The Influence of Antioxidant Nutrients on Viral Infection

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Introduction
Emerging viruses have risen to the forefront of attention, both in the popular press and the scientific literature. The explosive emergence of AIDS is, of course, the archetype example of an emerging virus. Recently, more attention has been focused on trying to understand the mechanisms of viral emergence. A number of areas have been discussed, including global climate changes, crossing species barriers, and increased worldwide travel. However, little or no attention has been focused on the influence of nutrition on emerging infectious disease.

It has long been observed that the nutritional status of the host can affect the illness outcome following a viral infection (Beck 1996). For example, measles infection in vitamin A–deficient children can lead to “severe measles,” which is associated with a lower respiratory tract infection and a high rate of mortality (Semba 1994, Ogaro et al. 1993). The Committee on Infectious Diseases of the American Academy of Pediatrics recommends vitamin A treatment for infants and children (up to age 2) hospitalized with severe measles. The association between famine and epidemics of infectious disease and high rates of mortality has also been noted throughout history. Clearly, the nutritional status of the host is an important consideration when studying the pathology of a viral infection.

This review will summarize recent work in our laboratory which demonstrates that the nutritional status of the host can influence the genome, and hence the virulence, of a coxsackievirus. This work developed from an interest in Keshan disease, a selenium-responsive endemic cardiomyopathy. The mechanism by which the genomic alterations occur is not known, but the immune status of the deficient host may play a role. I hope to convince the reader that nutritional status of the host plays an important role in the emergence of viral disease. Understanding the links between nutrition and viral infection will assist our ability to optimize strategies for prevention and/or treatment of viral diseases.

Keshan Disease
In China during the early 1930s, a cardiomyopathy named Keshan disease occurred in individuals living in specific regions of the country. The disease was characterized by acute or chronic heart function insufficiency, enlargement of the heart, arrhythmia, atrial fibrillation, and tachycardia. Histologically, the disease was characterized by multiple focal necrosis and myocardial parenchymatous degeneration (Ge et al. 1983). Epidemiologists in China found that the disease occurred only in areas with low selenium (Se) soil content, and thus, individuals residing in Keshan disease endemic areas were of low Se status (Keshan Disease Research Group 1979). Supplementation with Se to normal nutritional levels significantly decreased the occurrence of Keshan disease in endemic areas of China. However, the Se deficiency alone did not explain all aspects of the disease. Keshan disease has a seasonal and annual incidence, which suggests that an infectious cofactor may play an etiologic role in its development. Indeed, virologists in China have isolated several coxsackievirus strains from blood and tissue specimens of Keshan disease victims (Su et al. 1979, Bai et al. 1980). Using the technique of RT-PCR, investigators have found coxsackievirus sequences in archived heart tissue from Keshan disease victims (Li et al. 1995).

Thus, it appeared that in addition to the Se-deficiency, a viral infection, possibly a coxsackievirus, was responsible for the development of Keshan disease. Coxsackieviruses, and particularly coxsackievirus B3 (CVB3), are known etiologic agents of myocarditis and are suspected agents of dilated cardiomyopathy (DCM) (Fuster et al. 1981, Leslie 1989). DCM is the second leading indication for heart transplantation in this country (O’Connell and Robinson 1985). Coxsackieviruses are nonenveloped RNA viruses in the Picornaviridae family, subgroup enterovirus (of which the most commonly known enterovirus is polio virus).

CVB3-induced Myocarditis: Effects of Se or Vitamin E Deficiency
With over 3 decades of research, the mouse model of CVB-induced myocarditis is widely accepted as an appropriate animal model for human myocarditis (Woodruff 1980). Inoculation with coxsackievirus B3 (CVB3) induces a myocardial inflammatory infiltrate 10–14 days later (El-Khatib
by the viral infection, the heart pathology is widely believed to be due to an immunopathologic process. Evidence for an immunopathologic basis for enterovirus-induced cardiomyopathy is provided by the lack of or diminished disease in CVB3-infected athymic (nude) mice and lack of disease in X-irradiated adult mice which were not reconstituted with T cells (Woodruff and Woodruff 1974). In addition, at the peak period of cardiac inflammation, virus is not generally detectable in mouse heart tissue (Woodruff 1980). Although the immune system clearly contributes to the pathogenesis of Keshan disease and viral infection, a collaboration was initiated with Orville Levander at the United States Department of Agriculture. C3H/HeJ male mice were obtained at 3 weeks of age and fed one of five diets: (1) lard-based with Se and vitamin E; (2) lard-based with Se but without vitamin E; (3) lard-based without Se but with vitamin E; (4) menhaden oil (MO)-based with Se and vitamin E; or (5) MO-based with Se but without vitamin E. The basal diet consisted of torula yeast (30 g), tocopherol-stripped lard (4 g), tocopherol-stripped corn oil (1 g), AIN-76 salt mix without selenium (3.5 g), AIN-76A vitamin mix of calcium deposits in the nutritionally deficient mice when compared with nutritionally adequate mice (Table 1). Of particular interest, CVB3/0-infected mice fed either Se- or vitamin E-deficient diets developed a moderate level of myocarditis in contrast to CVB3/0-infected mice fed adequate diets, which did not develop myocarditis. Thus, a dietary deficiency in either Se or vitamin E permitted a viral phenotype change: CVB3/0 developed enhanced virulence, and CVB3/0 changed from an avirulent virus to a virulent one.

We examined virus titers in various organs to determine if virus replication patterns were altered as a result of replication in a nutritionally deficient animal. Virus titers in the heart, liver, spleen, and serum were 10-100-fold higher in the Se- or vitamin E–deficient CVB3/20- or CVB3/0-infected mice as compared with infected Se- and vitamin E–adequate mice, although the kinetics of the response were similar, and the deficient mice were able to clear the virus (data not shown).

Because both Se and vitamin E deficiency enhanced CVB3/20-induced myocarditis and allowed a normally benign CVB3/0 infection to become virulent, we hypothesize the existence of a common mechanism of oxidative stress induced by a dietary deficiency of either Se or vitamin E.
Table 1. Cardiac Histopathological Scores 10 Days Post-infection with CVB3

<table>
<thead>
<tr>
<th>Diet</th>
<th>Histopathological Score Post-CVB3/20 (Myocarditic)</th>
<th>Histopathological Score Post-CVB3/0 (Amyocarditic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard: +Se/E</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>Lard: -Se/E</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>Lard: +Se/E</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>MO: +Se/E</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>MO: +Se/E</td>
<td>4+</td>
<td>3+</td>
</tr>
</tbody>
</table>

1Mice were fed the indicated diets for 4 weeks prior to infection. MO = Menhaden oil. Each group represents 20 animals.

The extent of inflammatory lesions within the myocardium was graded without knowledge of the other experimental variables. The grading was performed in a semiquantitative manner according to the relative degree (from heart to heart) of mononuclear cell infiltration and the extent of necrosis. Pathologic score: 0 = no lesions, 1+ = foci of mononuclear cell inflammation associated with myocardial cell reactive changes without myocardial cell necrosis, 2+ = inflammatory foci associated with myocardial cell reactive changes, 3+ = inflammatory foci clearly associated with myocardial cell necrosis and dystrophic calcification, 4+ = extensive inflammatory infiltration, necrosis and dystrophic calcification. Each score represents the majority of the animals (in each case, hearts from a minimum of 11 animals in the group were scored at the indicated level).

Se is an essential constituent of glutathione peroxidase, an antioxidant enzyme that plays an important role in removing hydrogen peroxide and organic hydroperoxides (Chaudiere et al. 1984). Vitamin E acts a scavenger of oxygen radicals and, thus, inhibits lipid peroxidation (Packer 1992). Vitamin E and Se have been demonstrated to spare host and/or the virus in a manner that enhances virulence. Because the immune system is involved in the inflammatory response to the virus as well as in viral clearance, we chose to examine several immune functions: (1) B cell activity, measured by neutralizing antibody production, (2) T cell proliferation against both antigen and mitogen, and (3) expression of mRNA in the heart for interleukin (IL)-1, IL-6, and tumor necrosis factor-beta (TNF-β).

As shown in Figure 1, spleen cell responses to both mitogen and antigen were decreased in nutritionally deficient mice when compared with nutritionally adequate mice. These results suggest that T cell activity is altered by the Se or vitamin E deficiency. The precise defect in T cell responsiveness, however, is not yet known. T cell responsiveness could be affected at several steps in the activation pathway, including recognition of antigen and the ability to produce cytokines post stimulation.

To determine if cytokine responses were altered, we looked at three different cytokines that are thought to play a role in inducing inflammation post viral infection: IL-1, IL-6, and TNF-β. Using RT-PCR, we examined heart tissue for the presence of mRNA coding for the cytokine proteins at various times postinoculation. Rather than relying on cell culture, the RT-PCR technique allows one to study the message of interest at the site of inflammation. As shown in Figure 2, mRNA levels for IL-6 and TNF-β in the Se-deficient animals were decreased at each time point when compared with Se-adequate animals, except at day 4 post infection.

Table 2. Vitamin E Levels and Heart Histopathology of CVB3/20-infected Mice Fed Diets with and Without DPPD

<table>
<thead>
<tr>
<th>Diet</th>
<th>α-Tocopherol (μmol/L)</th>
<th>Heart Histopathology</th>
</tr>
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<tbody>
<tr>
<td>+Vitamin E</td>
<td>4.5 +/- 0.6</td>
<td>2+</td>
</tr>
<tr>
<td>-Vitamin E</td>
<td>0.6 +/- 0</td>
<td>4+</td>
</tr>
<tr>
<td>-Vitamin E +DPPD</td>
<td>0.8 +/-0.1</td>
<td>1+/2+</td>
</tr>
</tbody>
</table>

1All diets were Se-sufficient.
Figure 1. Mitogen- (solid bars) and antigen-specific (open bars) spleen cell proliferative responses from mice fed the indicated diets. Data are expressed as stimulation indices calculated as a ratio of counts per minute (cpm) in the presence of mitogen or antigen over cpm in the presence of medium or HeLa cell membranes (background). Background proliferation never exceeded 800 cpm. ConA = concanavalin A. Bars represent the mean±SD of 10 mice.

for TNF-β. However, IL-1 levels were similar between hearts from Se-adequate and Se-deficient mice. Thus, cytokine responses were altered in Se-deficient mice, indicating decreased T cell function.

Our results may indicate a suppression of T helper (TH1) cells preferentially to TH2 cells. TH2 cells are necessary for providing aid to B cells and secreting cytokines such as IL-4, IL-5, and IL-10. TH1 cells produce IL-2 and interferon-γ (INF-γ). Because antibody responses were intact (suggesting TH2 cells were functioning) and proliferation levels were decreased (suggesting a lack of IL-2), the nutritional deficit may have affected TH1 activity. We are currently examining levels of IL-2 and INF-γ in infected Se-deficient and Se-adequate mice.

To further examine the influence of antioxidant nutrients on the immune system, in collaboration with Oliver Smithies at UNC-Chapel Hill, we have infected macrophage inflammatory protein 1α (MIP-1α) knockout mice with CVB3/20 (Cook et al. 1995). MIP-1α is a member of a large superfamily of structurally related molecules called chemokines that induce chemotaxis in vitro (Miller and Krangel 1992, Loetscher et al. 1994). In vivo, chemokines may provide a necessary signal to direct mononuclear cells to sites of antigenic challenge and may also act to facilitate activation.

We found that MIP-1α knockout mice do not develop any inflammation post CVB3/20 infection, whereas 50% of the wild-type mice develop moderate to severe inflammation (Cook et al. 1995). This finding suggests that MIP-1α plays a central role in the development of inflammation post CVB3 infection. However, if MIP-1α knockout mice are placed on a diet deficient in vitamin E and Se, myocarditis will develop post CVB3/20 infection, demonstrating that the protective effect of a lack of MIP-1α can be

Figure 2. mRNA levels of various cytokines in the hearts of mice fed either a Se-adequate or Se-deficient diet. One-half of the heart was homogenized, and nucleic acids were recovered using a phenol-chloroform extraction method. Using RT-PCR, PCR fragments for IL-1, IL-6, and TNF-β were identified. The fragments were normalized with α-tubulin and scanned using a laser densitometer. Levels of mRNA for each sample were determined by measuring the area under the curve (AU) for each PCR fragment.
to be regulated by oxidative stress through regulation of nuclear factor \( \kappa \) (NF-\( \kappa \)B). NF-\( \kappa \)B is an inducible cellular transcription factor that regulates a wide variety of cellular and viral genes. We propose that the oxidative stress imposed by the nutritional deficiency may have upregulated NF-\( \kappa \)B, thus leading to increased expression of other chemokines in order to compensate for a lack of MIP-1\( \alpha \). This increase in chemokine expression then leads to cardiac inflammation in CVB3/20-infected nutritionally deficient knockout mice.

**Changes in the Viral Genome**

The increased pathology seen in nutritionally deficient mice may have been due to changes in the host that allowed for the virus to cause increased damage, such as a decrease in immune function or changes in cardiac cell physiology that led to increased susceptibility to viral damage. A second possibility is that the virus itself was affected as a consequence of replication in a Se- or vitamin E-deficient host. In order to determine if host oxidative stress status directly affected the CVB3/0 virus, we passaged virus obtained from Se- and vitamin E-deficient donors back into Se- or vitamin E-adequate recipients. The experiment was performed as follows: at 10 days post-inoculation with CVB3/0, hearts from mice fed either a Se-deficient or adequate diet or a vitamin E-deficient or adequate diet were processed for virus isolation. The viruses recovered from the hearts were passaged onto HeLa cell monolayers then isolated and titered. Next, these viruses were renamed to reflect the host from which they had been isolated. Virus, which was isolated from a Se-adequate host, was designated CVB3/0se+, while virus isolated from a Se-deficient host was designated CVB3/0se−. The recovered viruses were inoculated into 7-week-old male C3H/HeJ mice fed the lard-based Se- and vitamin E-adequate diet. At 7 and 10 days postinoculation, mice were sacrificed, and their hearts were examined for pathology.

The results demonstrated that CVB3/0 from either Se- or vitamin E-deficient mice underwent phenotypic change in the deficient animal, such that passage into Se- and vitamin E-adequate recipient mice also induced disease (Beck 1994a, 1994b, 1994c). Control animals, which were inoculated with virus obtained from Se- and vitamin E-adequate mice, did not develop any myocarditis, demonstrating that passage alone did not induce the changes. Thus, the Se or vitamin E deficiency was able to change the phenotype of the myocarditic CVB3/0 virus from benign to virulent.

Was the phenotype change due to a change in virus genotype? To answer this question, we sequenced four separate virus isolates obtained from CVB3/0-infected, Se-deficient mice, two isolates from vitamin E-deficient mice, and four separate isolates from CVB3/0-infected, Se- and vitamin E-adequate mice. We also sequenced the input virus, CVB3/0, for comparison. We found six nucleotide differences between virus obtained from the deficient animals as compared with the input virus (Beck et al. 1995). No nucleotide changes were found in virus isolates obtained from the nutritionally adequate mice. Of note, all six nucleotides were identical to nucleotides found in the virulent CVB3/20 virus, as well as another virulent strain, CVB3/M1. Interestingly, one nucleotide (nt 2690) in the avirulent strain, which is different from the cardiovirulent strains, did not change. Thus, the viruses isolated from the nutritionally deficient animals were identical to each other and represented a hybrid between known virulent strains and the avirulent input strain. To our knowledge, this is the first description of a specific host nutritional deficiency permitting an avirulent virus to develop virulence due to a change in the viral genotype.

The change in genotype of the virus isolated from Se- or vitamin E-deficient mice may be related to enhanced ability to replicate in deficient hosts. Because the immune system was altered in the Se- and vitamin E-deficient mice, the virus was able to replicate to a higher titer, thus increasing the chances for a mutation to occur. The most common causes of mutation in proliferating viruses are thought to be errors in polymerase transcription and endogenous damage to nucleic acid bases. Many virologists have noted that viruses have a tremendous capacity for change, particularly the RNA viruses. RNA viruses have a high rate of mutation, on the order of \( 10^3 \) to \( 10^5 \) substitutions per copied nucleotide. This rate is at least \( 10^2 \)-fold to \( 10^6 \)-fold larger than the mutation rate for cellular DNA. The high mutation rate of RNA viruses is due to the RNA replicase lacking efficient proofreading and post-replicative repair activities (Steinhauer et al. 1992, Eigen and Schuster 1979). It has been suggested that RNA viruses replicate near the minimal fidelity compatible with maintaining their genetic information. However, not all mutations will be viable. The success of a mutation depends on its ability to complete an infectious cycle as well as its overall fitness. Therefore, in an individual virus population, individual genotypes that differ in one or more nucleotides will form the average or consensus sequence of the population. Thus, viruses exist as populations or swarms of mutants, which have been termed quasi-species (Domingo et al. 1995). Quasi-species are enormous and dynamic mutant distributions that have great adaptability. Our results suggest that in a nutritionally deficient host, selection of new viral variants with altered pathogenic properties can occur. Once the newly selected viral variant becomes the dominant genotype, even animals of normal nutritle are susceptible to its enhanced pathogenic potential.
Conclusion

The relationship between nutrition and viral infection has been postulated to be one of inducing changes in the host’s immune response. This generally accepted hypothesis suggests that because the nutritional deficiency impairs the immune response, exposure to a viral pathogen will result in an increased opportunity for infection to occur and an increase in the severity of disease. The observations from our laboratory, however, suggest that this unidirectional model of nutrition-host immunity–virus interaction may not be entirely accurate. As outlined above, we have demonstrated that a normally avirulent coxsackievirus becomes virulent in a nutritionally deficient host due to point mutations in the viral genome. Once the viral mutations have occurred, even nutritionally adequate hosts are now susceptible to viral-induced disease. Thus, nutrition of the host not only affects the host but can affect the viral pathogen as well. What allows for the change in viral genotype to occur? Our work has demonstrated that the increased oxidative stress status of the host impairs the immune response. The decreased immune response may allow for the selection of a more virulent viral strain. The other possibility is direct oxidative damage of viral RNA, leading to increased mutations that result in enhanced virulence. Work is in progress to distinguish between these two possibilities. Our work points to the importance of increasing collaborations among nutritionists, virologists, and immunologists.


of measles. Trop Geog Med 45:283–6