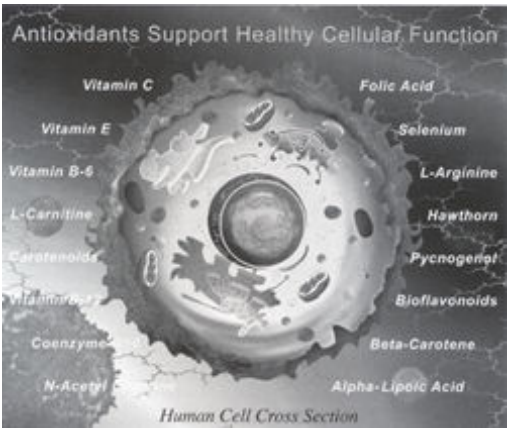
Functional Intracellular Analysis of

Nutritional and Antioxidant Status



Paula Boerner, Ph.D., PA-C*
Research Director, SpectraCell Laboratories, Inc.

*Correspondence:
Paula Boerner, Ph.D., PA-C
7051 Portwest, Suite 100
Houston, TX 77024
Phone: 713621-3101 Fax: 713-621-3234
Email: pboerner @ AOL.COM

INTRODUCTION

Reliable analysis of nutrient status has been a goal of health providers since the discovery that well-known diseases, such as beri-beri, pellagra, or osteoporosis, can result from a vitamin or mineral deficiency. Knowledge of the essential role nutrients play in health maintenance has led to eradication of most overt deficiency diseases in developed countries. However, the high incidence of chronic disease, often having no discernable cause, has led health professionals to ask whether or not sub-clinical deficiencies can contribute to the genesis and/or progression of

chronic states of ill health, including cancer and heart disease. An observation confusing this issue is that, of the many people who live in similar environments and eat similar diets, not everyone develops chronic disease. Moreover, some will develop cancer while others succumb to heart disease, and yet others will develop progressively debilitating diseases such as multiple sclerosis, fibromyalgia, or age-related dementia.

The lack of clear connection between cause and effect makes identification of precipitating factors difficult, but lends credence to the idea that individual genetic differences can tilt one person towards chronic disease, while other individuals in similar environments remain healthy. The notion that genetic individuality can influence nutrient requirements and increase the probability of disease when the elevated nutrient requirements are not met was first suggested 100 years ago by a British physician, Archibald Garrod. In 1902 Garrod1 studied Alcaptonuria (a relatively harmless, inherited metabolic disorder characterized by lack of the enzyme homogentisate oxidase and consequent production of black urine) and concluded that variations of chemical behavior are probably present everywhere, and "... as no two individuals of a species are absolutely identical in bodily structure, neither are their chemical processes carried out on exactly the same lines." Fifty years later Roger Williams pursued this idea in his book Biochemical Individuality,2 in which he stated that "…nutrition applied with due concern for individual genetic variations, which may be large, offers the solution to many baffling health problems." He also posed his hypothesis that "…practically every human being is a deviate in some respect…with an important bearing upon
the susceptibility of the individual...to disease later in life."

These ideas received substantial support when the explosion of gene-and-protein- sequencing information led to correlation of genetic changes with alterations in cellular biochemistry/physiology and to subsequent altered nutritional needs. Sauberlich3 correlated the single base change underlying sickle cell anemia with increased production of oxygen-based free radicals in red blood cell membranes and with the subsequent increased oxidative stress for the patient. Half of 18 patients with sickle cell anemia had sufficiently reduced white blood cell ascorbic acid levels to be considered deficient, an example supporting the hypothesis that a single base pair change can lead to an elevated nutrient requirement.

In another illustration of a single mutation altering a nutritional requirement, Whitehead et al.4 and van der Put et al.5 discovered that approximately 5-6% of the general population carries a mutated form of methylenetetrahydrofolate reductase, an enzyme responsible for conversion of homocysteine to methionine. The frequency of the mutation increases to 10-20% for parents of patients and patients with neural tube defects. This mutation increases the level of folic acid required by the reductase enzyme to achieve normal activity. Carrying the mutations, an individual who received the amount of folic acid provided in a typical diet would be at high risk of elevated serum homocysteine and decreased amounts of S-adenosylmethionine (SAM), required for SAM-dependent methyltransferase reactions. If a woman with this genetic defect were pregnant, the reduced level of reductase activity would negatively impact neural tube development, significantly increasing the risk of neural tube defects in the fetus. Elevated serum homocysteine, another consequence of this single mutation, is a strong risk factor for heart disease6,7 and stroke.8 The increased risk of heart disease associated with elevated homocysteine can often be reduced by supplementation with folic acid and vitamins B6 and B12,9,10 suggesting that at least some of the biochemical abnormalities underlying high homocysteine concentrations may be corrected by appropriate supplementation.

Assimilating the rapidly-accumulating information published in this area through the year 2000, Eckhardt11 illustrated the scope of human diversity for nutrient requirements. When one assumes a need for only 30 nutrients and if the metabolic pathway influencing these requirements is affected by only two alleles at a single locus (probably an underestimate), then the number of alternative genotypes would be greater than 200 trillion (330). In addition to nutrient deficiencies resulting from genetic variation, deficiencies may also be caused by inadequate intake, disease unrelated to known genetic changes, medications, unusual dietary practices, or stressors such as pregnancy, trauma, surgery, or infection. In the past, many

medical practitioners have not taken into account the possibility of sub-clinical nutrient deficiencies in their diagnostic/therapeutic repertoire, unless the case involved well-known, clear cut clinical pathology, such as protein malnutrition, pernicious anemia, or alcohol-related B vitamin deficiencies. One reason for this medical default has been the inability to reliably test for functional deficiencies.12

METHODS OF NUTRITIONAL ASSESSMENT

The most commonly used methods of nutritional assessment involve quantitative measurements of nutrients in accessible body fluids13-16. This static approach, however, provides a one-time snapshot of a dynamic, complex situation. It provides no integrated view of an individual's capacity to localize that nutrient into appropriate compartments or to optimally use it. Optimal function of a nutrient requires 1) absorption from the gut, 2) transport in the bloodstream, often requiring appropriate levels of functional binding proteins, 3) uptake into cells, which can require appropriately-functioning membrane transport systems, and 4) either one or, in many instances, multiple metabolic enzymes with appropriate co-factors. Thus, serum levels of a nutrient may be "normal," but the functionality of that nutrient may not be sufficient to maintain optimal health over a normal lifespan.

For example, Joosten et al.17 used assays for serum metabolites, such as homocysteine and methylmalonic acid, to identify deficiencies of vitamin B6, vitamin B12 and folic acid. These assays detect abnormally high levels of the metabolites, which can accumulate when reactions dependent upon vitamins B6, B12, or folic acid are impaired. Using this approach, the authors found specific vitamin deficiencies in a high percentage of an elderly population, many of whom were originally assessed as having "normal" vitamin levels in their serum. The prevalence of deficiencies identified by the conventional serum vitamin assays was less than 10% in a normal elderly population, but the prevalence was 63% when evaluated by serum metabolite assays.

Similar deficiencies for individuals identified as having "normal" serum levels by conventional testing were also found by Lindenbaum et al.18 when metabolite assays were used. It was also found that approximately 5% of patients with hematologic or neurologic syndromes, secondary to B12 deficiency, had normal serum B12 values.19 In a subsequent study, Naurath et al.20 demonstrated that vitamin B6, B12, and folic acid supplementation of individuals, identified as deficient by metabolite testing, reduced the elevated metabolite levels within 5-12 days. The authors suggested that the discrepancy between the "normal" serum values for the tested vitamins and their findings of deficiency using metabolite testing may be due to age-related impairment of an enzymatic reaction associated with an essential function of the vitamin, such as suggested by Brattström et al.21 for the reduced activity of cystathionine beta-synthetase measured in skin fibroblasts. The argument for identification of deficient individuals, even though serum levels are found to be "normal," is supported by findings that atherosclerotic thrombosis and neuropsychiatric disorders may be associated with elevated levels of these metabolites, especially homocysteine and methylmalonic acid. These clinical conditions, moreover, may be prevented by specific vitamin supplementation.22-25 However, metabolite tests can be expensive and may not be accurate under conditions of renal dysfunction23,26 or therapy with certain drugs.27,28

Analyses are available for quantification of nutrients in cells and tissues such as red and white blood cells, hair, nails, and soft tissue biopsies.13-16 These tests offer deeper insight into nutritional status than analysis of body fluids, but do not always detect increased need secondary to genetic polymorphisms. Erythrocyte enzyme stimulation tests are available, but only for selected B vitamins. These are limited because they measure only one or two of the many enzymes dependent on each vitamin, and results may be obscured by deficiencies of other nutrients. Erythrocyte enzyme activity tests are available for selenium (glutathione peroxidase), zinc, and copper (superxoide dismutase), but these tests suffer the same limitations as the stimulation tests. Microbial growth assays tend to be more sensitive and accurate than chemical analyses, but these are primarily used with body fluids and suffer the same limitation of

providing short-term views of the presence, but not the functionality, of the nutrient. Hair analysis, which is limited to minerals, is subject to interference from handling and processing, and interpretation can be complex, since deficiencies can be indicated by either high or low values.

A desirable analysis for nutrient deficiency would, therefore, be one which depended upon the correct operation of every step required for optimal function of the tested nutrient: absorption, transport to the appropriate tissue, transport into the cells of the tissue, and the presence of all factors required for the nutrient to perform its role. This approach would not identify the source of a deficiency, but, unlike other tests, a deficiency due to any cause, whether from reduced intake or a genetically altered, reduced affinity co-factor, would be revealed.

DEVELOPMENT OF THE FUNCTIONAL INTRACELLULAR ANALYSIS (FIA) METHOD FOR ASSESSMENT OF SELECTED NUTRITIONAL DEFICIENCIES

The basic methods underlying the Functional Intracellular Analysis (FIA) were developed at the University of Texas, Austin, by Dr. William Shive. The driving force for his research came from his chairmanship in 1975 of a Nutrition Board mandated by a Joint Presidential and Congressional Commission to investigate medical applications of nutrition. The conclusion of the commission was that clinicians appreciated the large amount of scientific research involving nutrition, but felt that clinical application of this knowledge was limited by lack of a method for reliable evaluation not only of individual nutritional needs, but especially of responses to nutritional therapy. Motivated by knowledge of this deficiency of interaction between clinical medicine and nutrition, Dr. Shive and his co-workers12 commenced "…to develop a method by which the limiting nutritional and biochemical factors of an individual could be identified." Dr. Shive began with the idea of creating a human cellular assay analogous to the quantitative microbial assay developed by Dr. Roger Williams2 who used a growth assay to assess the specific nutritional and metabolic characteristics of microbial cells.

The human tissue chosen by Dr. Shive's laboratory needed to be representative of the entire human body. Initial attempts utilized epithelial cells, but lymphocytes were found to have more advantages, especially their greater availability and their ease of manipulation in culture. Lymphocytes make up about 1% of total body weight.29 And, unlike erythrocytes, which lack a nucleus, lymphocytes represent the genetic identity of the donor and, therefore, possess the general metabolic pathways identical to most of the donor's cells.30,31 An additional useful feature for the use of lymphocytes for nutritional testing is that results gained from testing the capacity of these cells for activation and growth can be directly related to immune system function. The function of the immune system and lymphocytes are more closely related to the overall state of health of the individual than any other cell type.

Lymphocytes are produced in lymphoid organs such as bone marrow, where their nutritional reserve, based on whole body nutritional status, is supplied.29 They then circulate between blood, lymph and tissues, but remain metabolically inert until activated by mitogens, lymphokines, or antigens.32 A major advantage, therefore, of using lymphocytes isolated from peripheral blood is that, at the time of their activation in culture, their nutritional content represents an average of the body's nutritional status over their lifetime of approximately 3-12 months.16,33 Thus, lymphocytes are not significantly influenced by fasting or short-term nutritional status, but represent a time-averaged history of nutrient availability. A rough analogy to this property would be the use of glycosylated hemoglobin to estimate serum glucose levels over an integrated 1-3 month period.34

An initial obstacle to the use of lymphocytes for nutritional analysis was that lymphocyte growth in culture, at the time of Dr. Shive's initial work, depended upon supplementation of the growth medium with fetal calf serum. Growth of cells in media supplemented with serum prevented reliable nutritional analysis because serum is relatively undefined, because specific serum lots may contain trace contaminants, possibly related to the nutrients being tested, and because sera-containing media have traditionally had problems with variable growth results, not

only from one laboratory to another, but also from one serum lot to another.35 Thus, Shive et al.12,36-38 carried out a systematic study, beginning in the late 1970s, to develop a fully-defined, serum-free, protein-free, chemical medium that would support growth of human lymphocytes at a level equivalent to that obtained with sera-containing media.

Since a defined medium was to be the basis for the study of nutritional and metabolic status, it had to be a minimal growth medium with respect to the amounts of each constituent. In painstaking fashion, the amount of each nutrient found to be necessary for optimal growth, relative to serum-containing medium, was changed until an amount of that nutrient, just sufficient to support optimal growth, was achieved. After examining one component, the entire medium was reexamined in a reiterative fashion to account for possible interactions between new concentrations of nutrients. Thus, each adjustment of an individual component required reevaluation of the dose response curves of the remaining components. In this fashion, a new medium that would support optimal growth of human lymphocytes in the absence of serum or even of purified protein factors was developed: CFBI 1000, named after the Clayton Foundation Biochemical Institute.

The CFBI 1000 medium consisted of the minimal amounts of glucose, essential amino acids plus glycine and serine, vitamins, inorganic salts, inositol, choline, pyruvate, and adenine that would support maximal growth, in addition to HEPES buffer and phytohemagglutinin (PHA), a lectin that provides the activation stimulus for T-lymphocytes. Until the lymphocytes are exposed to PHA, they are metabolically inert and incorporate no measurable 3H-thymidine into DNA.39 Short-term growth of human lymphocytes in this medium was equivalent to or superior to that that observed with some sera-containing media.36 In fact, comparison of growth in this medium with other media revealed that other formulations have had levels of amino acids so high that growth was actually inhibited. Thus, the heart of the FIA technology is the patented CFBI 1000 medium, which supports reproducible human lymphocyte growth and can be manipulated in vitro. Manipulation of individual components of the medium, as described below, allows individual nutritional/metabolic defects of the lymphocyte donor to be identified.

Following mitogenic stimulation in the CFBI 1000 medium, the cells undergo blastogenesis, synthesize DNA and divide, functions that can be accurately quantified by measurement of radiolabeled thymidine incorporation into DNA.39 Since cell division and growth, measured by 3H-thymidine incorporation, represents an integration of the donor's common metabolic pathways, this integrated function represents a sensitive endpoint for evaluation of the donor's personal genetic make-up, physiology, and relatively long-term (3-6 months) nutritional history. Therefore, measurement of cell growth in various formulations of the basic CFBI 1000 medium by quantification of 3H-thymidine incorporation represented a method for evaluation of the nutritional and metabolic status of individual lymphocyte donors. Thus, it could be concluded that the extent of any decrease in 3H-thymidine incorporation, observed when the nutrient being tested was omitted from the medium or otherwise manipulated (see below), was related to at least one of the following: 1) the capacity of the cell to transport that compound to its site of utilization, 2) the ability of the cell to utilize the compound, and 3) the availability of cellular reserves of that compound. If the cells grew normally when a component being tested was deleted from or otherwise manipulated in the medium, it could be concluded that no deficiency was present.

FIA PROCEDURE AND TEST DESCRIPTIONS

For the basic procedure, 8-16 cc of blood are collected into 1-2 Vacutainer Cell Preparation Tubes (CPTTM) by venipuncture from a donor who has not been required to fast. The ambient temperature samples are received by the laboratory and processed within 24-30 hours of collection. Mononuclear cells are separated by centrifugation of the CPT tubes, which contain a polyester gel in a density gradient liquid. The tubes are centrifuged for 30 minutes at 2800 rpm in a Beckman GS-6 centrifuge equipped with a swinging bucket rotor and GH 3.8 buckets. The surface layer of mononuclear cells is decanted and washed twice with 12 cc of phosphate-

buffered saline (PBS) containing 72 mg/L glucose. Washed cells are diluted with the wash buffer and counted in a Coulter T-540 cell counter. Ninety-six-well microtiter plates containing 0.2 cc of individual formulations of the CFBI 1000 medium are inoculated with 25,000 cells/well contained in 0.02 cc wash buffer. After incubation at 370 in 5% CO2 for 4 days, 0.057 uCi 3H-thymidine (6.7 Ci/mM) is added, the cell are incubated for an additional 24 hours, the DNA is collected onto glass fiber filter mats using a Packard Filtermate 196 cell harvester, and the 3H-thymidine incorporated into DNA is counted using a Packard Matrix 9600 beta radiation counter.

Twenty-two tests are performed in triplicate wells on a 96-well plate for each blood sample received by the laboratory. Nine control wells containing 100% medium are scattered throughout the plate. Most of the test results are expressed as a percentage of the average of the 9 control wells. Thus, each subject is his/her own control, represented by the extent of growth in 100% medium. The results of each test are expressed as percentiles of reference values obtained from a large reference population.

Many applications offered in SpectraCell's FIA are carried out by measurement of cell growth in medium from which the component being tested has been removed. Growth of lymphocytes in the deficient medium is compared with growth in the basic 100% medium. Subjects who have lymphocyte growth lower than the normal range in the absence of the nutrient being tested are considered to have a decreased functional status of that nutrient. This test design is used for vitamins B1, B2, B3, B6, pantothenic acid, biotin, choline, and inositol.

The status of nutrients involved in growth-essential pathways that can be metabolically isolated can be assessed by manipulation of medium components. Thus, optimal growth becomes dependent upon a specific pathway, and, hence, upon the nutrient required for that essential pathway to function. For example, growth of human cells requires methionine. The functional status of vitamin B12 can be evaluated because the enzyme methionine synthetase requires B12 as a co-factor to convert homocysteine to methionine in the presence of folic acid. If homocysteine and folic acid are added to CFBI 1000 medium, which for this test is deficient in methionine and B12, functional, endogenous B12 is required to produce sufficient methionine for normal growth.12,38 If optimal growth is not observed, then the cells are considered to have a functional deficiency of vitamin B12.

Another example of a nutrient that can be made dependent upon a specific metabolic pathway by manipulation of medium components is serine. Although not an essential amino acid, severe health problems can arise from defective or even reduced endogenous serine synthesis because this amino acid participates in protein and phospholipid synthesis, energy production, and one-carbon metabolism required for nucleic acid synthesis. Defects in the serine synthetic pathway can lead to neuropathy, neuritis, or behavioral disorders, and can mimic folate or vitamin B12 neurological deficiency symptoms. Thus, defects in serine metabolism need to be analytically distinguished from functional deficiencies of folic acid or B12. The serine test is performed in medium lacking serine and glycine, but with excess B6 and folic acid added to saturate their respective enzyme systems, in case an individual is deficient in these nutrients.12,38 Under these conditions, lymphocyte growth depends completely upon the cell's ability to synthesize and use serine. If serine synthesis or utilization is absent or reduced, cell growth will be reduced. Similarly, manipulations of serine, glycine, B6 and B12 can be used to isolate metabolic pathways dependent upon folic acid.12,38,40,41

Still other deficiencies are identified by addition of a selected nutrient to the complete medium. Addition of zinc, magnesium, or increased calcium to the medium normally does not stimulate growth above the levels seen for the subject's cells in the 100% medium, because cells from normally replete individuals contain an endogenous supply that supports optimal growth. If growth is stimulated by addition of any of these minerals, it suggests that the cells do not contain optimal functional levels. A deficiency of oleic acid, the most common monounsaturated fatty acid in human cells, is also tested in this manner. The CFBI 1000 basic

medium is supplemented with oleic acid and a deficiency is suspected when growth is stimulated by this addition.42 In general, no deficiency symptoms are clearly defined for oleic acid, since a dietary intake is not known to be essential. However, a functional deficiency for oleic acid observed in this assay may reflect a need for essential fatty acids, including the omega-3 series, or could indicate an excessive intake of trans-monounsaturated fats, found in margarines and shortenings.

The FIA has also been used to identify individuals with abnormal glucose utilization. Addition of insulin does not normally affect lymphocyte growth in CFBI 1000 medium. However, growth of lymphocytes from some individuals was stimulated by insulin, suggesting that these cells were not utilizing glucose optimally. Subsequent glucose tolerance testing frequently showed that the subjects whose lymphocytes responded to insulin suffered from hypoglycemia or from an untreated diabetic condition.43

APPLICATION STUDIES

Several lines of evidence support the contention that lymphocyte growth responses in CFBI 1000 medium reflect a person's nutritional status. Matthews et al.44 modified the CFBI 1000 medium to support optimal growth of murine lymphocytes, labeled it CFBI 2000, and used it for nutritional depletion and repletion studies using mice. They found that lymphocytes from mice, fed a diet deficient in a specific B vitamin grew at a reduced rate, relative to controls in 100% medium, when cultured in medium deficient in the same B vitamin deleted from their diet. Lymphocytes from these mice exhibited normal growth when cultured in medium deficient in B vitamins that had not been removed from their diet. Subsequent dietary repletion of the vitamin that had initially been deleted from their diet was followed by a return to optimal growth when lymphocytes taken from the repleted mice were cultured in medium lacking the B vitamin that had been restored to the diet. The authors suggest that "….examination of proliferation of lymphocytes/splenocytes in culture may provide a novel tool for the assessment of nutritional status."

Results using the FIA method with human lymphocytes have been compared with results using other methods of nutritional assessment. Shive12 found that lymphocytes taken from subjects who excreted reduced levels of vitamin B2 in their urine grew at a reduced rate, relative to controls grown in 100% medium, when cultured in medium lacking B2. He also reported that the decreased lymphocyte growth responses, observed when the cells were grown in the absence of vitamin B6, correlated with a standard erythrocyte glutamate-oxalacetate transaminase test for B6 status.12 In addition, results of FIA testing correlated with a nutritional disorder found in several subjects tested. Growth of lymphocytes from patients with pernicious anemia or macrocytic anemia was inhibited when tested for B12 and folate-dependent utilization of glycine.12

Using a slightly different assay protocol for serine synthesis in cultured lymphocytes, Ellegard and Esmann40 detected folate deficiencies in 17 of 23 patients with suspected deficiency secondary to malnutrition (5 patients), malabsorption (14 patients), and phenytoin use (4 patients). Measurement of folic acid levels in erythrocytes of the same patients identified only 11 cases of deficiency. Twelve patients treated with oral folic acid supplementation for 4 weeks showed an approximate 2-fold increase in lymphocyte serine synthetic activity.41 Bucci noted that specific nutrient repletion was found to normalize 33 of 42 abnormal FIA tests, originally observed in five subjects, when retested a year later. Seven of the 42 tests showed improvement, though test subjects did not reach the normal range, while 2 of the 42 test subjects scored lower than the original test score.45

Dr. Shive's experience with lymphocyte testing for nutritional deficiencies included numerous case studies that illustrate the benefits of identification of deficiencies followed by appropriate supplementation.12,46,47 An 86-year-old patient with severe mental deterioration was found to be deficient in vitamin B2 and biotin by FIA testing. These deficiencies were confirmed with the finding of reduced urinary output of both vitamin B2 and biotin. After supplementation, the

patient was again able to communicate meaningfully with others. A second patient showed a long medical history of lassitude, paresthesia, muscle pain, mild depression, and anxiety. After FIA testing revealed a deficiency of biotin, supplementation was begun with injections followed by maintenance on oral biotin, which alleviated his severe symptoms. It was later discovered that the patient had been consuming a drink containing raw egg whites and had been eating barely heated eggs for breakfast on a daily basis: e had most likely suffered from an avidin-induced depletion of biotin. A third patient, who presented with a neurological disorder, was found to have defective serine synthesis. She was given serine or placebo supplements, alternated randomly at 2-week intervals for 10 successive intervals. During this time she was able to correctly identify serine or placebo at each interval, based on improvement in clinical symptoms by her neurologist.

Once the CFBI 1000 medium and the tests utilizing it were established, a large number of subjects (>5000) were tested to build a database for reference range determination. During this early phase of testing it was noted that subjects with similar disease states did not exhibit consistent patterns of nutrient deficiencies. This finding may help explain why clinical trials of single-nutrient supplementation of subjects with specific conditions seldom achieve a high level of clinical response. It also became clear that, of the 22 tests applied, many individuals exhibited not only single nutrient deficiencies, but a significant number were found to have multiple deficiencies. Table 1 illustrates the number of tests performed by SpectraCell and the prevalence of nutrient deficiencies observed during the two years from August, 1997 through July 1999. Out of 89,747 individual tests performed, 12,087 deficiencies were found. Calcium, antioxidant capacity (SpectroxTM - a test of the capacity of the donor's lymphocytes to resist growth inhibition by free radicals) and glutathione deficiencies were the most prevalent, whereas choline and pantothenic acid deficiencies were the least prevalent. SpectroxTM and glutathione values indicate lymphocyte antioxidant capacity (see below).

B1		4178	17
B2		4131	5
B3		4103	10
B6		4748	7
B12		4748	7
Folate		4743	5
Pantotheine		4098	3
Biotin		4095	5
Serine		3483	10
Gln		3505	11
Asn/Gln		3487	12
Choline		3650	2
Inositol		3568	7
Ole/BSA		3534	8
Insulin		3548	18
Fructose		3530	18
Calcium		4564	28
Zinc		4570	20
Mg		4541	16
GSH		4249	27
Cysteine		3619	21
Spectrox		5055	33

APPLICATION OF THE FIA METHOD TO THE EVALUATION OF CELLULAR ANTIOXIDANT STATUS

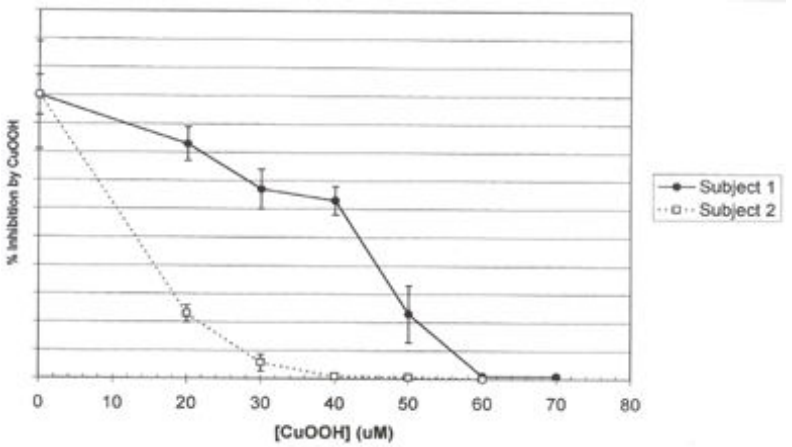
Oxidative stress is rapidly becoming recognized as a significant contributor to chronic disease.48-50 Its contribution to heart disease and stroke51,52 are well recognized, as is its relationship to cancer53-55 through genetic mutation and perhaps even cell membrane damage. Evidence is also rapidly accumulating for association between elevated oxidative stress and pre-eclampsia in pregnancy,56 cognitive decline with aging,57 the process of aging itself,58 many of the complications of diabetes,59 and depressed immune function.60,61 The broad range of physiological damage and potential chronic disease associated with a prolonged imbalance of oxidants and antioxidants supports the need for a reliable, sensitive, rapid, and inexpensive method for measuring internal cellular redox imbalances and antioxidant deficiencies.

Current methods of assessment of redox state fall into two major categories.62 One approach is the quantification of lipid peroxidation products such as isoprostanes, eicosapentaenoic and docosahexaenoic acids, oxysterols, hydroxy fatty acids, lipid peroxides and aldehydes in serum or urine.63 These approaches have been widely investigated for their potential to predict risk of cardiovascular disease.52,64,65 Formation of protein carbonyls has been used to estimate oxidative damage to proteins.56 These approaches estimate recent molecular damage, but suffer from the drawback that assay of one type of peroxidation product may not represent the overall redox balance in an individual. Comparisons of different methods of redox assessment did not

always show a high level of agreement.66,67 A second approach assesses antioxidant capacity.66 Tests in this category include erythrocyte resistance to experimental oxidative stress, as well as measurement of antioxidant defenses in serum, such as the ferric-reducing ability of plasma (FRAP) and superoxide dismutase activity in plasma.

Adaptation of the FIA to the estimation of antioxidant status falls into the category of resistance to oxidative stress, but the FIA SpectroxTM assay is unique among the tests available: it is an integrated functional assay, not a biochemical assay of end products or of isolated activities. Antioxidant capacity is assessed by 2 independent assays, assessment of glutathione (GSH) cellular reserves, and by SpectroxTM. The GSH assay uses L-buthionine-[S.R.]-sulfoximine68 (BSO), inhibitor of an enzyme required for GSH synthesis, gamma-glutamylcysteine synthetase, to inhibit endogenous synthesis of GSH in culture. If cell growth is inhibited relative to control growth by preventing new synthesis of GSH, the cells are considered to have deficient GSH stores.

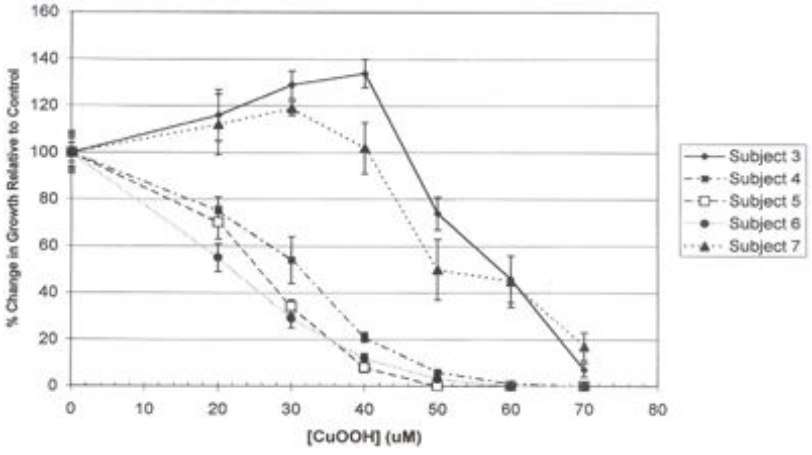
Figure 1. comparison of Spectrox TM assay results for two subjects. all values were normalized to growth of cells in 100% CFBI 1000 medium without added factors.



The second component of the antioxidant assessment involves culture of lymphocytes in the 100% medium in the presence of a free radical-generating compound, cumene hydroperoxide (CuOOH). Antioxidant capacity of the lymphocytes is measured as resistance of the lymphocytes to inhibition of cell growth by free radicals generated from the CuOOH. Inhibition of cell growth is evaluated relative to growth in 100% medium. The capacity of the lymphocytes to resist inhibition by a free radical assault presumably depends upon their level of antioxidants and the normal function of their intracellular antioxidant enzymatic systems. Superoxide dismutase requires zinc, copper, and manganese, while glutathione peroxidase requires selenium and GSH.

Cell growth of lymphocytes from most individuals is 100% inhibited at high concentrations of CuOOH. However, at lower concentrations of CuOOH, different individuals vary in the capacity of their lymphocytes to resist oxidative damage and inhibition of cell growth. Thus, the CuOOH concentration required for 50% inhibition of growth can vary widely from one person to another. This difference is illustrated in Figure 1, where the CuOOH resistance of a subject with no known adverse health conditions (subject 1) is compared to a person with cancer (subject 2), who had been avoiding antioxidant supplements because of individualized therapy. Growth of cells from both individuals was completely inhibited at 60 uM CuOOH, as is the case for most individuals tested. However, 50% inhibition of the growth of cells from subject 2 required only 13 uM CuOOH, whereas 50% inhibition of the growth of cells from subject 1 required 42 uM CuOOH, indicating a 3-fold higher capacity of cells from subject 1 to resist oxidative damage than cells from subject 2.

Figure 2. Growth responses of lymphocytes isolated from 5 donors to cumene hydroperoxide (CuOOH), a free radical generaor. All values were normizlized to growth of cells in 100% medium without added factors.



One application of the Spectrox test (capacity of lymphocytes to resist free radical assault) was communicated by Dr. Rita R. Ellithorpe.69 Twelve subjects with relatively low Spectrox scores were given supplements of Juice Plus+TM, a preparation of dehydrated fruit or vegetables in capsule form. The average Spectrox score before supplementation was 35%, with a range of 13 to 64%. After three to five months of supplementation, there was an average increase of 41% in the scores, with a range of post supplementation values from 55% to 95%. The extent of the increase observed ranged from 11 to 73%. These results illustrate the potential of the Spectrox test for use in evaluation of antioxidant supplementation.

A surprising finding from the application of the Spectrox test to a wide variety of individuals was that growth of lymphocytes from a small number of individuals was stimulated by exposure to CuOOH. Figure 2 illustrates CuOOH inhibition curves for five random subjects tested on the same day. Lymphocytes from subjects 4, 5, and 6 showed normal inhibition curves. The CuOOH concentrations required for 50% inhibition of growth were 31, 27, and 22uM, respectively. However subjects 3 and 7 not only showed strong resistance to CuOOH inhibition (58 and 50uM CuOOH required for 50% inhibition, respectively), but also showed some stimulation of lymphocyte growth at low to moderate levels of CuOOH.

The meaning of this response is unclear, and its prevalence is low, but it is usually observed in at least 1-2% of the individuals tested. This paradoxical response indicates that low levels of free radicals can stimulate rather than inhibit growth of lymphocytes from a small number of individuals. Perhaps lymphocytes from these subjects harbor a viral infection that is inhibited or killed by in vitro exposure to free radicals, thus improving lymphocyte function in vitro. Perhaps patients identified as such may benefit from hydrogen peroxide therapy. Conversely, these subjects may have an oxidation-reduction imbalance such that they are not low in antioxidants, but are so high that immunological function, in terms of lymphocyte growth in vitro, is depressed. A physiological balance between oxidants and anti-oxidants may be more desirable than flooding the system with high doses of antioxidants. Clearly, much work remains to be done concerning the meaning of this response and its correlation with a subject's clinical state.

Since both the Spectrox and GSH tests provide a functional estimate of antioxidant load, the correlation of the results of these tests was examined. GSH values from two groups of samples evaluated on consecutive days were plotted as a function of their corresponding Spectrox values from the same patients (Figure 3). Regression analysis revealed a linear relationship with an R2 value of 0.604. The correlation appears to deviate slightly at both high and low Spectrox values.

When the Spectrox values approached 20-40%, several of the GSH values were higher than would be predicted by the regression line. More strikingly, when Spectrox values were high, 120-140%, a majority of the GSH values were also elevated more than predicted, indicating a stimulation of growth secondary to inhibition of GSH synthesis. Perhaps when antioxidant load is high, as perhaps suggested by the positive growth response to free radical exposure, inhibition of GSH production becomes beneficial for lymphocyte growth. This would again suggest that a redox balance may be more desirable than an imbalance, even if the imbalance is tilted in the favor of excess antioxidants.

A potential application of the FIA in the study of antioxidants is evaluation of the response of lymphocyte growth in culture to antioxidants, rather than to oxidizing agents. An intriguing question was whether lymphocytes from some subjects would respond to an antioxidant with a positive growth response, while lymphocytes from others would respond with a negative growth response, as observed with the oxidizing agent, CuOOH. Preliminary experiments have been carried out with N-acetyl-cysteine (NAC), a potent antioxidant used clinically as a mucolytic agent and as an antidote to acetaminophen poisoning, and, which has been recommended for use with AIDS patients because of studies showing inhibition of AIDS virus replication in vitro. NAC is also thought to enhance GSH formation because cysteine is a rate-limiting precursor to GSH synthesis. However, in many cases the mode of action of NAC has not been well defined.

Lymphocytes from five subjects were cultured in CFBI 1000 medium supplemented with concentrations of NAC from 0.5 to 8mM. As shown in Figure 4A, not every subject responded similarly. Growth of lymphocytes from subject 12 was stimulated by exposure to levels of NAC up to 4mM, whereas growth of lymphocytes from subject 9 was relatively unaffected at low levels of NAC. Growth of lymphocytes from subjects 8, 10 and 11, however, was inhibited from 10 to 40%, even in low concentrations of NAC. Either no significant effect or a minor degree of inhibition were the most prevalent responses, but Figure 5 illustrates two extreme, fairly rare responses observed. Subject 13 was inhibited approximately 60%, whereas subject 14 was stimulated more than 2-fold.

Figure 4B illustrates CuOOH responses by lymphocytes from the same subjects shown in Figure 4A. A very rough inverse correlation between positive growth responses to NAC and sensitivity to inhibition by CuOOH was observed. Patient 12, whose lymphocytes were stimulated by NAC showed the greatest sensitivity to inhibition by CuOOH, whereas lymphocytes from patient 10, which were the most resistant to inhibition by CuOOH, were among the most sensitive to inhibition by NAC. This correlation was not consistent for all samples compared. Figure 6 illustrates a regression analysis of 25 tests, which compares responses to NAC to concentrations of CuOOH required for 50% inhibition of growth. High sensitivity to inhibition by CuOOH (low I50) was related to a positive response to NAC by an R2 value of 0.249.

DISCUSSION AND CONCLUSIONS

The FIA approach to nutritional testing required approximately 25 years to reach its current stage of development. The heart of this technology is the fully-defined, protein-free CFBI 1000 medium.36-38 Reproducible nutritional studies would not be possible without complete definition of the culture conditions that support lymphocyte growth. Application of free radical exposure to estimate antioxidant capacity would also be precluded if a serum-containing medium were necessary, because the high concentration of serum proteins would buffer the cells from free radical effects. A variety of sera-free media have been developed, but most require addition of protein factors and many do not always support cell growth as effectively as a serum-containing medium does.36

Another feature that sets the FIA apart from other methods of nutritional testing is its strict dependence on functionality. With the FIA, the concept of evaluating integrated function replaces the concept of quantification of isolated compounds. For example, a nutrient may be

present in normal amounts in the serum, but may not be effectively transported into the cell. Or, the gene coding for a nutrient's co-factor may be altered such that binding affinity for the nutrient is lowered and, thus, higher than "normal" dietary amounts are required for effective function.4,5 In these situations, information about the quantity of a nutrient is not sufficient and is even likely to be misleading. The dependence of the test upon lymphocyte DNA synthesis and cell growth, which in turn depend upon the integrated functioning of the individual's general metabolic pathways, ensures that the assay will be sensitive to most, if not all, of the underlying potential causes of a specific nutrient deficiency. In general, the FIA cannot pinpoint the specific genetic, biochemical, or even the physiological location of a deficiency involving a specific nutrient. It can, however, indicate the presence of any one of a broad range of potential defects. The FIA also enables a medical practitioner to test the efficacy of a therapy targeted at specific nutrient deficiencies. Given the average lymphocyte lifespan of 3-12 months, retesting is commonly recommended 6 months after initiation of therapeutic intervention.

Large-scale application of FIA testing lends credence to the growing suspicion that many individuals harbor undetected and, therefore, untreated nutrient deficiencies.18,20,70,71 These deficiencies may arise from individual genetic deficits, chronically poor diets, poor lifestyle choices with persistently elevated stress levels, long-term depletion of nutrients from our soil and from our food, increasing levels of toxins in our environment, the aging process itself, or, most likely, individualized combinations of these factors. Aging is a particularly insidious process because our bodies imperceptibly begin to fail and damage can accumulate before a problem is detected. An aged digestive tract is often not able to absorb the quantities of nutrients required more than ever by older persons. This physiological deficit may underlie findings that elderly populations tend to be far more deficient in nutrients than younger populations.72-74 The subtle nature of sub-clinical, chronic nutrient deficiencies, especially when initiated in youth or middle age, may lay the groundwork for future medical problems. As our society ages, the corresponding rise in medical costs might be considerably moderated if nutritional deficiencies were corrected before significant damage occurred. However, the potential damage of subclinical deficiencies needs to be recognized as a general medical problem, then individual deficiencies need to be identified and corrected.

Figure 3. Correlation of Spectrox TM and glutathione test results.

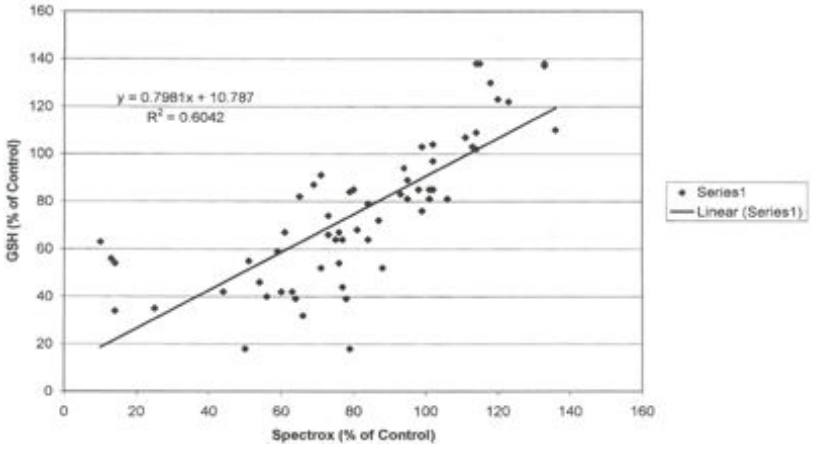


Figure 4. Growth responses of lymphocytes isolated from 5 donors to an antioxidant, N-acetyl-cysteine (NAC). All values were normalized to growth of cells in 100% CFBI 1000 Medium witout added factors.

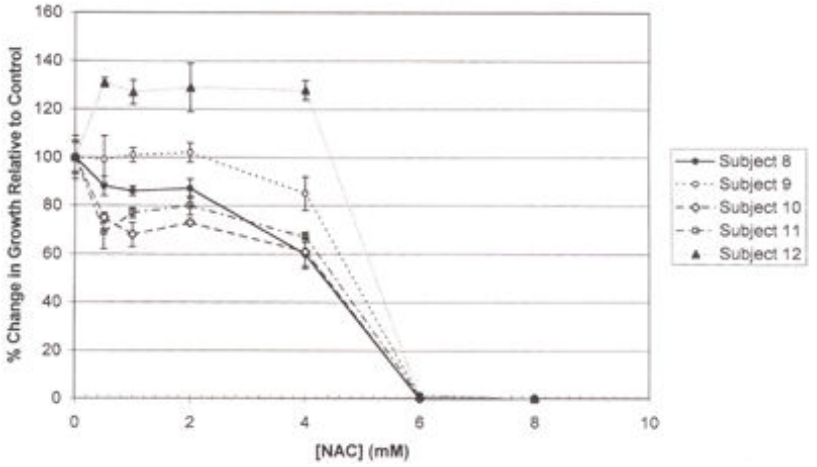
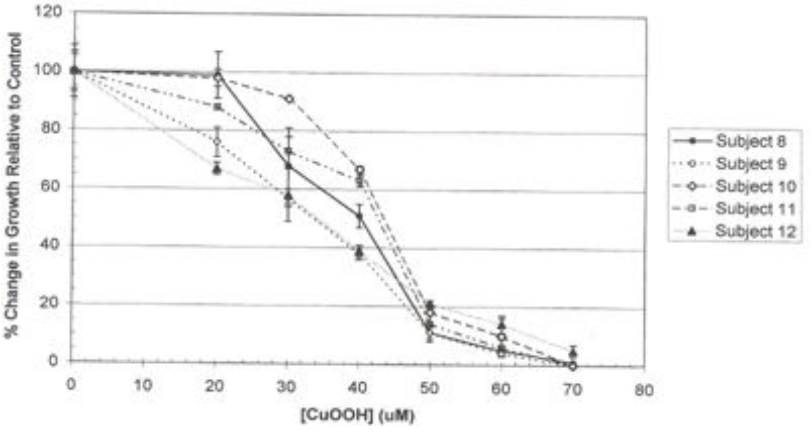


Figure 4B. Growth responses of lymphocyes isolated from 5 donors to cumene hydroperoxide (Cu))H), a free radical generator. All values were normalized to growth of cells in 100% CFBI 1000 medium without added factors

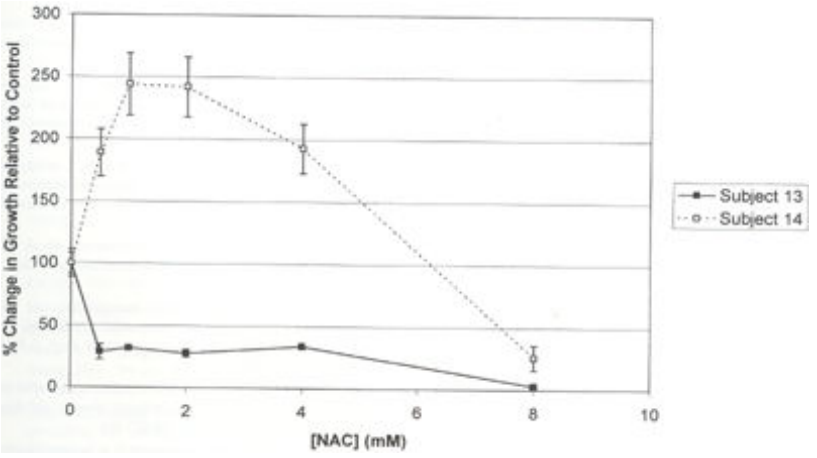


A health-related benefit of FIA testing is the use of immune system cells. Because lymphocytes were selected as the tissue to be evaluated,12 the results of FIA testing can be directly related to immune function, and immune function is critical to optimal health.61 The lectin used to activate the lymphocytes in culture, PHA, specifically stimulates T lymphocytes,30 which then produce interleukin-2, a major immune system mediator. Thus, correction of any nutrient deficiencies identified through negative effects on lymphocyte activation and growth will be likely to benefit immune function specifically, as well as to contribute to the correction of general metabolic imbalances.

The results of the FIA tests are reported as a percentile of a large population (>5000) of apparently healthy, "normal" controls. "Normal" in this usage represents a population statistic, not an absolute indication of the perfect state. This qualification can be applied to many of the formats used to assess nutrient levels. Unlike testing for serum potassium or magnesium levels, where both high and low healthy ranges have been firmly established, the high end of the optimal range for many nutrients is not known. Toxic levels have been identified for a few nutrients, such as vitamin A, but many nutrients, such as oleic acid, do not appear to be harmful, even when taken at high levels. Since humans are subject to aging, and some individuals seem to age more slowly than others, is the basis for this physiological difference in apparent rate of aging solely genetic, or have some individuals inadvertently stumbled onto a

nutritional regimen that is more beneficial than "normal," supposedly "good" dietary habits? Perhaps with more attention paid to evaluation of nutrient scores on the high end of "normal," combined with greater effort applied to clinical correlations, optimal levels of intake, rather than adequate levels of consumption, may eventually be defined.75,76

Figure 5. Growth respones of lymphcytes isolated from 2 donors to an antioxidant, N-acetyl-cysteine (NAC). All values were normalized to groth of cells in 100% CFBI 1000 medium without added factors.



Identification of nutrient and antioxidant deficiencies by FIA testing is currently used in many clinics. The potential applications of future, expanded FIA testing panels are broad. These include expansion of the range of the current test panel, identification of sensitivity to toxic effects of a range of substances, including nutrients and pharmaceuticals, evaluation of nutraceutical usage, and an expanded SpectroxTM test. Experiments are in progress to determine if it is possible to identify the specific antioxidants to which an individual's lymphocytes best respond. It may then prove feasible to use an expanded SpectroxTM panel to develop personalized antioxidant cocktails for each individual tested.

The SpectroxTM-FIA data have begun to suggest that usage of antioxidants, an area where more substantive information is needed, can be very complex. Two schools of thought concerning antioxidant usage are prevalent today. One approach recommends using multiple, purified preparations of individual antioxidants,77-79 whereas a second approach advocates obtaining a mixture of antioxidants from natural foods or food products.80,81 Given the widespread and increasing use of antioxidant preparations, application of the FIA to the evaluation of antioxidant balance represents a potentially valuable screening tool. Although much data supports Pauling's contention that large doses of vitamin C are beneficial,82 is this the optimal approach? Can we depress our immune function and, in general, reverse the known positive benefits of antioxidant intake by consuming too many or by taking the wrong combination of supplements? Dröge et al. provide support for this possibility with respect to immune function. They found that certain lymphocyte activities were potentiated by reactive oxygen intermediates, and that the intact immune system appeared to require a balance between prooxidant and antioxidant conditions.83,84 On the basis of data such as these, the finding that lymphocytes isolated from different subjects responded diversely to antioxidant (NAC) exposure as well as to free radical exposure in vitro (Figures 2,4, and 5) suggests the direction of these responses could reflect the past history of a subject's antioxidant intake and his/her current state of oxidation-reduction balance. Clearly, large scale clinical studies are needed to understand the significance of the preliminary data presented here.

If these new clinical correlation studies, directed to antioxidant usage, do identify connections among the following: 1) recent intake of antioxidants, 2) in vitro lymphocyte responses to antioxidants and oxidants via SpectroxTM testing, 3) results of other markers of oxidation-

reduction balance, and 4) clinical condition, then SpectroxTM-FIA testing may provide the clinician a relatively rapid means of optimizing a patient's antioxidant intake, as well as providing information concerning specific vitamin deficiencies.

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