly required for the inflammatory response found in the lungs of influenza-infected mice (34). Exposure of rat alveolar macrophage cells to H_2O_2 or menadione (a quinone compound that undergoes redox cycling, leading to an increase in

oxygen species) up-regulates the expression of mRNA for MIP-1α (105). Thus, increased oxidative stress in an influenza-infected host may lead to increased lung inflammation as a consequence of increased production of MIP-1α.

In a recent report by Hayek et al (56), supplementation with excess vitamin E (500 ppm for 6 weeks) of old (22 months) mice reduced influenza lung titers 25-fold when compared with old mice fed the control diet. The decrease in viral titer was accompanied by an increase in NK activity, although cytotoxic T cell activity was not affected. However, the effect on lung pathology was not reported in these animals. These results suggest that antioxidant status is important for an effective host response against influenza viral infection.

In our model of coxsackievirus-induced myocarditis, we found no differences in neutralizing antibody titers between mice fed a diet deficient in either Se or vitamin E and nutritionally adequate mice (10, 11). However, spleen cell proliferative responses to either mitogen or antigen were decreased in deficient mice. Mitogen responses were found to be much more depressed than antigen responses. Natural killer cells have been shown to be important in CVB3 clearance in normal animals (48). However, NK levels were only slightly depressed in our Se- or vitamin E–deficient mice, although mice fed diets containing menhaden oil, whether vitamin E deficient or not, had markedly depressed NK activity, confirming previous reports of the effect of fish oil on NK activity (49). Thus, in our model, NK cell activity was not critical for limiting CVB3-induced pathogenesis.

To determine if production of cytokines involved in inflammatory processes were altered, we isolated heart tissue from CVB3/20-infected mice fed either Se-adequate or Se-deficient diets at various times postinoculation. Using reverse transcriptase–polymerase chain reaction, we identified polymerase chain reaction fragments for interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-β. We found that mRNA levels for TNF-β and IL-6 were decreased in infected mice fed the deficient diet when compared with infected adequate mice. However, IL-1 levels were fairly consistent between the two groups. IL-6 is a costimulant for IL-2 production and is a growth and differentiation factor for cytotoxic T cells. TNF-β is also involved in inflammatory responses. The immune response to viral infection involves an array of cytokines that must be secreted and utilized at specific times during the inflammatory process—a so-called cytokine network. Our preliminary data suggesting that the cytokine network is altered in the infected Se-deficient animals need to be more intensively studied to determine the impact on the pathological outcome.

Recently, NF-κB has been demonstrated to be an important immune regulator. NF-κB is an inducible transcription factor that can rapidly activate the expression of genes involved in inflammatory responses (100). The protein is found in B and T lymphocytes, macrophages, monocytes, and other tissues.

Although NF-κB knockout (KO) mice do not show any developmental abnormalities, they are defective in basal and specific antibody production (104). NF-κB KO mice are resistant to infection with murine encephalomyocarditis virus (104), a cytopathic virus in the Picornavirus family, closely related to coxsackievirus. This increased resistance was postulated to be due to an increase in interferon-β production by an unknown mechanism.

NF-κB has been found to be up-regulated by a number of stimulants, including viruses, bacterial lipopolysaccharide, protein synthesis inhibitors, TNF-α, TNF-β, IL-1, T cell mitogens, lectins, calcium ionophores, and T cell receptor antibodies (5). Schreck et al (100) suggested that the common mechanism used by all of these different agents is the synthesis of reactive oxygen intermediates. These then serve as messengers to mediate the stimulation of NF-κB. Christensen & Pusey (31) demonstrated that dietary Se deficiency increases binding of nuclear proteins to the NF-κB regulator sequences, presumably because of increased oxidative stress as a result of decreased glutathione peroxidase activity. This suggests possible mechanisms through which Se may affect the immune response. Thus, regulation of NF-κB by oxidative stress status of the host can have an effect on the host response to viral infection.

USE OF GENETIC KNOCKOUT MICE TO STUDY THE ROLE OF DIETARY OXIDATIVE STRESS IN VIRAL DISEASE

KO mice provide a powerful tool for understanding the mechanisms involved in the host response to oxidative stress. Since the techniques of homologous recombination for the introduction of planned alterations in the genome of the mouse were first described (reviewed in 70), a number of investigators have developed KO mice for a variety of proteins. A particularly large number of KO models are of immunological interest because many of the immune genes are not essential for survival of an unchallenged host and because the KO models provide a window into the in vivo functioning of immune networks. Table 2 provides a small sampling of the many KO mice that have been reported. We encourage the use of these mice in nutritional studies to further delineate the effect of host antioxidant status on resistance or susceptibility to viral infection.

The KO immune models provide the means to determine whether a specific immune component is involved in protection from infectious disease. For example, IL-6 KO mice appear normal, but when challenged with vaccinia virus they exhibit impaired T and B cell responses (71). Another example is provided by interferon (IFN) receptor KO models. Type I (α-IFN, β-IFN) and type II (γ-IFN) IFN receptor KO mice demonstrate no overt abnormalities, but they display differential abilities to resist viral infections (111). Type I, but not

Table 2 Some cytokine knockout mice available for research into nutrition-infection interactions[a]

Gene deletion	Characteristic features
IL-2	Inflammatory bowel disease, autoimmune hemolytic anemia
IL-4	Reduced IgG1 and IgE
IL-5	Reduced eosinophilia
IL-6	Reduced hematopoietic precursor cells, reduced mucosal immunity
IL-10	Inflammatory bowel disease, increased TH1 responses
IL-12	Diminished TH1 response
GM-CSF	Normal hematopoiesis, lung pathology
G-CSF	Neutropenia, increased susceptibility to infections
IFN-γ	Macrophage dysfunction, increased susceptibility to infections

[a]IL, Interleukin; Ig, immunoglobulin; GM, granulocyte macrophage; CSF, cerebrospinal fluid; G, granulocyte; IFN, interferon.

type II, IFN provides resistance against vesicular stomatitis virus and Semliki Forest virus, whereas resistance to vaccinia virus and lymphocytic choriomeningitis virus involves both types of IFN. Thus, different viral infections will not necessarily affect KO mice in the same fashion.

Apolipoprotein (Apo) E KO mice provide an excellent in vivo model for the study of plasma and lipoprotein lipid peroxidation (57, 122). Apo E–deficient mice have highly oxidized plasma lipids compared with control mice and have increased susceptibility to undergo lipid peroxidation when exposed to the free radical generator 2,2-azobis-(2-amidinopropane) hydrochloride. To our knowledge, Apo E KO mice have not been studied in viral challenge experiments, with or without dietary oxidant stress.

Although a wide variety of KO models are available, little work has been done utilizing a nutritional manipulation. Below, we summarize two models from our laboratories that utilize KO mice to understand how dietary-induced oxidative stress affects viral pathogenesis. Both KO models were produced by homologous recombination in mouse strain 129–derived embryonic stem cells, which were then injected into C57Bl/6J blastocysts.

Macrophage Inflammatory Protein-1α Knockout

Macrophage inflammatory protein-1α (MIP-1α) is a member of the β chemokine family. Chemokines are involved in leukocyte trafficking in vivo, and MIP-1α induces chemotaxis of monocytes, CD8+ T cells (109), CD4+ T cells, and B cells in vitro (97). Although other chemokines, such as MIP-1β and RANTES, share many of the activities of MIP-1α, including an ability to bind to the same receptor (83), Cook et al (34) demonstrated an absolute requirement for MIP-1α for coxsackievirus-induced heart inflammation and a partial

requirement for influenza-induced pneumonitis. Thus, other chemokines could not functionally substitute for MIP-1α in vivo.

To determine the effect of a dietary deficiency on the MIP-1α KO, we fed the KO mice either a control or a Se- and vitamin E–deficient diet for 4 weeks. As reported earlier (34), wild-type mice fed a control diet developed myocarditis when infected with a myocarditic strain of coxsackievirus B3 (CVB3/20). In contrast, MIP-1α KO mice fed a control diet did not develop any inflammation post– CVB3/20 infection. However, approximately one half of the infected MIP-1α KO mice fed a diet deficient in vitamin E and Se developed myocarditis. Thus, the protective effect of a lack of MIP-1α from CVB3-induced myocarditis could be overcome by ingestion of a diet deficient in antioxidants. It is not clear what the mechanism for the result is, but it is not due to overcompensation by other chemokines, as the mRNA levels for MIP-1β, RANTES, and MCP-1 of infected KO mice were similar to what was found for infected wild-type mice (MA Beck, unpublished observations).

Cardiac virus titers of MIP-1α KO mice fed the deficient diet were significantly lower than virus titers of mice fed the adequate diet. This may be due to the presence of immune cells in the hearts of deficient KO mice, thus providing a mechanism for viral clearance. Because inflammation does not occur in KO mice fed an adequate diet, virus titers may be elevated due to a lack of localized immune cells responsible for viral clearance.

MIP-1α KO mice fed either an adequate or a deficient diet produced equivalent levels of neutralizing antibody, similar to the levels found in wild-type mice. However, spleen cell reactivity to either mitogen or antigen was decreased in mice fed the deficient diet, whether wild type or KO. The mechanism of the T cell impairment in the oxidatively stressed animals is not known.

Glutathione Peroxidase 1 Knockout

Glutathione peroxidase 1 (GPX-1) is a selenium-dependent enzyme with antioxidant properties. As previously described, mice that are Se-deficient develop myocarditis when infected with a normally benign strain of CVB3/0. To determine whether the mechanism for myocarditis susceptibility was mediated through GPX-1 activity, we inoculated both wild-type and GPX1 KO mice with CVB3/0, the benign strain of coxsackievirus. None of the wild-type mice developed any myocarditis. However, slightly over half of the GPX-1 KO mice developed mild-to-moderate myocarditis. The fact that GPX-1 KO mice were susceptible to developing myocarditis when infected with the benign strain of CVB3 suggests that the similar susceptibility to CVB3/0 of mice fed a Se-deficient diet is due to the common mechanism of a reduction in GPX-1 activity. However, there are differences in viral loads and in the immune response between infected Se-deficient and infected GPX-1 KO mice.

Heart virus titers in GPX-1 KO mice are equivalent to wild-type mice, although Se-deficient mice have increased heart titers when compared with Se-adequate mice. Several immune functions are also altered. Se-deficient mice have decreased T cell proliferative responses against both mitogens and antigens when compared with Se-adequate mice. However, neutralizing antibody responses are not affected. In contrast, GPX-1 KO mice have normal T cell proliferative responses and greatly diminished antibody responses. This difference in immune response may be due to differences in developmental exposure. GPX-1 KO mice are deficient in GPX-1 activity throughout development, whereas Se-deficient mice are exposed to the diet at 3 weeks of age, a time at which the immune system is matured.

Because our previous study (12) had demonstrated changes in the viral genome of CVB3/0 recovered from the hearts of Se-deficient mice, we sequenced virus recovered from the hearts of both wild-type and GPX-1KO mice. As shown in Table 3, seven nucleotides of the CVB3/0 genome had changed as a consequence of replication in GPX-1 KO mice. These nucleotide changes in the virus were found only in GPX-1 KO mice that developed cardiac pathology. No genomic changes were found in virus recovered from the hearts of infected GPX-1 KO mice without heart pathology, nor from infected wild-type mice. The strict association between changes in the viral nucleotide sequence and induction of cardiomyopathy in GPX-1 KO mice suggests that some, if not all, of these changes are required for virulence.

Six of the seven changes in the viral genome obtained from the hearts of GPX-1 KO mice are identical to genomic changes found in virus recovered from the hearts of Se-deficient mice (Table 1), which suggests that GPX-1

Table 3 Nucleotide composition of possible virulence-determining genomic sites of virus recovered from the hearts of CVB3/0-infected wild-type (+/+) and GPX-1 KO (−/−) mice

Mouse genotype	Virus-induced heart pathology score	Genomic nucleotide number (5′–3′)						
		234	788	2271	2438	2690	3324	7334
+/+	0	C	G	A	G	G	C	C
+/+	0	C	G	A	G	G	C	C
−/−	0	C	G	A	G	G	C	C
−/−	0	C	G	A	G	G	C	C
−/−	2+	T	A	T	C	A	T	T
−/−	3+	T	A	T	C	A	T	T
−/−	2+	T	A	T	C	A	T	T
CVB3/0Se−[a]	3+	T	A	T	C	G	T	T

[a]CVB3/0Se− is the designation for virus recovered from the hearts of CVB3/0-infected Se-deficient mice (see Table 1). Mouse strain is C3H/HeJ.

activity is required for protection against viral mutations and that the mutations may be driven by oxidative stress (see below).

POSSIBLE MECHANISMS FOR THE EFFECT OF OXIDATIVE STRESS ON THE VIRAL GENOME

Direct Oxidative Damage to Viral RNA

Oxidative damage to DNA is a well-documented phenomenon and its biological significance is widely recognized (15, 25), whereas the biological importance of RNA base oxidation is relatively unexplored. Rhee et al (95) used a reverse transcriptase procedure to detect sites in RNA oxidatively damaged by dye photosensitization. Wamer & Wei (113) found that irradiation of skin fibroblasts with ultraviolet A radiation resulted in significant levels of guanine hydroxylation in RNA. Oxidative damage is observed in both liver RNA and DNA in rats administered the hepatocarcinogen 2-nitropropane, and the products of oxidative damage of nucleic acids, 8-hydroxyguanosine as well as 8-hydroxydeoxyguanosine, can be detected (33). Thus, it seems at least theoretically possible that the RNA of a virus replicating in a pro-oxidant milieu, such as a cell of an oxidatively stressed host, could be oxidatively damaged in some way, thereby leading to new mutated forms. Additional research, however, is necessary to validate this concept.

Viral Selection Via Quasispecies

An RNA virus is really a collection of closely related mutants ("quasispecies") rather than a single uniform molecular entity (61). That is, the nucleotide sequence of an RNA viral genome represents a consensus or average base composition derived from a population distribution of viruses and does not mean to convey the notion of a fixed identical nucleotide chain for each and every individual strand of viral RNA. The occurrence of such pre-existing microheterogeneous genomic structures would allow the RNA virus to adapt quickly to changes in its environmental conditions (43).

Application of this principle to our coxsackievirus model would mean that the shift in host cell redox status occasioned by dietary deprivation of Se or vitamin E would favor the emergence of a new, more virulent (in this case) virus that is somehow more suited for growth under the altered conditions of increased host cellular oxidative stress. It seems reasonable to suppose that host nutritional status may exert a powerful (though heretofore largely unexplored) influence on the in vivo evolution of RNA viral pathogens (41). In fact, it appears possible that host oxidative stress could have more than one effect on viral evolution, perhaps first by accelerating the already rapid mutation rate of such viruses (42) by rendering their genome more susceptible to oxidative damage (see

section above), and then by favoring the selection of such mutated viruses over whatever viral strain was originally dominant. Only further research will allow us to distinguish between these two mechanisms.

ROLE OF NUTRITION IN EMERGING VIRAL DISEASES

Emerging viruses have risen to the forefront, both in the popular press and in the scientific literature. The explosive emergence of AIDS is, of course, the archetypal example of an emerging virus. Recently, more attention has been focused on trying to understand the mechanisms for viral emergence. A number of possibilities have been discussed: global climate changes, jumps across species barriers, increased worldwide travel disseminating viruses, etc. However, little or no attention has been focused on nutrition. It has long been observed that the nutritional status of the host can affect the outcome of illness following a viral infection (102). For example, infection of vitamin A–deficient children with measles can lead to severe measles associated with a lower respiratory tract infection with a high rate of mortality (86, 103). The association between famine and epidemics of infectious disease and high rates of mortality has been noted throughout history. Clearly, the nutritional status of the host is an important consideration when studying the pathology of a viral infection.

Below, we describe two nutritionally related diseases, Keshan disease in China and optical neuropathy in Cuba, which are strongly correlated with viral infection as a cofactor. These two examples provide evidence that viral diseases are affected by host nutritional status and that nutritional status of the host may have a profound effect on the emergence of a viral disease. In addition, we speculate on the possibility that other viral diseases, i.e. influenza and HIV, may be influenced by host nutritional status.

China

As the data from our murine myocarditis model suggest, nutritionally induced oxidative stress may have played a role in the emergence of a virulent coxsackievirus responsible for the etiology of Keshan disease in the low-selenium regions of China. It is unproven, but molecular epidemiology surveys conducted in areas adequate and deficient in Se in China might provide evidence to confirm or refute this idea. Virologists have available the tools required to sequence the genome of virus isolated at different stages of an epidemic to determine the evolution of a pathogen with time, even in samples that have been archived for long periods. Indeed, using the technique of polymerase chain reaction, enteroviral RNA has been detected in paraffin-embedded myocardial specimens from Keshan disease victims (73).

Influenza is another viral disease that should be discussed in the context of China. The flu virus can evolve by two different mechanisms (98): by the relatively slow process of stepwise mutation and selection (genetic drift) and, because of the segmentation of the influenza virus genome, by sudden genetic reassortment to produce new pandemic strains (genetic shift). Generally, flu viruses tend to stay within their natural reservoirs and do not cross species barriers readily. However, the barrier to infection in pigs is relatively low, so when coinfected with avian and human flu viruses, swine can function as "mixing bowls," to allow for the production of pandemic reassortants.

The mechanism by which these "new" flu viruses suddenly appear in the human population is not known, but the three major antigenic shifts that have taken place since the virus was first isolated all appear to have occurred in China (72). The survey of Shu et al (106) seems to support the idea that close contact among ducks, pigs, and humans on Chinese farms provides an excellent opportunity for the production of new and potentially hazardous strains of the flu virus.

Can Se status or dietary oxidative stress in general have any effect on the reassortment process? We simply do not know, but it may be more than a coincidence that so many of the reassortant flu strains have originated in a part of the world that also encompasses the planet's most Se-deficient soils. No one can predict exactly when the next global influenza pandemic will occur, but all flu experts agree that it will nonetheless take place (115). Such concerns led to the establishment of an international network of collaborating laboratories to monitor the emergence and spread of new epidemic and pandemic strains of influenza (35).

Optic and Peripheral Neuropathy in Cuba

An epidemic of optic and peripheral neuropathy affected more than 50,000 people in Cuba between 1991 and 1993. Illness was associated with the dietary limitations and increased physical demands accompanying the shortages of food and fuel experienced in Cuba since 1989. Most patients responded to parenteral vitamin therapy, and the epidemic began to subside when oral supplementation with vitamin A and B complex vitamins, including folate, was begun for the entire Cuban population in May, 1993. The number of cases reached 50,466 by the end of 1993, an incidence of 462 per 100,000 population. There were no fatal cases, but some patients had residual visual impairment, particularly those whose symptoms were far advanced before treatment was begun. Disease was rare in children, pregnant women, and the elderly; these groups are favored in the Cuban system of food distribution (90, 96). New cases have continued to accumulate at a low rate, with 52,406 reported by the end of 1995.

Extensive epidemiologic studies carried out with international cooperation (36, 50, 81) have established that the disease was associated with an unbalanced

diet—low in animal protein, fat, and B-group and other vitamins—and an increased consumption of sugar. In particular, impairment of protective antioxidant pathways was suggested, because patients had lower levels of riboflavin, vitamin E, selenium, α and β carotenes, and especially the carotenoid lycopene compared with matched controls. Smoking, especially of cigars, was a related risk factor that intensified the effects of the nutritional deficiencies and was thought to cause injury through oxidative damage. Vitamin E was included in the parenteral therapy given to epidemic neuropathy patients but not in the oral vitamins given for prevention to the entire population (110).

Attempts to isolate virus from the cerebrospinal fluid (CSF) of neuropathy patients, undertaken in 1993 to rule out an infectious agent, unexpectedly yielded in 84% (105 of 125) of the specimens cultivated viruses resembling enteroviruses (78). Five of these isolates were typical strains of coxsackievirus A9 (CVA9), identified by the standard technique of neutralization. The other 100 isolates produced a slowly progressive cytopathic effect (CPE) on Vero cells and were designated light CPE virus. They were antigenically related to both CVA9 and CVB4. In Western blot experiments, they lacked the capsid proteins typical of enterovirus, which contain the major epitopes for neutralization. This virus resembles CVA9 antigenically and in its pathogenic potential for mice yet differs from it in the VP1 capsid protein. Mutations in this specific region have been associated with acquisitions of virulence in CVB4 (26) and of the ability of poliovirus to produce persistent infection in human neuroblastoma cells (32). Light CPE virus persisted in the CSF of some patients for 1–12 months. The CSF of one patient yielded CVA9 on the first culture attempt and a virus of the variant type from a second culture 1 month later. Thus, the epidemic neuropathy in Cuba may have been the result of an emergence of a new strain of coxsackievirus as a consequence of replicating in a nutritionally compromised host.

Africa

A mean serum Se concentration of 27 ng/ml was reported in schoolchildren living in the northern Zaire town of Karawa, and some of the children had nondetectable levels of red blood cell glutathione peroxidase activity (112). This serum Se level is somewhat higher than the plasma Se level observed in the Keshan disease areas of China (119), but these children, nonetheless, have to be regarded as being at risk of Se deficiency. A later survey indicated marginally or moderately decreased mean serum Se level ($<$55 ng/ml) among pregnant Zairean women living in certain rural areas (84). Dietary Se intakes as low as 17 μg/day were found in the neighboring country of Burundi (19), almost exactly the minimum amount of Se that had to be consumed by adults in order to protect residents in the endemic areas of China against Keshan disease (120).

Like other RNA viruses, retroviruses such as the human immunodeficiency virus (HIV) responsible for AIDS mutate rapidly and occur as quasispecies (93). One possible explanation for the emergence of AIDS as a human health problem is the horizontal transmission of a closely related simian retrovirus to humans (47). Did impaired Se nutriture in central Africa have anything to do with the mutational event(s) that presumably allowed the AIDS virus to cross over from monkeys to man?

Regardless of whether Se had any role in the initial emergence of HIV, increasing evidence suggests that this trace mineral may have some value in the treatment of AIDS. Chronic oxidative stress appears to accompany infection with HIV (88), and in general, declines in the status of several antioxidants seem to correlate with the severity of the disease (46). Glutathione is consumed during HIV infection and progression (7), and glutathione deficiency in CD4 T cells and low total serum thiol levels are associated with decreased survival in HIV-infected subjects (58, 77).

Early work suggested that supplementation of HIV-infected patients with Se might cause symptomatic improvement and possibly also slow the course of the disease (99). More recently, Look et al (74) found that serum Se, plasma glutathione, and red cell glutathione peroxidase activity were decreased further at each successive stage of HIV-1 infection. Moreover, Baum et al (8) showed that Se deficiency (defined as a serum Se concentration less than 85 ng/ml) was a significant predictor of HIV-1–related mortality. Such in vivo results obtain some support from in vitro studies with lymphocytes, which indicate that Se supplementation decreased NF-κB activation (which stimulates HIV-1 replication) by tumor necrosis factor (TNF) (76) and suppressed TNF-induced HIV-1 replication (62). The rate of progression to AIDS varies greatly among HIV-1–infected persons, and those who experience rapid CD4 T cell loss have a quasispecies virus population in relative evolutionary stasis; however, those with more moderate rates of CD4 loss have virus populations still undergoing genetic evolution (117). Would it be possible to alter the evolution of HIV in vivo through manipulation of Se nutriture, thereby retarding the course of disease progression and having a beneficial impact on the health of AIDS patients?

CONCLUSIONS

ED Kilbourne found it "not surprising" that new viruses are continually evolving (67). This is particularly true for the RNA viruses with their high mutation rates and lack of proofreading capability. As pointed out by Nathanson et al (82), the evolution of viruses and virus diseases is subject to the complex interplay among the agent, the host, and the environment, the three components of the so-called epidemiologist's triangle. The unpredictable nature of RNA virus

evolution due to random mutation and recombinant events, as well as the subtle effects of chance host/environmental factors, was emphasized by Duarte et al (45). Several environmental variables have been discussed in the context of viral evolution (global warming, changes in industrial or agricultural processes, improvements in personal hygiene, etc), but little has been said about the impact of host nutritional status. One wonders how many outbreaks of disease attributed to a nutritional deficiency are actually the result of a viral infection that has changed its pathogenesis as a result of replicating in a nutritionally deficient host. It is hoped that the information presented in this review will persuade others of the need to examine the nutrition factor and will stimulate additional collaborations between nutritionists and virologists to investigate some of these problems.

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