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Interacting Nutritional and Infectious Etiologies of Keshan Disease

Insights from Coxsackie Virus B-Induced Myocarditis in Mice Deficient in Selenium or Vitamin E

ORVILLE A. LEVANDER*,[1] AND MELINDA A. BECK[2]

[1]*Nutrient Requirements and Functions Laboratory, Beltsville Human Nutrition Research Center, U.S. Department of Agriculture, ARS, Beltsville, MD 20705-2350;* [2]*Frank Porter Graham Child Development Center, University of North Carolina at Chapel Hill, NC. E-mail: cwoods@asrr.arsusda.gov*

ABSTRACT

In 1979, Chinese scientists reported that selenium had been linked to Keshan disease, an endemic juvenile cardiomyopathy found in China. However, certain epidemiological features of the disease could not be explained solely on the basis of inadequate selenium nutrition. Fluctuations in the seasonal incidence of the disease suggested involvement of an infectious agent. Indeed, a coxsackievirus B4 isolated from a Keshan disease victim caused more heart muscle damage when inoculated into selenium-deficient mice than when given to selenium-adequate mice. Those results led us to study the relationship of nutritional status to viral virulence. Coxsackievirus B3/0 (CVB3/0), did not cause disease when inoculated into mice fed adequate levels of Se and vitamin E. However, mice fed diets deficient in either Se or vitamin E developed heart lesions when infected with CVB3/0. To determine if the change in viral phenotype was maintained, we passaged virus isolated from Se-deficient hosts, designated as CVB3/0 Se-, back into Se-adequate hosts. The CVB3/0 Se- virus caused disease in Se-adequate mice. To determine if the phenotype change was due to changes in the viral genome, we sequenced viruses isolated from Se-deficient mice and compared them with the input CVB3/0 virus. Six point mutations differed

*Author to whom all correspondence and reprint requests should be addressed.

between the parent strain and the recovered CVB3/0 Se- isolates. When the experiment was repeated using vitamin E-deficient mice, the same 6 point mutations were found. This is the first report of a specific host nutritional deficiency altering viral genotype. Keshan disease may be the result of several interacting causes including a dominant nutritional deficiency (selenium), other nutritional factors (vitamin E, polyunsaturated fatty acids), and an infectious agent (virus).

Index Entries: Selenium; vitamin E; cardiomyopathy; myocarditis; Keshan disease; coxsackie virus; viral evolution; quasispecies; oxidative stress; infection.

INTRODUCTION

In 1957, Schwarz and Foltz *(1)* reported that traces of the mineral selenium added to the diet could prevent nutritional liver necrosis in vitamin E-deficient rats. This observation proved to be a seminal discovery that quickly led to the establishment of an entirely new area of trace element nutrition research. This discovery derived from basic research also found almost immediate practical agricultural application in the prevention of several economically important diseases of production animals, such as exudative diathesis in poultry or white muscle disease in livestock (*2*).

It was not until 1979, however, that the importance of selenium was demonstrated in human nutrition when two papers appeared that showed beneficial responses to selenium supplementation. One paper dealt with the ability of sodium selenite to alleviate muscle pain in a patient being fed intravenously *(3)*. That patient resided in the south island of New Zealand, a known low-selenium area, and thus presumably had low selenium stores to begin with that allowed relatively rapid development of a selenium-depleted state when fed with solutions that were probably nearly devoid of selenium. The other paper dealt with the epidemiologic relationship between poor selenium status and the incidence of Keshan disease, an endemic cardiomyopathy that affected primarily infants and young children, but also affected women of child-bearing age *(4)*. This endemic disease was distributed in a broad geographic zone stretching from northeastern to southwestern China. The evidence for an etiological role for selenium deficiency in Keshan disease was strong:

1. The disease occurred only in those regions of China with low-selenium soils and, hence, low levels of selenium in the food chain;
2. The disease occurred only in those individuals of poor selenium status as indicated by low blood or hair selenium content; and

3. The disease was prevented in an at-risk population by supplementation with selenium.

Despite the convincing linkage between selenium deficiency and Keshan disease, there were certain epidemiological features of the disease that could not readily be explained solely on the basis of inadequate selenium nutrition. For example, there were significant fluctuations in the seasonal and annual incidence of the disease, which suggested the likely involvement of an infectious agent *(5)*. Indeed, a number of viruses were isolated from Keshan disease victims, including a Coxsackie virus B4. As reported by Chinese scientists in 1980, this virus was much more cardiotoxic (i.e., caused more heart muscle damage) when inoculated into selenium-deficient mice than when given to selenium-adequate mice *(6)*. These results served as the starting point for our studies regarding the relationship of nutritional status to viral pathogenesis and virulence.

SELENIUM AND INFECTION WITH A MYOCARDITIC COXSACKIE VIRUS

Our first objective was to duplicate with Coxsackie virus B3 the earlier Chinese results showing that selenium deficiency exacerbated the heart muscle damage in mice caused by infection with Coxsackie virus B4. For that purpose, we used a myocarditic strain of the virus (i.e., a strain that normally causes heart muscle damage) called Coxsackie virus B3/20 (abbreviated CVB3/20). We found that selenium deficiency indeed potentiated the cardiotoxicity of CVB3/20 *(7)*. The viral-induced lesions occurred earlier and were more severe in the selenium-deficient mice compared to the selenium-adequate mice. Moreover, peak postinoculation virus titers were higher in the hearts, livers, spleens, and sera of selenium-deficient mice than in the corresponding tissues of selenium-adequate mice. Although some deficits in immune response were noted in the selenium-deficient mice, these relatively minor impairments in immune function were not thought sufficiently severe to account for the relatively great differences observed in virus titers between the two dietary treatments. This conclusion led us to test the hypothesis that the virus itself may have changed phenotype as a result of replication in a selenium-deficient host.

A viral passage experiment in which viruses were harvested from hearts of either deficient or adequate primary hosts and then reinoculated into a secondary selenium-adequate host revealed great differences in the virulence of the viruses depending on whether they had been allowed to replicate in a selenium-deficient or a selenium-adequate primary host. Viruses isolated from selenium-adequate primary hosts (now

called CVB3/20se+) caused little or no heart damage within seven days when inoculated into selenium-adequate secondary hosts. On the other hand, virus isolated from selenium-deficient primary hosts (CVB3/20se-) caused severe myocarditis in the same time when inoculated into selenium-adequate secondary hosts. The increased heart damage owing to CVB3/20se- was accompanied by 10^3-fold higher cardiac virus titers than those observed in hearts of secondary mice infected with CVB3/20se+.

SELENIUM AND INFECTION WITH AN AMYOCARDITIC COXSACKIE VIRUS

Having demonstrated that selenium deficiency markedly increased the heart damage caused by the myocarditic CVB3/20, we next turned our attention to the influence of selenium deficiency on host response to an amyocarditic strain (i.e., not causing heart damage under normal conditions), Coxsackie virus B3/0 (CVB3/0). As expected, the CVB3/0 caused no heart damage when inoculated into selenium-adequate mice *(8)*. However, when CVB3/0 was inoculated into selenium-deficient mice, a moderate degree of heart damage was seen, and virus titers were higher in the hearts, livers, spleens and sera of the deficient mice compared to the adequate mice. Inoculation of a selenium-adequate mouse with virus harvested from a selenium-adequate mouse (CVB3/0se+) resulted in no heart damage within 7 d, but inoculation of a selenium-adequate mouse with virus harvested from a selenium-deficient mouse (CVB3/0se-) caused moderate heart damage. To the authors' knowledge, this was the first report showing that a specific nutritional deficiency in the host allowed a benign virus to become virulent, apparently as a result of change in the viral genome.

VIRAL GENETIC CHANGE INDUCED BY HOST SELENIUM DEFICIENCY

The genomes of the virulent and benign strains of the Coxsackie virus B3 differ at several nucleotide positions *(9)*. When we found that the benign CVB3/0 could be converted to a virulent form (CVB3/0se-) by replication in a selenium-deficient mouse, we sequenced the entire viral genome (about 7500 nucleotides) to determine which base changes were responsible for the newly acquired virulence. Of the seven sites thought to be important determinants of virulence, six in the CVB3/0se- had changed to resemble the wild-type virulent strain, CVB3/20 *(10)*. No other nucleotide changes were seen along the length of the genome. Thus, replication of the benign CVB3/0 in a selenium-deficient host had altered its genetic makeup to resemble more closely that of the virulent

wild-type CVB3/20, thereby converting an avirulent strain to a virulent one. As far as the authors are aware, this was the first report to show that the nutritional status of a host can alter the genetic constitution of an infecting pathogen.

VITAMIN E AND INFECTION WITH COXSACKIE VIRUS

An analogous series of experiments was carried out in mice with vitamin E as the nutritional variable, rather than selenium *(11)*. In all cases, it was found that vitamin E deficiency had effects similar to those observed with selenium deficiency:

1. The myocarditic CVB3/20 caused greater heart damage in vitamin E-deficient mice than in normal mice;
2. The benign CVB3/0 converted to virulence in vitamin E-deficient mice;
3. In both cases above, the Coxsackie virus achieved higher cardiac titers in vitamin E-deficient mice compared to vitamin E-adequate controls;
4. The newly virulent virus obtained as a result of passage through a vitamin E-deficient host had exactly the same six genomic changes as those seen in the newly virulent virus obtained by passage through a selenium-deficient host *(12)*.

The extent of heart damage caused by the virus in vitamin E-deficient mice was not as great as that seen in selenium-deficient mice unless fish oil, a tocopherol antagonist *(13)*, was included in the vitamin E-deficient diet. This is probably because it takes longer to deplete mice of vitamin E than it does of selenium, unless polyunsaturated fat is added to the vitamin E-deficient diet as a pro-oxidant stressor.

DIETARY OXIDATIVE STRESS AND COXSACKIE VIRUS INFECTION

What do dietary deficiencies of selenium and vitamin E have in common so that they both might be expected to have similar effects in intensifying the cardiotoxicity of the Coxsackie virus? One obvious possibility is that since both nutrients are known to have antioxidant properties *(14)*, their lack in the diet would result in an oxidative stress in the host. Such host oxidative stress could affect viral virulence in a number of different ways. For example, the cardiomyocytes themselves could become more vulnerable to direct viral cytopathogenic attack because of their compromised antioxidant defense mechanisms, or the host's immune system could be sufficiently impaired, thus permitting an

increased viral load and more severe damage to the heart. Any increased ability of the virus to replicate by evading the host's immune response could favor the emergence of viral mutants with heightened cardiovirulent properties. On the other hand, host oxidative stress might stimulate any tendency of particular nucleotide sites in the viral genome to undergo rapid mutation, thereby leading to the evolution of more cardiovirulent progeny, or the altered redox milieu in the host could allow for dominance of an already mutated form of the virus by selectional pressures. Whatever the exact biochemical and/or genetic mechanism whereby dietary selenium and/or vitamin E deficiency alters the expression of Coxsackie viruses, these experiments show the powerful influence that host nutritional status can have on viral pathogenicity and point out the need for additional research in this area.

IMPLICATIONS FOR THE ETIOLOGY OF KESHAN DISEASE

Keshan disease clearly has a complex etiology, presumably encompassing both nutritional and infectious factors. Selenium deficiency, of course, is the nutritional condition most clearly associated with Keshan disease as outlined earlier. Nonetheless, the metabolic relationship between selenium and vitamin E is so close that it would be inappropriate to rule out any role for the latter in Keshan disease. Nutritional myopathies observed in cattle, sheep, and swine respond to either vitamin E or selenium *(2)*, and such animal diseases provided some of the first hints that selenium might be involved in human nutritional cardiomyopathy (i.e., Keshan disease) *(15)*. Human vitamin E nutriture has been reported to be marginal in some Keshan disease areas *(16)*, and the nutritional requirements for vitamin E and selenium are known to be mutually interdependent *(17)*. Selenium and vitamin E prevented the histochemical and ultrastructural alterations induced in the livers of rats fed grains from a Keshan disease endemic area, and it was concluded that the relative deficiencies of vitamin E and selenium may be closely related to the occurrence of hepatic damage in Keshan disease as well *(18,19)*. In this context, it should be recalled that the first nutritional disease shown to be responsive to selenium, dietary liver necrosis in rats, is actually a dual-deficiency disease preventable by either selenium or vitamin E *(1)*. Thus, the Chinese studies with rats fed grain from Keshan disease endemic areas imply that such foodstuffs may be lacking in both selenium and vitamin E.

The fact that highly unsaturated fish oil exacerbated the heart damage caused by Coxsackie virus B3 in vitamin E-deficient mice emphasizes the need to consider dietary pro-oxidants and other factors as well as antioxidants when attempting to gain a comprehensive understanding of the etiology of Keshan disease. Others, however, were unable to find any

substantial difference in the fatty acid pattern of the plasma or erythrocyte lipids of Chinese children from Keshan or non-Keshan disease areas vs their Western counterparts with the notable exception of the erucic acid content *(20)*. The dietary source of this cardiotoxic fatty acid apparently was a local rapeseed oil, but no difference was observed in the erucic acid content of blood from children from endemic and nonendemic areas. Erucic acid was also not considered an etiological factor in an idiopathic cardiomyopathy observed in a Keshan disease area of Sichuan Province *(21)*.

If infection is indeed an etiological component of Keshan disease, then some sort of enterovirus, such as Coxsackie B or ECHO virus, would seem to be the most likely candidate agent *(22)*. Mild fever was often reported before the appearance of heart symptoms *(23)*, and such enteroviruses were among those isolated from blood and tissue samples taken from Keshan disease patients *(24)*. Recently, powerful molecular biology techniques have been used to obtain evidence for the presence of enteroviral RNA in blood and heart muscle specimens collected from patients with Keshan disease *(25,26)*. On the other hand, the histopathological picture of Keshan disease differs from that of viral myocarditis *(27,28)*, and the representative lesion of Keshan disease is a myocytolysis apparently initiated by mitochondrial damage. Impaired respiratory capability was noted in heart muscle mitochondria from Keshan disease patients or laboratory animals fed cereals from Keshan disease-endemic areas *(29,30)*, and the so-called respiratory decline was a prominent feature of liver mitochondria prepared from selenium and vitamin E-deficient rats originally described in early work by Schwarz *(31)*. Later work showed that selenium was a strong catalyst for the reduction of cytochrome c by thiols, particularly glutathione *(32)*. In some cases of Keshan disease, pancreatic septal atrophy involving the acini is observed *(33)*, and acinar atrophy of the pancreas has been reported in chickens fed a severely selenium-deficient diet of corn and soybeans from northeastern China *(34)*. Although Coxsackie virus has most often been linked to β-cell damage and consequent diabetes *(35)*, under certain circumstances, infection with the virus will lead to severe acinar necrosis *(36)*. Atlantic salmon (*Salmo salar*) afflicted with the infectious pancreatic necrosis virus suffer extensive necrosis of exocrine pancreatic tissue and exhibit significantly lower plasma α-tocopherol levels than healthy fish sampled from the same cages *(37)*.

Our results with the mouse model of viral myocarditis suggest a new way to look at the complex etiology of Keshan disease. If these experiments are applicable to humans, then people should be able to exist comfortably in an environment surrounded by benign viruses (Coxsackie or otherwise) as long as they are well nourished. However, once their nutritional status is compromised (selenium, vitamin E deficiency, and maybe others), they become vulnerable to the action of newly virulent viruses either produced within their own bodies or transmitted from

other malnourished individuals. Perhaps the best approach to Keshan disease is to acknowledge its complicated etiology and accept the possible role of several interacting factors, including a dominant nutritional deficiency (selenium), additional nutrients (vitamin E, polyunsaturated fatty acids, and others) and an infectious agent (virus).

IMPLICATIONS FOR NUTRITION

In their extensive monograph, Scrimshaw et al. *(38)* pointed out that only 7% of the reported interactions between malnutrition and bacterial infection could be classified as antagonistic (i.e., the simultaneous presence of malnutrition and infection results in an interaction that is **less** serious for the host than would be expected from the combined effect of the two working independently), whereas 37% of the reported interactions between malnutrition and viral infection could be so classified. The relatively common occurrence of antagonistic interactions between malnutrition and viral infection was thought to be owing to the intracellular nature of these agents and their consequent high degree of dependence on the intact metabolism of the host cell. That is, if the intermediary metabolism of the host cell were disrupted by a nutritional deficiency, this would also have an unfavorable impact on the ability of the viral pathogen to replicate and complete its infectious cycle. Thus, malnutrition could have the somewhat unexpected consequence of improving the ability of a host to resist viral infection by "starving" the virus at the cellular level, thereby presumably restricting viral replication.

On the other hand, Woodruff and Kilbourne *(39)* found that graded underfeeding (marasmus) exerted a detrimental effect on the ability of mice to resist infection with Coxsackie virus B3 that was proportional to the severity of the malnutrition. The criteria of increased severity of infection in the malnourished groups included persistence of increased virus titers, increased cardiac lesions, and increased mortality. Although the mechanism whereby severe food restriction (i.e., withholding of the total diet) increased the susceptibility of mice to Coxsackie virus B3 infection was not clear, there was a stepwise involution of the lymphoid tissues that paralleled the decreasing amounts of diet consumed *(40)*. Refeeding the nutritionally deprived mice such that their lymphoid tissues returned to normal resulted in a restoration of the mice to a normal susceptibility to viral infection.

An unusual feature of the Coxsackie virus infection observed in this study was a massive hepatic necrosis in the infected, severely malnourished mice. The reason for that necrosis was unknown, but was thought to be related to the "enormously increased replication of virus" seen in the livers of the starved animals. It should be noted that although the selenium content of the diet was not given, the diet was clearly deficient in vitamin E at least for part of the experiment. Caloric restriction, such

as that used in gerontological research *(41)*, appears to decrease in vivo oxidative stress as indicated by decreased ethane production or lipofuscin accumulation *(42)*, but perhaps under conditions of viral infection, partial fasting may tend to exacerbate oxidative stress. It might be worthwhile to re-examine the influence of food deprivation on the course of Coxsackie virus infection in mice that have been receiving diets of carefully controlled vitamin E and selenium content.

Woodruff and Kilbourne *(39)* were aware that their data conflicted with earlier results of increased resistance to viral infection in nutritionally compromised experimental animals, but believed that their observations were more in accord with practical experience, which demonstrated more severe viral infections in malnourished children. They stressed that their research differed from earlier work in that they:

1. Employed a "moderate" non lethal viral challenge;
2. Maintained a strict and sustained dietary deprivation; and
3. Used a quantitative assessment of viral replication and lesions.

Since deficiencies of either selenium or vitamin E exacerbate the heart damage caused by the Coxsackie virus, then the most straightforward explanation for this result is that oxidative stress in the host is somehow involved. The fact that dietary fish oil, a known pro-oxidant stressor *(13)*, worsened the cardiopathology in the infected vitamin E-deficient mice, whereas supplementing the deficient diet with *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), a synthetic organic antioxidant that bears no structural relationship to vitamin E, but nonetheless acts as an excellent tocopherol mimic *(43)*, protects against such damage bolsters this idea. If host oxidative stress is indeed involved in Coxsackie virus pathogenesis, other nutrients known to have antioxidant properties should also be investigated in animals infected with the virus. Some examples of such "antioxidant nutrients" would include copper and zinc by virtue of their role in cytoplasmic superoxide dismutase, manganese because of its involvement in mitochondrial superoxide dismutase, iron not only as a constituent of catalase, but also because of its inherent pro-oxidant catalytic capability, riboflavin as a component of glutathione reductase, vitamin C, and β-carotene. Indeed, copper and zinc have already been studied in an indirect way, since polyethylene glycol conjugated with superoxide dismutase exerted some protective activity against Coxsackie virus-induced myocarditis in mice *(44)*. Chen et al. *(45)* pointed out that the heart has relatively weak antioxidant defenses, which make this organ a target for damage in copper-deficient rats.

In the context of host oxidative stress, another aspect of nutrition that should be considered is severe protein deficiency (kwashiorkor). The possibility of a link between selenium deficiency and kwashiorkor was recognized early, since selenium tends to follow protein in food so that, other things being equal, diets low in protein would also tend to be low

in selenium. Schwarz reported a beneficial effect of selenium supplements in stimulating the growth of two Jamaican infants with kwashiorkor, although the results provoked some controversy *(46,47)*. Another report showed a favorable effect of supplemental selenium in stimulating the reticulocyte response in three malnourished Jordanian children *(48,49)*. Blood selenium levels were indeed depressed in samples taken from Guatemalan children with kwashiorkor, and red blood cells from these patients took up more radioselenium in vitro than red cells from normal controls *(50)*. Golden and colleagues *(51,52)* postulated that formation of free radicals might play a role in the pathogenesis of kwashiorkor. These workers were able to frame a set of clinical criteria based on pro-oxidant/antioxidant balance in vivo that could predict whether or not a given patient would die from protein malnutrition: those individuals whose red cell glutathione peroxidase activity was below 17 U/g hemoglobin and whose plasma ferritin was above 250 μg/L subsequently died. Some workers have suggested that specific selenium replacement may be required in the nutritional rehabilitation of children with kwashiorkor *(53)*, whereas others have questioned whether low selenium status and low selenium intake exhibit a health risk inhibiting further rehabilitation of patients with severe protein-calorie malnutrition *(54)* or have found no reliable evidence of harmful consequences to living tissue of the decreased antioxidant protection of such patients *(55)*. In any case, for the purpose of this article, it should be recalled that infection is almost ubiquitous in kwashiorkor and that the disease is often precipitated by an infection such as measles *(52)*, the pathogen of which, like Coxsackie virus, is an RNA virus.

Although the effects of dietary selenium supplementation at nutritional levels in limiting Coxsackie virus-induced myocardial pathology can be reasonably explained on the basis of increasing host glutathione peroxidase activity thereby minimizing in vivo oxidative stress, some work indicates effects of additional selenium even in mice nutritionally replete with the element. For example, some evidence was found of protection by small bolus doses of selenium (0.04 μg in a 20-g mouse) injected into mice fed a diet containing 0.14 ppm selenium but a marginal level of vitamin E *(56)*. At that dietary selenium intake, one would expect tissue glutathione peroxidase activities to be maximized, so why the additional bolus dose should have any protective effect is not clear. In a later report, 5 ppm selenium in the drinking water, generally regarded as a toxic dose *(57)*, improved survival of Coxsackie virus-infected mice fed an unspecified diet, but had no effect on myocardial inflammatory or necrotic lesions *(58)*.

Certain heavy metal poisons that have strong metabolic interactions with selenium, such as methyl mercury *(59)* or cadmium *(60)*, also interact with the Coxsackie virus: methyl mercury increased the viral-induced inflammatory and necrotic myocardial lesions *(61)* and Coxsackie virus

infection markedly altered the in vivo distribution of cadmium *(62)*. These results suggest that three-way interactions among nutrients, toxicants, and infectious agents should be studied systematically under controlled conditions of exposure to each. Parizek and coworkers hypothesized more than 25 years ago that one of the biological functions of selenium could be the protection of the organism against the toxicity of trace amounts of metals that even under "normal" conditions enter the body from the environment and remain *(63)*.

With the exception of our work showing that highly unsaturated fat in the form of fish oil worsens the cardiac damage caused by Coxsackie virus infection in vitamin E-deficient mice, relatively little work has been done on the effect of dietary lipid on the outcome of Coxsackie virus infection. Loria et al. *(64)* found that 97% of adult male mice fed a hypercholesterolemic test diet and infected with the Coxsackie virus B5 died, whereas none of the infected mice fed a control diet succumbed. However, the test diet was a purified diet based on casein, whereas the control diet was a crude diet (Wayne Lab Blox, Allied Mills, Chicago, IL). Therefore, any direct comparisons between these two diets is inappropriate, since a great variety of nutritional factors would be different. Although the test diet contained luxus levels of vitamin E, the selenium content may have been limiting, depending on how much selenium was in the casein. The salt mix used in this study (Hegsted IV, ICN Life Sciences, Cleveland, OH) contains no added selenium, and the casein content of the test diet was relatively low (15%) especially for a diet containing 20% fat. Thus, the marked difference in the mortality of the infected test and infected control groups could be explained by many nutritional variables, so that the experiment is difficult to interpret. A later study carried out under more carefully controlled dietary conditions showed that mild hypercholesterolemia had no effect on the histological lesions, lethality, or immune cell activity in mice infected with Coxsackie virus B3 *(65)*. However, cholesterol accumulation was noted in certain organs, including aorta, in infected mice fed the normal diet, thereby suggesting a possible role for Coxsackie virus infection in the development of atherosclerosis.

IMPLICATIONS FOR VIRAL DISEASES

Thus far, our research has shown that host deficiency in either of two metabolically related antioxidant nutrients (vitamin E or selenium) causes changes in the genome of Coxsackie virus replicating within the host that result in increased virulence of the pathogen. At the present time, we simply do not know the scope of this phenomenon. That is, we do not know how many different deficiencies in the host might have a similar effect on the virus, nor do we know how many different viruses

might be affected by such deficiencies. However, it would seem prudent to keep ourselves open to all possibilities and to examine systematically the impact of a variety of nutritional deficits on the virulence of several viral pathogens. Not only would such a survey possibly provide practical information about which deficiencies might be important in determining resistance to disease, but theoretical insights gained thereby might also lead to greater understanding of the molecular mechanisms by which pathogen virulence is altered by changes in host nutriture.

RNA viruses, such as the Coxsackie virus (i.e., viruses that use RNA as their genetic material), have the capability of evolving rapidly because of their high mutation rate. This high frequency of mutation is owing to the inherently error-prone nature of RNA synthesis, which may be 10^6-fold greater than that of the DNA synthesis that occurs in their animal, plant, or human hosts *(66)*. As pointed out by Holland, this characteristic gives rise to so-called quasispecies populations in which the RNA viruses must be regarded as clusters of related mutants rather than as discrete entities *(66)*. That is, "a particular virus, which we think of as homogeneous ... really is a statistical consensus of a genetically heterogenous population, which, again, is in constant flux" *(67)*. When the environmental conditions of the virus change (e.g., because of alterations in the level of oxidative stress owing to changes in the nutritional status of the host), it can be expected that a new quasispecies population will evolve depending on the relative adaptability and fitness of the various viral strains present.

Since most plant, animal, and human viruses are in fact RNA viruses, the potential impact of malnutrition as a determinant of viral pathogenicity seems large. Even if one limits oneself to the picornaviruses, the same family that includes the Coxsackie viruses, several diseases of human and animal interest come to mind, such as poliomyelitis, foot and mouth disease, and the common cold, among others. The heated debate surrounding the choice of the best approach to vaccination against poliomyelitis is an illustrative example. Currently recommended practice in the US is to use the "live virus" oral Sabin vaccine because of its great effectiveness, rather than the "killed virus" inactivated Salk vaccine *(68)*. Unfortunately, use of the Sabin vaccine results in a rare number of cases of "vaccine-associated paralytic polio," presumably because of a back-mutation of this weakened vaccine virus to the more virulent wild-type. Since 1979, 130 such occurrences have been reported *(69)*. Why these reversions occur in a small fraction of the vaccinations is not understood, but one wonders whether improved nutrition of the host could help guard against such tragic events.

Another situation in which it might be critical to ensure adequate host nutrition is during the use of viral pathogens to eradicate undesirable target species. In Australia, for example, a plan was devised to release the Rabbit Hemorrhagic Disease Virus (RHDV) as a way of helping to control that country's large population of feral rabbits *(70)*. How-

ever, fears that RHDV, a potent calicivirus, might spread to other species led to a delay in implementing this plan *(71)*. Knowing now that host selenium deficiency can markedly alter the pathogenicity of a virus, that Australia has zones of low-selenium soils (primarily in Western Australia *[72]*, but also in South Australia where the virus has already escaped *[73])*, and that rabbits, being herbivorous, might readily consume selenium-deficient forages, perhaps deferral of that decision was the wisest course of action. Rabbits are an unusual species in that, like hamsters and guinea pigs, they exhibit no disease signs that respond to selenium *(17)*. Nonetheless, rabbits require dietary selenium for full expression of glutathione peroxidase activity and show specific signs of vitamin E deficiency. Perhaps the soundest approach would be to infect selenium- and/or vitamin E-deficient rabbits with RHDV to see if nutritional deficiency in the host might have any effect on the properties of the virus.

Our work shows the value of interdisciplinary approaches to research problems. The real world is comprised of many interacting factors (nutrition, toxicology, infection, and others) that need to be examined *in toto*. The unpredictability of metabolic interactions among different substances was dramatized by the recent finding that simultaneous exposure to two common pesticides markedly multiplied their ability to act on estrogen receptors *(74)*. Thus, such combinations of "environmental estrogens" may be potent enough to increase significantly the risk of cancer and birth defects, and this research led one high-ranking EPA official to declare that the results will make "synergistic effects a long-term research priority" for the EPA *(75)*. Observations like these will not make life any easier for unifactorial experimentalists who study toxicants (or nutrients, infectious agents, or whatever) one-by-one, since these data emphasize the need to design appropriate multifactorial, multidisciplinary studies when complex problems are addressed. The relatively simple nutrition/virus interaction discussed here is only the first of many steps needed to obtain a comprehensive picture of the overall relationship between diet and infection, and much more interdisciplinary work needs to be done.

REFERENCES

1. K. Schwarz and C. M. Foltz, Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration, *J. Am. Chem. Soc.* **79,** 3292–3293 (1957).
2. Subcommittee on Selenium, *Selenium in Nutrition*, National Academy Press, Washington, DC (1983).
3. A. M. Van Rij, C. D. Thomson, J. M. McKenzie, and M. F. Robinson, Selenium deficiency in total parenteral nutrition, *Am. J. Clin. Nutr.* **32,** 2076–2085 (1979).
4. Keshan Disease Research Group, Epidemiologic studies on the etiologic relationship of selenium and Keshan disease, *Chin. Med. J.* **92,** 477–482 (1979).
5. G. Q. Yang, Keshan disease: an endemic selenium-related deficiency disease, in *Trace Elements in Nutrition and Children*, R. K. Chandra, ed., Raven, New York, pp. 273–290 (1985).

6. J. Bai, S. Wu, K. Ge, X. Deng, and C. Su, The combined effect of selenium deficiency and viral infection on the myocardium of mice, *Acta Acad. Med. Sin.* **2,** 29–31 (1980).
7. M. A. Beck, P. C. Kolbeck, Q. Shi, L. H. Rohr, V. C. Morris, and O. A. Levander, Increased virulence of a human enterovirus (coxsackievirus B3) in selenium-deficient mice, *J. Infect. Dis.* **170,** 351–357 (1994).
8. M. A. Beck, P. C. Kolbeck, L. H. Rohr, Q. Shi, V. C. Morris, and O. A. Levander, Benign human enterovirus becomes virulent in selenium-deficient mice, *J. Med. Virol.* **43,** 166–170 (1994).
9. N. M. Chapman, Z. Tu, S. Tracy, and C. J. Gauntt, An infectious cDNA copy of the genome of a non-cardiovirulent Coxsackievirus B3 strain-its complete sequence analysis and comparison to the genomes of cardiovirulent Coxsackieviruses, *Arch. Virol.* **135,** 115–130 (1994).
10. M. A. Beck, Q. Shi, V. C. Morris, and O. A. Levander, Rapid genomic evolution of a non-virulent coxsackievirus B3 in selenium-deficient mice results in selection of identical virulent isolates, *Nature Med.* **1,** 433–436 (1995).
11. M. A. Beck, P. C. Kolbeck, L. H. Rohr, Q. Shi, V. C. Morris, and O. A. Levander, Vitamin E deficiency intensifies the myocardial injury of coxsackievirus B3 infection in mice, *J. Nutr.* **124,** 345–358 (1994).
12. M. A. Beck, Q. Shi, V. C. Morris, and O. A. Levander, From avirulent to virulent: vitamin E deficiency in mice drives rapid genomic evolution of a coxsackie B3 virus, *FASEB J.* **10,** A191 (1996).
13. H. Dam, Interaction between vitamin E and polyunsaturated fatty acids in animals, in *Vitamins and Hormones*, R. S. Harris and I. G. Wool, eds., Academic Press, Orlando, FL, pp. 527–540 (1962).
14. W. G. Hoekstra, Biochemical function of selenium and its relation to vitamin E, *Fed. Proc.* **34,** 2083–2089 (1975).
15. G. Q. Yang, J. Chen, Z. Wen, K. Ge, L. Zhu, X. Chen, and X. Chen, The role of selenium in Keshan disease, in *Advances in Nutritional Research*, vol. 6, H. H. Draper, ed., Plenum, New York, pp. 203–231 (1984).
16. G. Q. Yang, K. Ge, J. Chen, and X. Chen, Selenium-related endemic diseases and the daily selenium requirements of humans, *World. Rev. Nutr. Diet.* **55,** 98–152 (1988).
17. G. F. Combs, Jr. and S. B. Combs, *The Role of Selenium in Nutrition*, Academic Press, Orlando, FL (1986).
18. S. Liu and F. Wang, Study on hepatic damage induced by pathogenic factors of Keshan disease in the grains from endemic area. *Chin. J. Control Endemic Dis.* **5,** 196–199 (1990).
19. S. Liu, L. Wang, Z. Zhao, and F. Wang, Alterations of histochemistry and ultrastructure in liver of rats fed with grains from a Keshan disease endemic area. *J. Norman Bethune University Med. Sci.* **18,** 521–523 (1992).
20. M. D. Laryea, Y. F. Jiang, G. L. Xu, and I. Lombeck, Fatty acid composition of blood lipids in Chinese children consuming high erucic acid rapeseed oil, *Ann. Nutr. Metab.* **36,** 273–278 (1992).
21. M. L. Bierenbaum, Y. Chen, H. Lei, and T. Watkins, Relationship between dietary fatty acid, selenium, and degenerative cardiomyopathy, *Med. Hypotheses* **39,** 58–62 (1992).
22. G. Q. He, On the etiology of Keshan disease, *Chin. Med. J.* **92,** 416–422 (1979).
23. K.-Y. Ge, J. Bai, X.-J. Deng, S.-Q Wu, S.-Q. Wang, A.-N. Xue, and C.-Q. Su, The protective effect of selenium against viral myocarditis in mice, in *Selenium in Biology and Medicine*, G. F. Combs, Jr., J. E. Spallholz, O. A. Levander, and J. E. Oldfield, eds., AVI, New York, pp. 761–768 (1987).
24. G. Su, G. Gong, J. Li, C. Chen, D. Zhou, and Q. Jin, Preliminary results of viral etiological study of Keshan disease, *Chin. Med. J.* **59,** 466–470 (1979).
25. Y. Li, H. Zhang, Y. Yang, H. Chen, and L. C. Archard, High prevalence of enteroviral genomic sequences in an endemic cardiomyopathy (Keshan disease) detected by nested polymerase chain reaction, *Progress in Clinical Virology 1995 Joint Meeting*, Prague, Czech Republic, September 10–14, p. 272 (1995).

26. P. D. Roath, M. A. Beck, X. Chen, A. Xue, S. Wang, and O. A. Levander, Detection of enterovirus RNA from heart tissues blocks and blood obtained from a Keshan disease endemic area of China using reverse transcriptase-polymerase chain reaction (RT-PCR), *FASEB J.* **10,** A3213 (1996).
27. K. Y. Ge, A. Xue, J. Bai, and S. Wang, Keshan disease-an endemic cardiomyopathy in China, *Virchows Arch. (Pathol. Anat.)* **401,** 1–15 (1983).
28. G. S. Li, F. Wang, D. Kang, and C. Li, Keshan disease: an endemic cardiomyopathy in China, *Hum. Pathol.* **16,** 602–609 (1985).
29. F. Y. Yang, Z. H. Lin, S. G. Li, B. Q. Guo, and Y. S. Yin, Keshan disease—an endemic mitochondrial cardiomyopathy in China, *J. Trace Element Electrolytes Health Dis.* **2,** 157–163 (1988).
30. F.-Y. Yang, Z-H. Lin, J.-R. Xing, S.-G. Li, J. Yang, S. San, and L.-Y. Wu, Se deficiency is a necessary but not sufficient factor required for the pathogenesis of Keshan disease, *J. Clin. Biochem. Nutr.* **16,** 101–110 (1994).
31. K. Schwarz, Vitamin E, trace elements and sulfhydryl groups in respiratory decline (an approach to the mode of action of tocopherol and related compounds), *Vitam. Horm.* **20,** 463–484 (1962).
32. O. A. Levander, V. C. Morris, and D. J. Higgs, Selenium as a catalyst for the reduction of cytochrome c by glutathione, *Biochemistry* **12,** 4591–4595 (1973).
33. D. Kang, G. Li, F. Wang, and G. Yang, Pancreatic lesions of Keshan disease and its significances, *Chin. Med. J.* **100,** 671–676 (1987).
34. G. F. Combs, Jr., C. H. Liu, Z. H. Lu, and Q. Su, Uncomplicated selenium deficiency produced in chicks fed a corn-soy-based diet, *J. Nutr.* **114,** 964 (1984).
35. M. Solimena and P. DeCamilli, Coxsackieviruses and diabetes, *Nature Med.* **1** 25–26 (1995).
36. R. M. Gomez, X. Cui, C. G. Castagnino, and M. I. Berria, Differential behavour in pancreas and heart of two coxsackievirus B3 variants, *Intervirology* **36,** 153–160 (1993).
37. T. Taksdal, T. Poppe, T. Sivertsen, and H. W. Ferguson, Low levels of vitamin E in plasma from Atlantic salmon *Salmo salar* with acute infectious pancreatic necrosis (IPN), *Dis. Aquatic Organisms* **22,** 33–37 (1995).
38. N. S Scrimshaw, C. E. Taylor, and J. E. Gordon, *Interactions of Nutrition and Infection,* World Health Organization, Geneva (1968).
39. J. F. Woodruff and E. D. Kilbourne, The influence of quantitated post-weaning undernutrition on coxsackievirus B_3 infection of adult mice. I. Viral persistence and increased severity of lesions, *J. Infect. Dis.* **121,** 137–163 (1970).
40. J. F. Woodruff, The influence of quantitated post-weaning undernutrition on coxsackievirus B_3 of adult mice. II. Alteration of host defense mechanisms, *J. Infect. Dis.* **121,** 164–181 (1970).
41. D. L. Snyder, ed., *Dietary Restriction and Aging,* Alan R. Liss, New York (1988).
42. Anonymous, Energy intake restriction and oxidant defense, *Nutr. Rev.* **49,** 278–280 (1991).
43. H. H. Draper and A. S. Csallany, Action of *N'N'*-diphenyl-*p*-phenylenediamine in tocopherol deficiency diseases, *Proc. Soc. Exp. Biol. Med.* **99,** 739–742 (1958).
44. Y. Hiraoka, C. Kishimoto, K. Masahiko, H. Ochiai, and S. Sasayama, Effects of polyethylene glycol conjugated superoxide dismutase on coxsackievirus B3 myocarditis in mice, *Cardiovas. Res.* **26,** 956–961 (1992).
45. Y. Chen, J. T. Saari, and Y. J. Kang, Weak antioxidant defenses make the heart a target for damage in copper-deficient rats, *Free Radical Biol. Med.* **17,** 529–536 (1994).
46. K. Schwarz, Development and status of experimental work on Factor 3-selenium, *Fed. Proc.* **20,** 666–673 (1961).
47. K. Schwarz, Selenium and Kwashiorkor, *Lancet* 1335–1336 (1965).
48. A. S. Majaj and L. L. Hopkins, Jr., Selenium Kwashiorkor, *Lancet* 592–593 (1966).
49. L. L. Hopkins, Jr. and A. S. Majaj, Selenium in human nutrition, in *Selenium in Biomedicine,* O. H. Muth, J. E. Oldfield, and P. H. Weswig, eds., AVI, Westport, CT, pp. 203–214 (1967).

50. R. F. Burk, Jr., W. N. Pearson, R. P. Wood, and F. Viteri, Blood-selenium levels and in vitro red blood cell uptake of ^{75}Se in Kwashiorkor, *Am. J. Clin. Nutr.* **20,** 723–733 (1967).
51. M. H. N. Golden and D. Ramdath, Free radicals in the pathogenesis of Kwashiorkor, in *Proceedings of the XIII International Congress of Nutrition,* T. G. Taylor and N. K. Jenkins, eds., John Libbey, London, pp. 597–598 (1985).
52. M. H. N. Golden and D. Ramdath, Free radicals in the pathogenesis of Kwashiorkor, *Proc. Nutr. Soc.* **46,** 53–68 (1987).
53. A. A. Sive, H. De V. Heese, W. S. Dempster, E. Subotsky, H. Malan, and R. Sacks, Protein energy malnutrition: selenium, glutathione peroxidase and glutathione in children with acute kwashiorkor and during refeeding, in *Trace Elements in Man and Animals* 7, B. Momcilovic, ed., IMI, Zagreb, pp. 19-15, 19-16 (1991).
54. H. M. Ahmed, I. Lombec, A. O. El-Karib, E. O. El-Amin, H. Menzel, D. Frosch, M. Leichsenring, and H. J. Bremer, Selenium status in Sudanese children with protein-calorie malnutrition. *J. Trace Elem. Electrolytes Health Dis.* **3,** 171–174 (1989).
55. R. Albrecht and M. A. Pelissier, About the oxidative stress status in children with Kwashiorkor, *Food Chem. Toxic.* **33,** 1081–1083 (1995).
56. N.-G Ilback, J. Fohlman, and G. Friman, Protective effect of selenium on the development of coxsackie virus B3-induced inflammatory lesions in the murine myocardium, *J. Trace Element Expl. Med.* **2,** 257–266 (1989).
57. National Research Council, Committee on Medical and Biologic Effects of Environmental Pollutants, *Selenium,* National Academy Press, Washington, DC (1976).
58. N.-O Ilback, J. Fohlman, and G. Friman, Selenium (Se) supplementation decreases myocardial injury and increases survival in coxsackie B3 (CB3) virus infected mice, *J. Invest. Med.* **43,** 269A (1995).
59. J. Parizek, J. Kalouskova, J. Benes, and L. Pavlik, Interactions of selenium-mercury and selenium-selenium compounds, in *Micronutrient Interactions: Vitamins, Minerals and Hazardous Elements,* O. A. Levander and L. Chen, eds., The New York Academy of Sciences, New York, pp. 347–360 (1980).
60. P. D. Whanger, J. W. Ridlington, and C. L. Holcomb, Interactions of zinc and selenium on the binding of cadmium to rat tissue proteins, in *Micronutrient Interactions: Vitamins, Mineral and Hazardous Elements,* O. A. Levander and L. Cheng, eds., The New York Academy of Sciences, New York, pp. 333–346 (1980).
61. N.-G Ilback, U. Lindh, L. Wessleo, J. Fohlman, and G. Friman, Heart trace elements are changed after viral infection (coxsackie B3) in methyl mercury exposed mice, *J. Invest. Med.* **43,** 269A (1995).
62. N.-G Ilback, J. Fohlman, G. Friman, and A. W. Glynn, Altered distribution of ^{109}cadmium in mice during viral infection, *Toxicology* **71,** 193–202 (1992).
63. J. Parizek, I. Ostadalova, J. Kalouskova, A. Babicky, and J. Benes, The detoxifying effects of selenium interrelations between compounds of selenium and certain metals, in *Newer Trace Elements in Nutrition,* W. Mertz and W. E. Cornatzer, eds., Marcel Dekker, New York, pp. 85–122 (1971).
64. R. M. Loria, S. Kibrick, and G. E. Madge, Infection of hypercholesterolemic mice with coxsackievirus B, *J. Infect. Dis.* **133,** 655–662 (1976).
65. N.-G Ilback, A. Mohammed, J. Fohlman, and G. Friman, Cardiovascular lipid accumulation with coxsackie B virus infection in mice. *Am. J. Pathol.* **136,** 159–167 (1990).
66. J. Holland, Replication error, quasispecies populations, and extreme evolution rates of RNA viruses, in *Emerging Viruses,* S. S. Morse, ed., Oxford Univerity Press, New York, pp. 203–218 (1993).
67. E. D. Kilbourne, Afterword: a personal summary presented as a guide for discussion, in *Emerging Viruses,* S. S. Morse, ed., Oxford University Press, New York, pp. 290–294 (1993).
68. P. Adler, Preventing polio: don't tamper with success, *Washington Post* April 2 (1996).
69. S. L. Katz, A vaccine riskier than the disease, *The Washington Post* April 26 (1996).
70. D. Drollete, Australia fends off critic of plan to eradicate rabbits, *Science* **272,** 191–192 (1996).
71. J. Kaiser, Australia postpones rabbit virus release, *Science* **272,** 341 (1996).

72. N. D. Costa, H. G. Masters, P. Steele, B. J. Hilbert, J. F. Hudman, and J. R. Bolton, The selenium status of horses in Western Australia, in *Trace Element Metabolism in Man and Animals*, J. McC. Howell, J. M. Gawthorne, and C. L. White, eds., Australian Academy of Science, Canberra, pp. 44–46 (1981).
73. K. O. Godwin, The role and the metabolism of selenium in the animal, in *Trace Elements in Soil-Plant-Animal Systems*, D. J. D. Nicholas and A. R. Egan, eds., Academic, New York, pp. 259–270 (1975).
74. S. F. Arnold, D. M. Klotz, B. M. Collins, P. M. Vonier, L. J. Guillette, Jr., and J. A. McLachlan, Synergistic activation of estrogen receptor with combinations of environmental chemicals, *Science* **272,** 1489–1492 (1996).
75. C. Suplee, "Environmental estrogens" may pose greater risk, study shows, *Washington Post* June 7 (1996).