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In this journal's issues during 2014, I wrote a few editorials regarding the rising tide of autoimmunity, including one showing the link with the gut microbiome. Autoimmune diseases and disorders now rank third in the United States after cancer and cardiovascular diseases, and they affect 53 million Americans. It is one of the leading causes of death in female children and women of all ages.1 There are more than 80 autoimmune disorders in which the immune system targets cells, organs, or tissues of its own body. We know that genetic predisposition plays a role as one of the triggers of autoimmune diseases in approximately 30% of the population. Other triggers are environmental factors such as gut dysbiosis, as well as infections and chemical exposures. Chemical exposures include those in foods. In a review article published in May, 2014, titled "Autoimmunity and the Gut in Autoimmune Diseases, Special Issue: Environmental Triggers and Autoimmunity," I discussed some of the environmental factors affecting not only the gut, but also mucosal immunity and the importance of detection via antibody testing to reverse the autoimmune reactivity by removing offending triggers.2

In the first 2 weeks of May 2014, I contacted via telephone and e-mail 16 laboratories in the United States that are known to provide testing for food immune reactions in serum for patients. I asked each laboratory if they would be willing to provide a manuscript for a special issue of this journal supporting their methodology and the effectiveness of their testing supported by the medical literature. These laboratories were contacted at least 3 different times, and the majority declined to write a paper. Three laboratories, however, did provide studies that were subjected to peer review and accepted for publication. Dr Vojdani provided 7 papers, which are all in this special issue. Studies by other authors will be published in subsequent issues of this journal. As we continue in 2015, we will be publishing more articles in this fascinating and very interesting area of science and medicine, essential to the well-being of patients.

This special issue is dedicated to food immune reactivities and their potential role in the development of autoimmunity. This is a common disorder that is rapidly increasing in prevalence for unknown reasons. All humans have one thing in common, and that is food. We all must eat to survive. The gut is continually and constantly in contact with food and food antigens, and most foods contain chemicals, even those that are labeled “organic.” These chemicals include not only artificial colorings, additives, flavorings, dyes, and preservatives, but also food contact materials, such as conveyer belts and food packaging materials. We must also take into consideration chemicals in agriculture, including pesticides, herbicides, fungicides, and artificial fertilizers.

The gastrointestinal mucosal immune system, as it relates to food, starts by being besieged by a wide variety of microorganisms, first from the mother's birth canal or skin if via Caesarean section and by the handling by medical personal, then by breast milk or commercial formula, and eventually by food and food antigens. The mucosal immune system is our first line of defense against chemicals, microbes, and dietary components, and it lines the intestinal tract and respiratory tract. This is why the gut mucosa consists of the largest assemblage of lymphoid tissue in the body. When in a state of balance, the microbiota, specific bacteria, and their products provide immune protection.3 Bacterial toxins, chemicals, foods, and undigested proteins and peptides can induce systemic food immune reactivity by causing failure of immune tolerance. Immune tolerance is the immune system's ability to recognize what is harmful and what is not. If immune tolerance is lost, then inflammation ensues and autoimmunity can occur. Factors that can affect immune tolerance and oral tolerance are the exposure to toxic chemicals and the diet of the mother during pregnancy, whether the child is born via the birth canal or via Caesarean section, breast-feeding versus commercial formula feeding, the timing of the introduction to solid foods, gut microbiota, digestive enzymes, use of medication or drugs by the mother during pregnancy and during breast-feeding, the child, and genetics. Therefore, the perinatal period is essential in establishing oral tolerance.

Approximately 1 ton of food goes through our gut every year, including more than 220 pounds of proteins, attesting to the fact of the effectiveness of the immune system in protecting us from adverse reactions. The disturbance of this homeostasis of the immune system by environmental factors can lead to food immune reactivities, bringing about the
penetration of dietary proteins and peptides into the submucosa. What can disturb this delicate but very effective balance? What we eat now as compared with the diet even 2 or 3 generations ago, and back to the beginning of human history, is vastly different. As mentioned earlier, we now have artificial sweeteners, artificial colorings, artificial flavorings, artificial preservatives, and a number of other food additives. The majority of Americans eat processed foods. Plastic containers are ubiquitous in our society for both foods and liquids. We microwave our foods and use coated cookware for food preparation. All these add chemicals to the foods and liquids we consume, which then bind to food antigens. These chemicals can bring about failure of oral tolerance, increased intestinal permeability, binding of food components to human tissue antigens, and molecular mimicry and cross-reactivity between food antigens and human tissues, resulting in autoimmunity.

The process of digestion of foods begins with the breakdown of proteins into peptides and then into amino acids. These are then absorbed by the gut. However, this process is affected by a multitude of factors: medications, processed foods, lack of digestive enzymes, and chemicals in foods. Think of the overuse in our society of antacids, antihistamines, histamine-2 blockers, and all available over-the-counter and antibiotics in our society. These interfere with the proper digestive processes and our gut is frequently presented with partially undigested foods, proteins, and peptides, which changes the microbiota and brings about the release of endotoxins by bacteria known as lipopolysaccharides. The lipopolysaccharides bring about inflammation, which opens up the tight junctions, damaging occludin, zonulin, and actinomycin, allowing these proteins and peptides to cross the mucosal layer, which then migrate into the regional lymph nodes and into then into the circulation. These peptides can bind to tissues, stimulating an attack by the immune system and causing autoimmunity.

Dr Vojdani describes why testing for both raw and cooked foods is necessary; why it is important to test for shrimp tropomyosin and shrimp protein; why a patient can react to pineapple proteins or pineapple bromelain, rice endochitinase, and rice protein; and the reason for serum testing for IgG and IgA food immune reactivity for all of these, as an example. Another important factor is the purity of each food antigen. For example, apple protein concentration is 0.2%; in other words, in 100 grams of apple, there are 200 mg of protein. Almond, on the other hand, has 20% protein; therefore in 100 grams of almond, there are 20 000 mg of protein, a vast difference and demonstrates that it is very important to “compare apples to apples,” and not “apples to almonds.” It is very interesting to read how some meats contain meat glue, a mixture of meat, transglutaminase, casein, chemical preservatives, and chemical colorings.

The clinician is faced with patients whose initial symptoms are vague and nonspecific, such as fatigue, joint aches and pains, sleep disturbance, brain fogliness, wide mood swings, cognitive function problems, changes in bowel habits, numbness and tingling, and a general feeling of malaise. The more specific symptoms of autoimmunity take longer to develop.

There is good news in all this: There are laboratory analyses that can detect these antibodies early, years before the reactions with the immune system appear that cause the irreversible and chronic damages that lead to autoimmunity. As we go forward and delve into this area of medicine, this publication gives you these first 7 articles, which describe the science and essence of food immune reactions.
The Evolution of Food Immune Reactivity Testing: Why Immunoglobulin G or Immunoglobulin A Antibody for Food May Not Be Reproducible From One Lab to Another

Aristo Vojdani, PhD, MSc, CLS

ABSTRACT
The gold standard for identifying food reactions is the elimination-provocation diet. Embarking on this long, tedious journey takes an expert practitioner and a completely dedicated patient, with a whole lot of patience from both. In the contemporary fast lane, microwave, “give me a pill” popping, I-want-satisfaction-now society, many clinicians have turned to laboratory assessments for quick answers to food reactivity. From the introduction of cytotoxic testing for food allergies in 1947 until today, food reactivity testing has evolved and branched; it has been both pseudoimproved and scientifically improved. With multiple available options for methodology, specimen types, and clinical lab, how is a clinician expected to find the one that fits the requirements of a particular practice? How, indeed, when one self-promoting paper supports a particular methodology, while another criticizes it? In this article, with the benefit of his years of training and experience as a research scientist and test development expert, the author, who is trained in both microbiology and immunology, discusses the history of food testing, analyzes the criticisms of it, reviews the scientific literature, and tours the methodologies. (Altern Ther Health Med. 2015;21(suppl 1):8-22.)

Aristo Vojdani, PhD, MSc, CLS, is the CEO of Immunosciences Labs, Inc, in Los Angeles, California, and a faculty member in the Department of Preventive Medicine at Loma Linda University in Loma Linda, California.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com

Health care practitioners have a variety of choices available for reactivity testing to detect food intolerance (ie, immunoglobulin E [IgE] or immunoglobulin G [IgG], in saliva or in blood, in vitro or in vivo, and a few or hundreds of antigens). The range of tests seems impressive. However, deciding which test is best for a given patient can be a daunting task. Adding to the confusion are nonpeer-reviewed, often self-promoting articles implying that the author’s test is better than any other. Multiple such articles have appeared, even in the same issue of a publication. This scenario only adds to the confusion of the reader.

To make an informed decision, the reader must consider the source. Is the author commercially connected to the test being reviewed? Further, the reader must consider the source’s sources. Are the references used to support the test relevant to the test methodology? In the current article, the author provides a guided tour through the labyrinth of food reactivity testing to help readers understand testing methodologies, recognize poorly written articles, and appreciate solid scientific publications and lab protocols, allowing them make informed decisions on testing for food reactivity.

THE CRITICISMS
In the past 3 decades, several articles have been published in scientific or alternative medicine journals and other magazines that question the clinical relevance of non-IgE-mediated testing for food allergies.\(^{1,4}\) The titles for some of these articles include "In-vitro Testing for Allergy”\(^{5}\); "IgG Food-Allergy Testing by ELISA (Enzyme-linked Immunosorbent Assay)/EIA (Enzyme Immunoassays): What Do They Really Tell Us?”\(^{6}\); "Evaluating the Clinical Relevance of Food-sensitivity Tests: A Single-subject Experiment”\(^{7}\); "Reproducibility and Reliability of Two Food-Allergy Testing Methods”\(^{8}\); "Testing for Food Reactions: The Good, the Bad,
and the Ugly "; and even the extreme title, "Should We Do Blood Tests for Food Allergy? ". This skepticism has led some national clinical societies to write position papers calling for a ban on the use of cytotoxic and ELISA/EIA testing for food reactivity.2-10 The 3 main categories of criticisms are (1) the tests lack reproducibility within a single lab and correlation from lab to lab; (2) cell-based measurements of food reaction or food IgG are clinically meaningless; and (3) contamination of the food antigen used in testing may interfere with test results. Each of these criticisms are reviewed and addressed in the following sections.

Reproducibility and Correlation
In the world of laboratory testing, if a test is not reproducible, it is considered invalid. Reproducibility in the lab means that testing gives comparable results when a patient's specimen has been split and run as 2 specimens. Comparable results, according to federal laboratory regulations, should be within a 20% variance. As early as 1998, the reproducibility of food reactivity testing was studied by drawing blood specimens from 1 patient, splitting it into 3 parts, and sending each to a different laboratory that already routinely performed the test.2 Additional specimens were collected from the patient on the same day as the first and immediately frozen. One week later, the frozen specimens were sent to the same 3 labs for food testing. After obtaining the results, it was concluded that 2 of the 3 labs had numerical variances of 49% to 73%,2 which was outside the acceptable lab standard of 20%. Further, the clinical interpretation (ie, whether the patient should avoid the food) varied from 7% to 59%.2 When a practitioner counsels a patient on the appropriate diet for his or her well-being, the recommendations must be based on reliable results. Clearly, many are not.

More than a decade later, a study was done that compared ELISA and cell size testing methodologies.4 Comparing test results from one lab to another lab that was performing the test using the same methodology is like comparing apples to oranges; comparing test results between 2 labs using 2 different methodologies is like comparing apples to sausages. Nevertheless, the study found the results of the IgG testing to be reproducible and to have exhibited results that were between 82% and 95% identical. Unfortunately, the way the data were presented did not allow the reader to determine definitively or accurately the reliability of the test.

From a scientific point of view, at least 10 individuals should have been tested for such a study, and the data should have been presented in the form of bar graphs to allow readers to examine the very reactive foods. For example, assume that a lab tested serum IgG for 100 foods, recording a positive IgG antibody against only 4 foods, with the other 96 foods showing a negative result. The next day, upon a repeated test of the same specimen, assume that only 2 of the 4 foods originally positive were recorded as positive again, and the other 98 foods were reported as negative. Calculation of test results would indicate the results were 98% identical, although in reality, the reproducibility of positive results for IgG foods on the 2 different days was only 50%.

Now, looking at the issue from another perspective, what if the test results were reproducible? Food-testing researchers know that several foods, such as ginger and cabbage, contain enzymes and many other factors that can react nonspecifically with reagents used in ELISA. Unless the food proteins are purified before being used in the assay, false-positive reactions could occur. In such a case, the patient could test positive to the nonpurified antigen each time, indicating reproducibility, but that would be a false-positive. Therefore, reproducibility of the results from day to day should be calculated only with the use of biochemically purified food proteins or peptides. The clinicians who wrote the articles mentioned previously1-6 may not have had the required level of understanding, nor may scientists who have not done extensive research with food antigens. Knowing such details requires many years of research experience and hands-on work with lab assays.

All companies that perform IgG or IgA antibody testing use their own in-house ELISA. This fact means that each lab has designed its own process from beginning to end, which is fine as long as all steps in the process are scientifically supported. In-house testing is quite common. These tests are called lab-developed assays (LDAs) or lab-developed tests (LDTs), and their validation processes are subject to federal laboratory regulations.

Remember the following piece of common sense: An ELISA test is only as good as the antigen used to coat the ELISA plate. Having developed more than 300 ELISA assays for the detection of various immune disorders, the author knows this fact very well. The antigen used on the plate results in the binding of circulating antibodies or nonspecific factors from the patient's blood to the coated wells. Many labs buy their food antigens in a lyophilized (freeze-dried) form from a company in the midwest. Two of the 3 labs in the aforementioned study admitted to buying antigens from an Oklahoma-based business.2 Having once been a client of this antigen-producing company, the author is very familiar with the company's process of food antigen preparation. The company obtains foods from the local markets to make antigens. The foods are chopped and first diluted in saline or other solution to make the suspension and then dried. The dried form of food is sold by weight. Other than rinses with acetone, the food antigens are not purified.3 Whether food antigens are obtained directly from the supermarket by the clinical testing lab, which then prepares them for use, or from companies producing them for sale, their purification is the responsibility of the lab developing the assay. This responsibility is why a very tedious and lengthy validation procedure was developed and recommended by the Clinical and Laboratory Standards Institute (CLSI) and the Food and Drug Administration (FDA) for all LDAs. If all required procedures are followed and if purified or synthetic food or tissue antigens are used in assays, undoubtedly the lab test will be specific to the antigen in question and the results will be reproducible.

Clinicians often run into scenarios where the results for a food antigen are positive from one lab but negative from
another. Which result should be used? Only one rule must be remembered in this situation. The practitioner cannot compare the results of one lab with the results of another lab. Many reasons exist for no correlation between labs. One factor, antigen purity, has already been discussed. Other aspects to consider include (1) specimen type, (2) the type of immune reaction that is tested, and (3) the time at which the specimens used in each test were collected (eg, on different days).

**Specimen Type.** If Lab A used saliva and Lab B used serum, the labs are testing 2 different specimen types as well as 2 different immune reactions.

**Immune Reaction.** If Lab A tested IgE or IgG4 and Lab B measured IgG, the labs are providing results for 2 different immune reactions. Both IgE and IgG can play a role in food immune reactivity.11 IgE functions via its high-affinity receptor, FcεRI, which is highly expressed on mast cells and basophils. IgG has several receptors: the high-affinity FcεRI and FcγRIV, and the low-affinity FcγRIIB and FcγRIII. All of these receptors are expressed on several types of cells involved in anaphylaxis, including mast cells, basophils, neutrophils, and macrophages. Inhibition of FcγRIII abolished temperature drops associated with shock in IgG-mediated, but not IgE-mediated, anaphylaxis.11

Five pathways are involved in food immune reactivity11:

3. IgG-basophil-PAF pathway.
4. IgG-neutrophil-PAF pathway via FcγRIV.
5. IgG-immune complex neutrophil pathway.

It may be of interest to note that the antigen doses required to trigger each mechanism may be related to the different pathways to systemic anaphylaxis, because the classical pathway or IgE production is activated by small doses, and the alternative pathways or IgG production are activated by large doses.11

**Specimen Collection.** If Lab A’s results are from a blood specimen collected in February and Lab A tested a new blood specimen from the same person that was collected in May, the tests occurred on 2 different collection dates, during which significant changes in immune responses could have occurred.

This section’s review of criticisms has been centered on ELISA testing. The reliability of cytotoxic food testing has also come under fire. The author will discuss reproducibility and other aspects of cytotoxic food testing in a later section.

**Clinical Value of IgG Food Testing**

The argument that IgG antibodies are an indication of exposure to antigens or a failure in immune tolerance has been made and repeated many times. This notion is based on the virus model, where levels of IgG are indicative of past exposure and high levels of IgG are indicative of superimmune protection.12 Contemporary immunologists have not applied this concept to other antigen reactivities, but many authors appear to be stuck in the virus model.

Elevated IgG antibodies against food antigens certainly have documented clinical significance. It is very well known that IgG against Helicobacter pylori toxin is an indication of H pylori-induced ulcers. Patients with systemic lupus erythematosus, who were negative on standard tests, have been shown to have high levels of antiprothrombin IgG.13 IgG testing for fibulin is used to differentiate osteoarthritis from rheumatoid arthritis.14

In general, elevated IgG antibodies against various tissue antigens can be used for the diagnosis of many autoimmune disorders. In fact, in a study on biopsy-confirmed celiac patients, antibodies to endomysial cell antigen, thyroid peroxidase, glutamic acid decarboxylase, insulin, and islet cell were elevated but normalized on a gluten-free diet.15 The author could cite many more studies supporting his point (ie, that IgG antibody testing holds clinical value in diagnostic medicine and that the removal of the identified antigen from the patient’s blood results in clinical improvement).

The human body is a constant reconstruction zone. Tissues are perpetually broken down and replaced. New cells are born every second. When the immune system is exposed to antigens, antibodies are formed. Because of the body’s regeneration activities, it is normal for the healthy adult human to produce small amounts of IgA or IgG antibodies to self-tissues.16-17 Likewise, it is normal for the human who eats potatoes twice a week, every week, to have some antibodies to potatoes. Laboratories are required to follow minimum standards set by the federal agencies that regulate laboratories, which establish reference ranges.18 A few laboratories, such as those under the author’s direction, use the higher standards set by the FDA as the minimum requirements for establishing reference ranges on LDAs.

What is not normal for a healthy individual is to have high levels of antibodies to a given antigen, with the exception of viruses.16-17 Even that notion is being challenged by emerging research.19 In fact, a few labs currently base their diagnosis of a herpes virus infection on the levels of the IgG antibody, measured against purified glycoprotein. When a lab assesses antibodies, whether to thyroid peroxidase or glutenin, it uses the reference ranges established for those specific antigens when reporting the results. If the patient’s numeric reading is above the reference range, the lab reflects that fact on the report. The practitioner receiving the report will use the results, together with other pertinent clinical information, to interpret a test’s meaning and provide a diagnosis and treatment protocol for the patient.

Is there clinical significance to IgG food testing? The aforementioned researchers of extraintestinal autoimmunity in celiac patients would say “yes.”20-22 Specific food testing has been used in studies with irritable bowel syndrome (IBS), showing that the IBS symptoms were alleviated by eliminating the IgG-positive foods.20-22 An in-house testing of a donor, who experienced a rash in the area where the elastic from
undergarments contacted the skin across the abdomen, resulted in extremely elevated IgG + IgA to banana. The individual had neither been suspected of having latex allergy nor tested positive to latex on a skin prick test. Further, she did not react to other foods known to be related to latex allergy, such as avocados, even though she regularly consumed that fruit. After she removed bananas from her diet, the skin condition disappeared.

In a clinical, double-blind, randomized, crossover trial of 30 migraine sufferers, elimination diets based on IgG food testing resulted in the reduction of the frequency of migraine attacks.23 As Shaw24 said in this quote from a recent Townsend Letter article,

In IgG-mediated, food-allergy testing, the goal is to identify foods that can cause inflammation, and thus, trigger a large number of adverse reactions. IgG1, IgG2, and IgG3 can all cause inflammation because these antibodies do not exchange heavy and light chains with other antibodies to form bispecific antibodies. Thus, IgG1, IgG2, and IgG3 antibodies to food antigens can and do form large immune complexes or lattices that fix complement and increase inflammation.

The author agrees with this statement. The formation of the antibody–antigen complex, however, depends on the type of antigens used, which may vary from one lab to another lab. The author will delve deeper into lab-to-lab antigen variances later in this article.

Despite all of the work that has been done with IgG immune responses, the literature fails to link IgG food reactions to clinical manifestations. In the context of the modalities used by many conventional and alternative practitioners, a well-respected scientist5 has written, “Immunoglobulin G (IgG)-based testing has shown promise, with clinically meaningful results.” Unless the author’s definition of clinically meaningful results pertains to a defect, a breach in oral tolerance, or disturbances at different steps in the path to oral tolerance that are known to result in food hypersensitivity,25,26 in reality, the direct clinical meaning of food IgG testing is not known. The industry needs more extensive clinical research in this area.

**Contamination of Antigen Poses an Interfering Factor**

One author has published an article questioning the validity of testing for food antibodies based on a theory that the microorganisms and pesticides on the food could cause false-positives.2 Indeed, microorganisms live on food. These organisms include common bacteria and fungi, and, possibly, some parasites and viruses. Pesticides or other chemicals may also be present on the food. Theoretically, if these components are in the food antigen well, the patient who has circulating antibodies to the microorganisms will test positive for the food. This finding would be considered a nonspecific response. The author posing this theory interviewed an unnamed technologist, after which she wrote that microorganisms, pesticides, and organic solvents are not rinsed away during test preparation.2

First, if a lab follows proper antigen purification processes, only a pure food antigen will be used to coat the ELISA plate wells. Second, having been educated and trained as a microbiologist and an immunologist, and thus being armed with an understanding of both the world of microorganisms and the human immune system, the author would have to disagree.

 Readers must understand the ratio between certain bacteria, fungi, or parasites in fruits or vegetables and their protein contents to see the error of this statement. This ratio is at least 1000:1, or even in some cases 10 000:1, in favor of food protein over microorganism protein. Usually each ELISA plate well is coated with 1 mcg or 1000 ng of antigen. If out of 1000 ng, 1 ng—or, in the worst-case scenario, 10 ng—is originated from bacteria or fungi, then only 0.01% of that immune reaction is going to be nonspecific, and 99.99% is going to be associated with the food antigen in the ELISA plate well. Therefore, a nonspecific reaction to 0.01% of the total antigen proteins is not enough to cause a false-positive result.

The critic goes on to say, “Numerous studies have shown high levels of IgG to pesticides and organic solvents in persons with high exposure rates”27 but does not provide the references for these studies. The current author searched scientific journals for such articles and found only 1 study that measured specific IgG against an organic pesticide in farmworkers.27

Chemical pesticides are hapten molecules. Haptons are too small to elicit antibody production by themselves. Pesticides applied to the food must first bind to food proteins to become a neoantigen and, thus, produce an immune response.28 Second, similar to the argument on bacterial and fungal contamination, the pesticide-to-food protein ratio is 1:1000 and, hence, not enough to result in a false-positive for food reactivity.

The author agrees that many false-positive results occur in food testing but not for the reasons reviewed above. The cause is not pesticide, bacterial, or fungal contamination of the food antigen in the well; rather, the truth is that many food proteins act like enzymes. In unpurified food antigens, many components act like substrate to the different enzymes that ELISA testing uses. Examples of these are peroxidase and alkaline phosphatase. Thus, coating the plate with these nonspecific factors plus the food antigens can result in a nonspecific reaction. In other words, the addition of antibody labeled with enzyme or the substrate to the antigen well during the testing process can cause these reagents to bind to the junk in the food-coated plates and result in color development, thus giving a false-positive. Or in some cases, the factors in nonpurified food antigen can prevent the binding of the antibody in the serum to the real antigens on the plate. In this manner, the use of nonpure food antigens can lead to both false-positive and false-negative results.

The literature that is critical of food IgG testing includes a negative editorial, “Should We Do Blood Tests for Food Allergy?”29 This editorial repeats most of the criticisms and conclusions made by the author of the previously mentioned article from the same issue,2 including the assertion that the presence of antibodies to food extracts could be indicative of
previous exposure to common bacteria or fungi. This conclusion is extremely naïve from the point of view of someone trained in microbiology and immunology. However, the article does contain a very important comment, with which the current author agrees:

"Some allergic reactions to food antigen are not due to the major proteins but rather to peptides produced during the process of digestion."

Examples include (1) cow’s milk versus α-casein, β-casein, or casomorphin peptides or (2) wheat protein versus α-, γ-, or ω-gliadin peptides. If the lab is testing only cows milk and not the peptides, and the patient is actually reacting clinically to the casomorphin peptide after the cows milk is digested, the test for cows milk may result in a negative finding, even though the patient clearly has a clinical response after consuming cows milk. Further, some food reactivity can occur against a raw food, such as a raw peanut, but not against the processed form, such as a roasted peanut. Other food reactivity can occur in the opposite fashion, such as a positive reaction to a hard-boiled egg and a negative one to a raw egg. Indeed, some food immune reactions are caused not by the native food proteins but by glycosylation products of these proteins that are produced during processing or cooking.28 This issue can result in both false-positive and false-negative results if the testing includes only raw foods rather than raw and processed foods simultaneously.

**Conclusion: Criticisms**

Of the criticisms reviewed above, the valid one is the one that criticizes the results due to lack of reproducibility within a single lab. Each lab should strictly follow antigen purification processes when preparing the food antigens used in testing procedures. If this purification is done, results will be reliable. Clinicians should challenge labs to ensure that tests are accurate. This challenge may entail secretly splitting a sample and sending it to the lab under 2 different patients’ names. If the results do not correlate by at least 80%, the test from that lab is not valid.

**THE PITFALLS**

Early attempts to develop serologic reactivity tests for foods were flawed. They lacked double-blind studies and quality control, made outrageous clinical claims, and were completely subjective based on the technician reporting the results. The following section looks at the pitfalls specific to food reactivity testing that have occurred for the last 65 years.

**Cytotoxic Testing**

When Bryan and Bryan29 widely popularized the food cytotoxicity test in the late 1950s, its existence was exciting and innovative for its time. During decades of use, the test proved to have many problems, and by 1985, it was officially banned in California and other states. An improved version and an automated version of this cytotoxic test are still in use in at least 3 laboratories under the names: ALCAT (antigen leukocyte antibody test); MRT (mediator release testing); and LRA (lymphocyte response assay) (Figure 1).

The process of cytotoxic testing entails the placement of a drop of the patient’s blood, including plasma, onto a plate well that is coated with a liquid or dried food extract. The prepared plate is examined microscopically after 10 minutes of incubation and, thereafter, at 30-minute intervals up to 2 hours. The technician looks for observable changes in leukocyte morphology. If the technician observes changes in the cell’s size, rounding, inactivity, or vacuolization and/or disintegration of cells, a positive result is recorded.28 In 1984, the author was involved, on occasion, in immunology research using ELISA testing methods but was really focused on classical cytotoxic testing to measure the effects of toxins on immune cells, specifically natural killer cells. He was approached by the CEO of a lab in west Los Angeles that
performed cytotoxic food testing. The lab was challenged by the California Department of Health to prove reproducibility, and the author was hired to refine the cytotoxic food testing method to meet the reproducibility demands of the state agency. After a couple of weeks observing the performance of this method, like Dorothy realizing she was not in Kansas anymore, he saw that cytotoxic testing of natural killer cells and cytotoxic testing of food antigens were worlds apart. The author found many flaws in cytotoxic food testing, including (1) use of lemon extract, (2) unique factors of food extracts, (3) pH of food extracts, (4) lack of consistent results with different technicians, and (5) different results from different times of testing for the same patient.

**Lemon Extract.** Testing lemon extract activity against a drop of blood was difficult. Because lemon is acidic, the acid naturally causes changes in the white blood cells. The effect is not necessarily due to a patient's immune reactivity but could reflect false-positivity.

**Food Extracts.** Each food extract has unique factors that can cause false-positives or false-negatives.

**pH of Food Extracts.** Under the author's direction, the lab changed the pH of all food extracts to human levels at 7.0 to 7.2. By making this change, the number of reactions dropped by more than 200%. For example, a patient originally reacted to 30 food extracts, but when the pH was matched to human levels, the same patient's specimen reacted to only 10 foods. This improved cytotoxic assay was presented in 1985 at the American Academy of Otolaryngic Allergy's annual scientific meeting. The author and his partner won the Best Presentation Award for that refinement of the assay.

**Lack of Consistent Results.** No matter what kind of improvement was implemented in the preparation of food extracts, the results were still read differently from technician to technician at the lab.

**Different Results From Different Times of Testing.** A patient's blood drawn once each day for 2 days in a row and tested for cytotoxic food reactivity produced reports that did not correlate with each other.

Even with the author's attempted refinements, reproducibility could not be achieved. The California Department of Health was not satisfied. Throughout California, labs had to stop performing cytotoxic food sensitivity testing, including the one in west Los Angeles. At that time, this scenario was being played out in other states as well.

Shortly after the banning of cytotoxic food testing, a scientist made a few improvements on the testing method and reintroduced it under a new name. Another scientist took some time and solved the subjective problems with cytotoxic food testing by developing a computerized machine that could observe and record cell size changes. Both of these versions of cytotoxic food testing are currently available. From both the scientific and business points of view, what is being done in these tests?

In contrast to the variety of food-IgG or other antibody-testing publications, there is a noticeable lack of peer-reviewed articles for any version of cytotoxic testing. For an immunologist to measure mediator release, such as cytokines, with 90 to 491 food antigens, a significant amount of blood is needed to release a sufficient amount of mediator. To assess one antigen, the lab needs 1 mL of blood per culture, or approximately 500 000 white blood cells, to obtain the requisite amount of mediators (cytokines, chemokines) to be measured. Thus, to perform this test on 491 food antigens, the lab would need to have 50 tubes of blood. Further, a lab's cost for the reagents used in the determination of these mediators, if the test were done properly, would be more than $2000 per patient run. In addition, those costs must be combined with the cost of the many hours of labor by skilled technicians to perform the culture of white blood cells. In reality, however, the price of the test to the patient appears to be much lower than a lab's costs could be.

Articles supporting the improved cytotoxic testing were published by a magazine marketed to practitioners and patients. In the article about lymphocyte response assays (LRAs), “Predictive biomarkers in personalized lab diagnosis and evidence-based-practice outcome monitoring,” the authors discuss various predictive markers for the assessment of health risk using the LRA. Although most of the biomarkers explained in the article—such as hemoglobin A1C (HbA1c), high-sensitivity C-reactive protein (hs-CRP), or oxidized low-density lipoprotein (LDL)—are scientifically based, the following claims behind the LRA test have not been proven: (1) the tests measure all 3 delayed-allergy pathways; (2) the tests avoid the false-positives common in tests of immunoglobulin reactivity, immune complexes, and direct T-cell activation; and (3) the tests' identification of a patient's specific sensitivities and delayed allergies is a clinical breakthrough. LRA cell cultures are reproducible within less than 3% variability when different readers read split samples on different days.

These claims are bold given that the authors have neglected to support them with peer-reviewed research publications, making readers ask, “Where are the science and/or clinical studies behind the claim that LRA measures all 3 delayed-allergy pathways? Where is the evidence that LRA measures sensitivity to chemicals?”

Culturing lymphocytes in chemical media and then measuring changes in cell size to determine if a specific chemical has a toxic effect on the lymphocyte, does not measure any type of allergy pathway. The same statement applies to reactions of lymphocytes to foods. A change in the cell size could be associated with hundreds of factors in the food extracts that are toxic to white blood cells, which is why this test was originally called a cytotoxic assay. In fact, according to an article published in *Allergy,* adverse reactions to food can be classified as toxic or nontoxic; however, many reactions to foods could be toxic to white blood cells. Nontoxic reactions that involve immune mechanisms resulting in IgE production are collectively termed allergy. If antibodies other than IgE are involved, the reaction is termed intolerance due to the breakdown in immunological tolerance. Patients with intolerance produce IgG, immunoglobulin A (IgA), or immunoglobulin M (IgM)
antibodies against these foods. An industry-wide proposed term for food intolerance could be non-IgE food immune reactivity.

In the same issue of the magazine in which the LRA article appeared, another article, “Mediator Release Test (MRT): A Comprehensive Blood Test for Inflammation Caused by Food and Food-Chemical Sensitivities,” was published. The message of the second article to the readers was that antibodies, such as IgG, IgM, or IgA, cannot identify reactivity to foods and food chemicals, and that antibody testing has not demonstrated an acceptable correlation with inflammation or clinical symptomatology in adverse food reactions. Thus, according to the author, the information provided by most commercially available food sensitivity tests was of limited clinical value. The author discussed the importance of white blood cells in food-induced inflammation, which he called an immunologic endpoint. To support this theory, a very nice graphic was presented describing an in vivo reaction of cells involved in the innate inflammatory reactions:

When sensitive food antigens cross the tight junctions, neutrophils and macrophages are typically the first cells to react. They engage in the destruction of offending pathogens or antigens, ultimately releasing various cytokines and other pro-inflammatory mediators.

The way the quote was presented would have led the reader to believe that the logical end action after exposure of white blood cells to different food chemical solutions was to measure the aforementioned cytokines and proinflammatory mediators. But on the next page, the author indicated that the described test measured volumetric changes in white blood cells, assumingly caused by food- or chemically-induced release of mediators. The clinical utility of this test as it relates to food and, in particular, to chemical sensitivity, however, has yet to be determined by scientific studies.

Although these 2 articles described tests that are based on volumetric changes in white blood cells (cytotoxic testing), each made the claim that the information provided by non-IgG antibody testing for food reaction has limited clinical value. In the same issue of the magazine, however, the clinical usefulness of IgG food allergy testing was discussed by another author.

Even with improvements in cytotoxic testing, recent reproducibility studies still have shown a lack of correlation of results for the method. One set of authors concluded, ...

... well-designed clinical trials should be published before patients are subjected to expensive testing for delayed hypersensitivities; eg, ALCAT and MRT testing, that offer little evidence of effectiveness. Disclosing the basis for food reactions continues to present a diagnostic challenge, and testing for food allergies in the context of an appropriate clinical history is paramount to making the correct diagnosis.

In a well-written paper by Hodsdon and Zwickey, the results of a small study on the repeatability of food-testing methodologies were revealed. The authors challenged both cell size and IgG ELISA labs with split specimens and specimens collected in the course of days. The study showed that cell size testing was not reproducible by split samples or by samples provided in the course of a week. In the split-sample challenge, only 17 of 50 foods were identical when using the cell size method (34%) versus 91 of 96 (95%) using IgG ELISA. Further, the split-samples challenge tested the internal reliability of the 2 labs compared the levels of reactivity between the cell size lab and the IgG ELISA lab (0, no reaction and 3, high reaction). The cell size method resulted in 14 cell-size foods of 50 (28%) showing a 3-level reactivity difference, whereas the IgG ELISA method showed 0%.

Compiling the results of blood samples collected on each of 3 days within the same week revealed an even more clinically disturbing result. Only 1 of 50 cell-size foods reported identical results. The corresponding IgG time challenge produced identical results of 82%. The difference in time-challenged, 3-level reactivity for cell size was a staggering 60%, or 30 of 50 foods; again, the difference for the IgG tests was. The study’s conclusion called into question the reliability of cell size testing for identification of food allergies but found that IgG testing was reproducible and reliable.

Lack of Quality Assurance

In 1986, the author developed the ELISA IgG food test after cytotoxicity food testing was banned in California. He opened the first clinical lab that offered IgG reactivity testing for food by ELISA based on the principles summarized in Figure 2. Soon other labs that had first used the author’s original lab as a reference lab copied the method and added it to their test menus. Unfortunately, those other labs did not then or even today fully understand the theories behind the test. One telltale is a lab reporting immune reactivity to sugar or sweeteners. The author uses the sugar well as quality control for ELISA testing. If the sugar well produces color, he knows that something has not gone right, and he needs to repeat the test. Humans do not make antibodies against sugars. Sugar is a very small molecule approximately the size of 200 to 300 Da, like the chemical haptenic principles described earlier. The human immune system does not recognize molecules smaller than 5000 Da, unless the hapten, in this case the sugar molecule, binds to food proteins during its processing or cooking, and thus forms glycosylation products. It is only after this process that the immune system recognizes sugar-bound protein (glycosylated protein) or advanced-glycation end products, but it will never react to unbound sugar or other sweetener molecules.

Recently a new test, the dried bloodspot test, has been promoted as an easy way to test for food allergies, even directly to patients on Amazon.com. For this assessment, the patient uses a lancet to prick a finger and drop a blood sample on a special blood spot collection card. The card is air dried and then shipped to the lab for the performance of either IgG or IgG4 ELISA testing of a varied number of food antigens. The report, depending on the lab, ranks the foods’ allergenicity either as borderline to mild, moderate, or severe, or as safe, moderately safe, or avoid. Without any unbiased, double-blind, published studies comparing
bloodspot food testing with venipuncture food testing, how does anyone know it is a reliable or reproducible improvement on the venipuncture IgG ELISA testing?

Scientifically, the promotion of this type of test is misleading. Measurement of IgG is not used for classical allergy testing. Rather, IgG measures food immune reactivities due to a breakdown in immunological tolerance. Serum specimens that can be accurately measured are the best for use in antibody measurement. In bloodspot testing, no lab can control how much blood is collected and, therefore, used in the testing process. IgG4 comprises only approximately 4% of total blood IgG, making the tests questionable if they are supposed to be measuring IgG4. A lab would need 2 to 4 mL of blood to accurately assess IgG4 reactivity to 45 to 95 foods. A couple of drops of blood do not equal 2 to 4 mL. Therefore, exactly what the tests that use the bloodspot method are actually measuring is seriously in question. Unless comparisons by scientific method are done between the serum and bloodspot methods and a standard operating procedure for bloodspot is established and published in peer-reviewed journals, clinicians cannot be encouraged to use this method.

**Lab Involvement in Therapeutics**

Labs have come under fire for suggesting that a patient must consume a wide range of foods in the 3 weeks prior to specimen collection to obtain accurate results. When a test is intended for allergy assessments (IgE or IgG4), why would anyone risk a patient’s life by making such an irresponsible recommendation? Allergists do not require their patients to consume potentially dangerous foods before performing an allergy test for IgE. The stated requirement is not only irresponsible on the part of the lab, but also immunologically inaccurate.

If the patient has a fully functioning, intact intestinal barrier, 1 meal of a specific food is not enough to elicit a serologic antibody response. For these patients, dendritic cell sampling, endocytosis, transcytosis, and retrotransport of secretory IgA are the routes that food antigens take to reach the blood stream where serologic antibodies are produced. Thus, it takes multiple exposures, over a period of time, for the immune system to produce antibodies to a food protein. Food challenges should never be taken lightly. Dangers are involved, and the patient needs to be forewarned. Some foods are known to cause anaphylaxis; others can exacerbate autoimmune conditions in some people. Rather than force a patient to consume foods he or she does not normally eat, why not find the most appropriate food test panel that closely reflects the foods that the patient consumes regularly?

As if a lab making clinical suggestions is not enough of a critical problem in itself, some labs provide diet protocols based solely on test results. Miller states,

> Included in these problems [associated with food allergy testing] are the distribution of therapeutics by a laboratory, the prescription of therapeutics based solely on the basis of laboratory testing, and the possibility that therapy recommendations are based on a lab test that may not be correct.

A single lab report is only one piece of the complicated puzzle of today’s multifaceted disorders. It can be interpreted one way when put in context with another lab report and another way when combined with the patient’s medical history. The lab is not involved with the patient, has not seen the patient, and does not have the patient’s other test results and clinical history. How, then, can a lab put together a therapeutic protocol for an individual patient? Are labs trying to replace health care practitioners and the vital patient-practitioner relationship?

**Conclusions: Pitfalls**

Over the years, lab testing has found itself in a few pitfalls. The cytotoxic testing for foods has time and time again failed reproducibility challenges. A lack of understanding of even the theories behind testing methodologies has led to false-positive results. The introduction of improvements has been done without unbiased control studies. And finally, the evidence that labs are crossing the line into the clinical setting, as if to replace the practitioner, leaves some critics cold. Laboratories should focus on providing the best, proven, reproducible results for

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**Figure 2.** Steps involved in the enzyme-linked immunosorbent assay (ELISA) procedure. A patient’s serum is added to a plate well that is coated with pure antigen. If antibodies are present in the sample after incubation, they will bind to the antigen. After washing an enzyme, a second antibody is added to the plate to make the blood and antigen react. After washing, the addition of the substrate, and incubation, the plate well’s degree of color development determines the presence and amount of antibody.

Abbreviation: ALKP, alkaline phosphatase.
each and every specimen coming into the lab. Nothing is more important than that responsibility.

**THE IMPROVEMENTS**

As long as critics and visionaries exist, clinical lab tests will continue to be tweaked, improved, or replaced as new technologies are born and old mysteries are solved. The evolution of lab testing has led to lactulose/mannitol currently being replaced by intestinal-tissue antibody testing as the gold standard for antigenic intestinal permeability. Salivary antibody testing of IgA alone produced too many false-negative results, but by adding the measurement of IgM, salivary antibody testing has become more sensitive and accurate. The traditional serologic, celiac test panel, which had been proven to give 50% false-negatives for patients with Celiac disease, has been improved significantly by measuring multiple proteins and peptides from wheat, together with multiple transglutaminases. Valid scientific improvements to food testing methods have been achieved in recent years.

**ELISA Replaces Cytotoxic Testing**

The article by Engvall and Perlmann in the *Journal of Immunology*, "Enzyme-linked Immunosorbent Assay, Elisa: III. Quantitation of specific antibodies by Enzyme-Labeled Anti-Immunoglobulin in Antigen-Coated Tubes," inspired the author during his postdoctoral studies. Earlier, when he was working on both his master’s and doctoral degrees, he and his classmates used the tedious lab test method called agglutination. Today this method is used in blood banks for typing blood. In the past for immunological testing, a separate tube was used for each determination of reactivity. The old method relied on the human eye to determine the level of agglutination, and the process was done manually, one sample at a time. By contrast, in the ELISA method, 96 to 384 wells are used simultaneously, and the color is generated due to an antigen-antibody reaction that can be measured by automated spectrophotometers called ELISA readers. For the author, this change was revolutionary because the aforementioned agglutination method would have taken months to measure antibodies against only 50 antigens. However, while he was doing postdoctorate studies in immunology at the University of California, Los Angeles in 1979 that a 2-day workshop on ELISA was being taught. The author immediately signed up for the expensive course. The cost of $350 then was the equivalent of more than $1100 today, a hefty sum for a postdoc researcher. He learned the ins and outs of ELISA testing, and to this day, he feels it was his best investment, not only for his own work, but also for the clinical world as well. Since the invention of ELISA, the method has seen a myriad of analytical and clinical applications.

When cytotoxic food testing was banned in California, the author began reading as many articles as he could on the immune mechanisms behind the type 2 immune reaction associated with cytotoxicity and found that antibodies in the blood could bind to food antigens and that the antigen + antibody could bind to a receptor on the surface of white blood cells, causing changes in the cell size. This receptor, which has since been named FcγRI, has a high affinity for the Fc region of IgG, as is shown in Figure 3. Therefore, the question that the author asked was, "If the antibody is IgG, which is responsible for the receptor’s binding to the white blood cells, then why aren’t clinicians measuring the IgG itself against specific foods rather than looking for changes in cell size, the tests for which have not been proven to be reproducible?" The ELISA workshop became the key to the answers.

The author made a scientific guess, because he did not know if IgG would be produced against every food. He did not know to which white blood cell receptor the IgG could bind. But he took a chance. The IgG ELISA test for food immune reactivity was produced, admittedly without doing

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**Figure 3.** The formation of IgG antibodies against various antigens can result first in the binding of antibodies to FcγRI on white blood cells and, then, to the formation of the antigen-antibody complex. The combination of antibody-antigen complexes with the functional receptor (FcγRI) leads to structural changes in the cell membrane and cell size.

Abbreviations: IgG, immunoglobulin G.
clinical studies and associating it with allergies or sensitivities. After his presentation in 1985 at the American Academy of Otolaryngic Allergy's annual scientific meeting, many visitors came to him to study the refinement of cytotoxic assays. Those individuals also became interested in ELISA. By 1988, many negative articles were being published about cytotoxic testing. In 1987, an article in the Journal of the American Medical Association (JAMA), "In Vitro Testing for Allergy," condemned cytotoxic testing, stating, "The National Center for Health Care Technology reviewed the evidence and concluded that this test is unproved, unreliable, and without scientific basis." It further stated, The Food and Drug Administration concurs and has developed a regulatory position that cytotoxic test kits marketed for use in the diagnosis of allergic diseases are adulterated and misbranded devices under the Federal Food, Drug, and Cosmetic Act.

The same article also included 3 sentences on IgG testing, one of which brought out the argument that IgG antibodies to food antigens do not correlate with food hypersensitivity. The author agrees with this statement. IgG is not a measurement of hypersensitivity; it is a measurement of loss of immune tolerance. Because of these publications, some labs abandoned cytotoxic testing and offered ELISA IgG instead. Eventually, many labs sprang up offering ELISA IgG food sensitivity testing across the United States.

Since its introduction, the ELISA method for IgG food reactivity has seen improvements. Despite a 2003 statement published by the American Academy of Allergy, Asthma, and Immunology that "Measurement of specific IgG antibodies to foods is also unproven as a diagnostic tool," most of the IgG testing on food reactivity throughout the world is done using the same ELISA technique. Indeed, many laboratories around the globe use the ELISA methodology to detect IgG reactivity to various food proteins and peptides; the labs must use food antigens from food products available in the United States and without scientific basis.

The author of the American Academy's statement goes on to say,

The clinical usefulness of IgG testing in an array of illnesses was illustrated in an early article published by an otolaryngologist who reported that the majority of his patients had substantial health improvements after an elimination of foods positive by IgG food allergy tests. The overall results demonstrated a 71% success rate for all symptoms, achieving at least a 75% improvement level.

This statement is correct, but the provided reference is about a completely different method (RAST), and different IgG subclasses were measured. This scenario represents a case in which the reader needs to check the sources to be able to determine the validity of the information.

**Antigen Selection and Purity Guidelines**

Some patients are vegans. Some patients are on Paleo diets. Some children have never knowingly consumed a vegetable. But the average patient tested for food immune reactivity consumes a variety of foods, both raw and cooked. If our diets are varied, why are the tests for food reactivities not? Few labs offer testing of food proteins that have been heated, yet only a few patients are completely raw foodists.

In a large-scale study by Zeng et al, variable serum levels of 14 different food-specific IgGs were assessed among healthy and symptomatic Chinese adults, and the possible association of the foods with chronic symptoms was investigated. The researchers indicated, Although testing for the presence of food-specific IgGs has been regarded as a potential tool for the diagnosis of food allergy/intolerance, it's the accuracy and clinical utility of such testing that remains unclear.

Interestingly, this study reflected that women had higher concentrations of food-specific IgG reactivity than men for most of the foods tested, which had been shown by previous research publications.

The food-group correlations with self-reported symptoms were shown. Participants who reported having eczema had higher concentrations of crab- and rice-specific IgG than subjects with no eczema. Another example was a participant with no gastrointestinal symptoms, who had higher concentrations of IgG against codfish, rice, mushroom, tomato, and wheat compared with an individual with chronic symptoms. Overall, these findings do not make sense and only add additional variables to many existing confusions associated with food-specific IgG testing.

Indeed, the authors admitted to several limitations in the study. First, IgG antibodies were tested against only 14 different foods consumed in China, but the kits were purchased from the United States. The kit company extracted food antigens from food products available in the United States and did not measure levels of specific IgGs against fruits, nuts, many vegetables, seeds, food additives, and more. Second, clinical symptoms were determined by self-reporting and were not confirmed by health care professionals. This procedure may have led to misclassification of chronic symptoms. Third, the study did not connect identified mechanisms of IgG food reactivity to clinical manifestations.

Additional limitations of the study were not mentioned by the authors. First, the researchers purchased kits for measuring antibodies against various extracts made from raw and unmodified foods. Any food reactivity testing performed must reflect the test subject's current diet, and the Chinese are not particularly known for eating solely raw food. Therefore, a study on the same topic should be repeated using modified food extracts. Second, in this extensive study on 5394 adult participants from different regions of China, only IgG was measured but not IgA or IgE antibodies.

It is a very well-established scientific fact that due to breakdowns in immune tolerance mechanisms, the first isotype antibody produced against food antigens is IgA, followed by IgG. Since 1985, when the author replaced cytotoxic testing with IgG testing, researchers have continued to study food-specific
IgG or its subclasses, but not IgA, or even IgM. Further elucidation of the role played by food-specific IgA in food immune reactivity (not food allergy) should be the first priority of future studies, followed by IgG and IgM.

The ELISA test, whether for food, bacteria, or human tissues, is only as good as the purity of the antigen on the plate. Both the degree of purity obtained in the extraction of different food proteins and other components of the foods tested, and the relative efficiency of the coupling of different extracted food components and antigens to the ELISA plate, are known to influence the accuracy of food-specific IgG measurements. When preparing an antigen that will be used to coat an ELISA plate, all interfering substances must be removed, leaving only the food-specific glycoproteins and lipoproteins. The process has been well described and is shown in Figure 4. Clements first developed a low-temperature, food extraction method for vegetables in 1965, which the author has modified. Centrifuge machines and 0.2 M filters are used to remove debris, bacteria, fungi, and viral particles. The remaining proteins are then dialyzed multiple times to remove molecules smaller than 6000 Da. Then the food antigens are fingerprinted using electrophoresis with sodium dodecylsulfate gel. This process for the preparation of quality food antigens is summarized in Figure 5. In a majority of cases, various researchers have determined the exact epitope—a combination of many amino acids—that is responsible for
allergenicity of food antigens. These peptides can be synthesized to a purity of more than 90% and can be used in IgE- and non-IgE-mediated food reactivities.28,64,65 As mentioned above, most people eat a mixture of both raw and cooked foods. The first documented case of immune reactivity to cooked food was reported in 1921.66 Commercial labs use food antigens prepared only from raw food. Research has shown that food can become more antigenic or less antigenic when it is heated.28 When a lab tests only raw-food antigens, it can fail the patient who reacts to the cooked or processed versions of the food rather than the raw form.

Cooking or processing and denaturization of food proteins may cause alterations in immunodominant epitopes, potentially affecting allergenic properties. This processing may destroy existing epitopes on a protein or may cause new ones to be formed (ie, neoallergen formation) as a result of changes in protein conformation. Neoallergen formation has been known for at least 30 years.67 The concept of neoallergens may explain why some individuals can tolerate a raw food or a raw-food ingredient but will react to the same food when it is heated in some way. For example, a person who is reactive to ketchup may not have an immune response to a raw tomato. If this patient is tested using traditional food sensitivity assays, the result would be negative for tomato, and the patient's problem with ketchup or other forms of cooked tomato would go unidentified.68-77 Researchers have found neoallergens from pecans67; wheat flour69; roasted peanuts74; lentils70; almonds, cashews, and walnuts76; soybeans71,72; shrimp, scallops, tuna, eggs, apples, plums, milk, and potatoes73,74,80-82; and many other foods that had not been previously tested.

Despite multiple publications in scientific journals demonstrating IgE-mediated reaction to many cooked or processed food antigens without reaction to raw foods, almost all commercial laboratories measure IgE and IgG antibodies primarily against raw-food antigens. Part of the findings of the author's own studies10 are reviewed in Figures 5 and 6. Data from only one patient is shown in Figure 6. This and other data published earlier28 clearly indicate that an individual may not show IgG, IgA and IgM antibodies against antigens prepared from raw food, but testing the same patient against antigens prepared from cooked food might result in a severe immune reaction.

One other component to food immune reactivity that is implicated in inflammation and autoimmunities is advanced glycation end products (AGEs). By reacting with AGEs and tissue proteins, antibodies against modified food antigens may cause perturbation in degenerative and autoimmune diseases, such as diabetes, atherosclerosis, inflammation, autoimmunity, neurodegeneration, and neuroautoimmunity. Thus, detection of antibodies against food antigens is not only an indication of immune reactivity to foods but also of the size of the role that these antibodies play in inflammation, autoimmunity, aging, diabetes, and neuroimmune disorders. The importance of IgG testing against different food antigens and their association with AGE human serum albumin, AGE hemoglobin, antioxidantized LDL antibody, and myelin basic protein antibody is shown in Table 1.

**Specimen Testing in Duplicates**

Many steps are involved in ELISA testing, from adding the patient's specimen to the plate, to washing the plate, incubating the specimen, adding substrate to the plate, and so on. With each step, a potential exists for error. One way to spot these potential errors is to run a patient's specimen in side-by-side duplicate (ie, split the specimen and run the halves side by side in duplicates).
side). If correlation occurs between the 2 side-by-side wells, then the results can be reported. If the side-by-side wells lack correlation, the patient’s specimen must be run again in side-by-side duplicate until correlation is achieved.

The Clinical Laboratory Improvement Amendments (CLIA) of 1988\(^1\) apply to all facilities that perform tests on human specimens. Laboratories employ CLIA guidelines for internal and external quality control or quality assurance. Internal quality testing entails pulling specimens that have already been run, and retesting them to see if reproducibility exists. Some food testing labs run as many as 100 specimens, each specimen on an individual well, and then randomly choose only 2 to 5 wells with a single antigen for quality control. As the inventor of the IgG ELISA test for food, the author feels that this practice provides inadequate quality assurance. In his opinion, each and every specimen must be run in side-by-side duplicate, thereby guaranteeing quality assurance for each patient. In addition, each food antigen tested should have a negative and positive control on the same plate. Other internal quality-control techniques include splitting a patient’s specimen and labeling it as belonging to 2 patients, without the knowledge of the technician running the test. The ideal is that the split specimens should have correlated results. Again, without doing quality control every day that patients’ samples are run, labs cannot assure the user of reproducible, quality test results.

### Conclusions: Improvements

Throughout the years of laboratories offering IgG ELISA testing, the literature has had a dearth of research papers showing quality controls and testing versus clinical manifestations and outcomes. This lack has led to IgG ELISA testing being discredited by researchers, clinicians, and even countries. For this reason, as the developer of the original assay, the author has kept his distance from IgG food testing. In fact, for many years, he prevented his marketing manager from promoting the food testing offered at his clinical lab. He did not whole-heartedly believe that the labs doing the test were doing the full and necessary steps required for reporting reproducible and reliable food antibody assays. As was discussed earlier, reporting of truly reliable test results is highly dependent on the purity and quality of the antigens used and the steps taken for the validation of the assay, which should be documented in the assay’s standard operational procedure. Because of this issue, as the inventor, the author felt a weight on his shoulders and lost much sleep each time the IgG ELISA test for food came under attack.

Despite his misgivings about the implementation of the ELISA IgG food test, the author has continued to research, study, and amend the process to yield better test results. This work entailed perfecting the food extraction method for obtaining only pure food proteins to coat his ELISA plates.

### Table 1. Comparison of Results

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<th>IgG Against AGE-Hb in EU</th>
<th>IgG Against anti-ox-LDL in mU</th>
<th>IgG Against MBP in EU</th>
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<td>185</td>
<td>170</td>
<td>3538</td>
<td>322</td>
</tr>
</tbody>
</table>

Abbreviations: IgG, immunoglobulin G; EU, equivalent units; AGE-HSA, advanced glycation end products-human serum albumin; AGE-Hb, advanced glycation end products-hemoglobin; anti-ox-LDL, antioxidanted low-density lipoprotein; MBP, myelin basic protein.

\(\text{Comparison is for 8 patients with IgG reactivity > 30 EU against many modified food antigens with those for 8 controls with low reactivity to a few modified food antigens, in relation to possible cross-reactivity with AGE-HSA, AGE-Hb, anti-ox-LDL, and MBP.}\)
The author has insisted that each specimen be run in side-by-side duplicate to ensure correlation and has embraced the concept of assessing not only raw food antigens but also processed ones to develop immune reactivity panels for food that better reflect the diet of the patients being tested. Finally, the testing of each individual antigen should be validated separately and properly documented.

What is really the cause for the low opinion some have for ELISA IgG food testing? Is the issue the test itself or the labs that have failed and continue to fail to implement and follow proper procedures for the validation process before reporting test results?

CLOSING REMARKS

While writing this manuscript, the author received the January 2014 issue of the Townsend Letter. It featured 4 different, self-promoting articles about the issue of food allergy and sensitivity. Each article promoted the author's methodology for assessing food immune reactivity. Each article implied, "My test is better than all the other tests." This message may have confused many readers. Although these articles are very well written, none of them dealt with the root causes of the problem, which are as follows: (1) What are labs really measuring? (2) What is the scientific mechanism behind the testing? and (3) Where is the clinical correlation of the test results with patients’ symptomatologies that should have been published in peer-reviewed, scientific journals?

So what do scientists need to do to change this industry trend that began in 1985 after the banning of cytotoxicity testing and the introduction of ELISA food reactivity?

First, the field has to admit that currently it has a huge problem, and unless labs get together to standardize testing methodologies, then thousands of false-positive and false-negative results will continue to be reported by many laboratories.

Second, scientists must classify the lab test simply as being cell-based reactivity or antibody (IgG, IgM, IgA) reactivity assays.

Third, labs that perform the cell-based assay must unite to standardize procedures and try to use the same reagents, in particular the food antigens, that are as close to the reality of human consumption as possible.

Fourth, labs that perform antibody-based assays should standardize procedures and try to use the same food antigens that reflect the dietary trends of the population being assessed.

Fifth, labs performing assays based on volumetric changes in cell size must come up with an acceptable resolution to the documented problems with the methodology and present the clinical significance of their tests.

Sixth, if such clinical significance is not available, the labs must immediately start clinical studies and present the raw data to the industry via publications in peer-reviewed journals.

Seventh, labs performing antibody-based assays need to present the clinical significance of this test to the industry through articles in peer-reviewed journals.

Eighth, scientists must stop referring to these tests as food allergy assessments. None of these tests measure specific IgE against foods, which is the measurement that is associated with food allergy.

Finally, during the validation process, each food antigen should be tested separately according to CLIA and FDA guidelines rather than tested using the shortcut process of validating antibodies to 90 foods simultaneously.

Until standardization of the antigens and procedures for testing occur, no one in the industry can compare one IgG lab to another IgG lab, one cell-based lab to another cell-based lab, or one IgG lab to a cell-based lab. Otherwise, scientists are just talking about comparing apples to oranges or apples to sausages.

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Oral Tolerance and Its Relationship to Food Immunoreactivities

Aristo Vojdani, PhD, MSc, CLS

EXCLUSION OF ANTIGENS
This exclusion occurs through secretory immunoglobulin A (SlgA) and immunoglobulin M (SlgM) antibodies to modulate or inhibit colonization of bacteria and yeast and dampen penetration by dangerous soluble luminal agents. Maternally acquired immunity is essential for the survival of newborns until endogenous immunity develops. These exogenous antibodies are acquired both prenatally through transplacental transfer and postnatally via breast-feeding and colostrum.1 In fact, when breast-fed infants were compared with formula-fed babies, a more rapid increase in SlgA1, SlgA2, and total salivary immunoglobulin A (IgA) was observed during the first 6 months.2 Further, breast-fed infants also produced higher levels of SlgA in urine than did formula-fed infants.

Therefore, the importance of infant-feeding practices cannot be underestimated, because a significant association exists between feeding patterns, bacterial colonization, and...
immunological maturation, particularly for IgA- and immunoglobulin M (IgM)-containing plasma cells in the gut lamina propria, a component of the mucosa. For this reason, intravenously-fed, fully developed infants lack these IgA- and IgM-producing plasma cells in their gut tissues, whereas orally fed infants have adult proportions of those immunocytes. Further, the initial bacterial colonization and subsequent antigenic challenge in the GI tract differ between breast-fed and formula-fed infants.

In addition to providing SlgA and SlgM, breast milk reinforces mucosal defenses by delivering antigens, immune complexes, regulatory cytokines, growth factors, and prebiotics, such as oligosaccharides, that promote the proliferation of friendly bacteria, which are part of the neonatal intestinal microbiota. Transfer could be an explanation for the protective role that breast-feeding plays in preventing the development of inflammatory bowel disease later in life. This protection emphasizes the impact of perinatal immune development, particularly of IgA and IgM antibodies, on mucosal homeostasis and chronic inflammation.

Maron et al demonstrated that oral administration of insulin induced efficient tolerance and protection from type 1 diabetes in nonobese diabetic neonates. Indirect administration of an oral antigen, ovalbumin (OVA) from hen eggs to neonatal mice through maternal milk was assessed for induction of oral tolerance. By day 7, tolerance to OVA had been induced, but the amounts of OVA required for the induction in that time frame were approximately 1000 times lower than those needed when the pups were force-fed instead. It has also been shown that the transfer of an antigen that was mediated by breast milk could prevent antigen-specific immune responses and development of allergic disease in rodents.

The possible induction of tolerance to antigens present in breast milk is clearly illustrated by the tolerance that develops from a child’s exposure to cells and molecules of soluble human leukocyte antigen (HLA) through the mother during pregnancy and through breast-feeding. The antigens that the child does not inherit genetically from the mother are called noninherited, maternal, HLA antigens (NIMA). Studies with rodents have shown that transfer of these HLA antigens from the mother to the pup in utero and through breast milk improves the acceptance of heart, skin, or bone marrow, semiallogeneic transplants that express NIMA.

In the case of a bone marrow transplant, the transfer of HLA antigen through breast milk was sufficient to prevent allogeneic reactions. When much of the maternal IgG received at birth has been catabolized at around 2 to 3 months of age, the infant becomes dependent on antibodies in breast milk. The IgA-producing immunocytes are detected in human intestinal mucosa at around 10 days of age, followed by a rapid increase of IgM immunocytes for up to 1 month. It is important to note that by 3 months after birth IgA1 to IgA2 immunocytes reach a ratio of 2:1 in the salivary glands, which is equivalent to an adult’s value. This increase in IgA1 and IgA2 in salivary glands almost parallels the catabolization of transferred maternal IgG, as if an exchange of guards has occurred, in the progression from transferred IgG and IgA to the adaptive immune defense. This gradual development of the mucosal immune system, in particular SlgA, from prenatal to adult concentrations is shown in Figure 1.

This secretory IgA serves as the first line of defense against various microorganisms through a mechanism called immune exclusion. This consists of the SlgA coating the bacterial invader, thereby neutralizing its activity. The neutralization of bacterial antigens by SlgA results in the blocking of bacterial attachment to mucosal glycolipids and glycoprotein receptors, preventing the entrance of bacteria into the submucosae. In the context of immune homeostasis, both maternal and newly synthesized SlgA, free or in the form of immune complexes, can be seen as part of an arsenal that shapes the GI immune system, both in terms of an immune exclusion defense and of immune tolerance induction, during initial exposure to nonself bacterial and food antigenic structures.

For many years, it has been acknowledged that oral feeding of proteins abolishes the subsequent responses to the same antigen after its systematic administration. This phenomenon is referred to as oral tolerance and has been well characterized in animal models and, to some degree, in humans. It would appear that SlgA-based immune complex formation with an orally fed antigen contributes early in life to the education of the mucosal immune system toward a tolerogenic profile. SlgA antibodies in maternal milk that are bound to innocuous antigens pass through the epithelium to activate dendritic cells (DCs) and to prime the CD4+ cells for the establishment of tolerance, either by helping them evolve to the regulatory T-cell phenotype or by deleting them. In the next section, this article discusses the mechanism of the induction of oral tolerance and its breakdown by various environmental triggers in relation to food immune reactivities.

TOLERANCE AND SUPPRESSIVE MECHANISMS

Development of oral tolerance or suppressive mechanisms allows an individual first to avoid local immune reactivity and, thereafter, to prevent peripheral reactivity against a variety of microbial and dietary antigens. Oral tolerance, which was characterized by Chase in 1946, refers to active inhibition of immune responses (ie, a lack of immunoreactivity) to an antigen by means of prior exposure to the same antigen through the mucosal surfaces. Tolerance occurs through cell deletion or an immune suppression mechanism.

In 1968, while the author was doing research toward his master’s degree, he was amazed by the results of his experiments performed with fish as a model. When fish were injected intramuscularly with 1 mg of antigen on days 1 and 25, a strong immune response to the antigen occurred, and high levels of IgM antibodies were detected on day 35 (Figure 2). When fish were first injected on day 1 in the gills (ie, the mucosae of the fish) with a low dose of antigen (50 μg) and then immunized intramuscularly on day 25 with 1 mg of...
antigen, no antibodies were detected in the blood drawn on day 35 (Figure 3). When fish were first injected on day 1 in the gills with a high dose of antigen (2 g) and then immunized intramuscularly on day 25 with 25 mg of antigen, no antibodies were detected in the blood drawn on day 35 (Figure 3).

Similar results were obtained when mice were first fed an antigen orally and then injected subcutaneously. These results showed that mucosal exposure to a low-dose antigen, followed by an immunogenic dose, resulted in active inhibition of the immune response to the specific antigen.17 Later, in additional experiments with the same fish that had developed a tolerance to an antigen, the author showed that the tolerance induction could be broken, first by injecting a very high dose of the same antigen, such as 1 g, and then by injecting an immunogenic dose of 1 µg of the antigen. Any disturbance in the path to oral tolerance can result in immune reactivity to food and autoimmunity.16,18

The generation of the immune response occurs in 2 forms through costimulatory signals provided either by (1) soluble cytokines, such as interleukin 2 (IL-2); or (2) interaction between costimulatory receptors on T cells, CD28, and counter-receptors on antigen-presenting cells (APCs), CD80, and CD86, as shown in Figure 4. Oral tolerance can be induced in animal models after administration of a single high dose, such as 100 mg per mouse, or repeated lower doses of 1 mg or less per mouse.19,20 The 2 forms of tolerance are mediated by 2 corresponding mechanisms: (1) high doses of antigen—signals provided by soluble cytokines, and (2) low doses of antigen—signals provided by interactions between costimulatory receptors and counter-receptors.
Oral administration of a high dose of antigen, such as food, can induce lymphocyte deletion or anergy, which is the opposite of allergy. In the absence of costimulatory signals, high-dose tolerance is mediated by lymphocyte anergy. This anergy can occur through T-cell receptor ligation with the major histocompatibility complex peptide or through FAS-mediated cell death or apoptosis, as shown in Figure 5.

In comparison with high-dose tolerance and the involvement of FAS-mediated cell death, low-dose tolerance is mediated by regulatory T (T \(_{\text{REG}}\)) cells. These cells can be divided into 3 subgroups of CD4\(^+\) cells: T-helper 3 (T\(_{\text{H}3}\)) cells, type 1 regulatory (T\(_{\text{R1}}\)) cells, and CD4\(^+\)CD25\(^+\) cells, as shown in Figure 6. In addition to T\(_{\text{REG}}\) cells, CD8\(^+\) suppressor T cells play a role in the local suppression of immune responses. This process of immune suppression by CD8\(^+\) seems to be regulated by intestinal epithelial cells through a membrane glycoprotein (GP180) on the epithelial cells with CD1d and CD8 ligands.

Oral tolerance to dietary proteins is crucial to prevention of the development of food immune reactivity. The mode of antigen uptake in the gut and of different regulatory immune suppressors is crucial to prevention of the development of food immune reactivity.
Table 1. Factors Involved in Induction of and Disturbance in Oral Tolerance

- Genetics of the host
- Maternal exposure to xenobiotics
- Mother’s diet
- Manner of birth (ie, normal birth vs cesarean)
- Method of feeding (ie, breast-feeding vs baby formula)
- Baby formula vs protein hydrolysate formula
- Infant’s gut microbiota and its source
- Time of introduction of solid food (ie, exposure to food proteins after weaning)
- Integrity of digestive enzymes
- Use of drugs or medications

cells plays a role in its maintenance. In addition to intestinal epithelial cells acting as nonprofessional APCs, DCs, and CD8+ cells, the previously mentioned subgroups of regulatory CD4+ cells, namely T regulate, T suppressor, and CD4+CD25+ cells, also play an important role in maintaining oral tolerance to low doses of antigen through suppression of immune responses. Other mechanisms are important in response to high antigen doses, including induction of lymphocyte anergy or deletion.

This induction of oral tolerance to soluble antigens is not limited to the intestinal mucosa but can involve the entire body. The explanation is that an antigen can gain access to the blood through oral exposure via the lymphatic system. Indeed, food protein can be detected in the blood of mice and humans soon after eating. This entry of undegraded food proteins into the circulation at low levels is a normal process, but in the presence of inactive enzymes or resistance of some dietary proteins to degradation, the level of dietary proteins in the blood is enhanced.

Of course, this presence of food antigen in the blood does not go unnoticed by the immune system. If the antigen is taken up by the APCs in the blood, the result could be production of IgG or IgA antibodies. But if the liver sinusoidal endothelial cells efficiently sample these circulating antigens and act as APCs, then these cells induce tolerance rather than active immunity to the circulating dietary antigens.

Oral tolerance to these antigens can also be mediated by the liver-associated lymphocytes carrying the natural killer marker (NK1.1). Overall, in the absence of costimulation, antigen presentation by the DCs of the liver—Kupffer cells—with the help of the NK1.1 cell, favors tolerance over immunity. If the antigens reach beyond the liver into the peripheral lymph nodes and spleen, when they are presented by the resident DCs in the absence of costimulation, the result can be anergy through activation of T regulate cells. Overall, the concentration of antigens reaching the circulation is a major factor in the development of oral tolerance or active immunity. Any disturbance in the path to oral tolerance through mucosal tissue, liver, or spleen can result in food immune reactivity and autoimmunity.

Induction

Oral tolerance is induced by multiple cellular and molecular processes that ensure lack of immune reactivity to harmless, intestinaly derived antigens, both in the mucosa and in the systemic immune system. Together, tolerance induced mucosally and in a circulatory manner appears to prevent intestinal disorders, such as inflammatory bowel disease, food immune reactivity, and organ-specific and nonspecific autoimmunities.

This process is performed by a very special population of DCs found in the microenvironment of mesenteric lymph nodes. The presence of antigen-specific T cells and nodes and cytokines, such as transforming growth factor beta (TGF-β) and interleukin 10 (IL-10), induce the generation and differentiation of these DCs into forkhead box P3+ (FOXP3+) regulatory T cells. These committed T regulate cells move back to the intestinal lamina propria, where some of them may exit from the mucosa via the lymphatic system or blood stream and disseminate throughout the immune system, promoting systemic oral tolerance. The ability of oral tolerance to maintain an inhibitory environment through the T regulate cells and the production of noninflammatory IgA against both dietary proteins and microbiota both can prevent hyperimmune reactivities in the mucosa and circulation.

The perinatal period is crucial for the establishment of oral tolerance or the induction of food immune reactivities.

Disturbance

These reactivities can result from many environmental factors that can disturb the homeostasis of the immune system, resulting in the penetration of dietary proteins and nontolerogenic peptides to the submucosa. To avoid immune reactivity to food antigens, the body employs inflammatory immune defenses, including SIgA antibodies and hyporesponsiveness to innocuous agents, particularly dietary antigens and the commensal gut microbiota. The induction of these homeostatic mechanisms depends on exogenous stimuli, and the neonatal period is particularly critical to this end. Both the intestinal surface barrier with its reinforcement by SIgA and the immunoregulatory network require adaptation.

In most cases, this adaptation is remarkably successful in view of the fact that a ton of food, perhaps including 100 kg of proteins, can pass through the gut of an adult human being every year without causing adverse reactions. Food immune reactivity reflects a lack of such homeostasis, either due to retarded immunological development with immaturity of the intestinal surface barrier or a persistently imbalanced immunoregulatory network.
Both homeostatic deficiencies may be associated with immune reactivity, in particular IgA and IgG production against innocuous antigens, such as food proteins. The mechanism of oral tolerance to food antigens and microbiota is shown in Figure 7.

Many variables influence the induction of oral tolerance and productive, SIgA-dependent, mucosal immunity. Some of these variables are reciprocally modulated to achieve mucosal immune homeostasis. Increased epithelial permeability for exogenous antigens is clearly an important primary or secondary event in the pathogenesis of many diseases, inducing food allergy. Postnatal epithelial barrier function is determined by a newborn’s age (eg, preterm versus full-term); genetics; mucus composition; interactions between mast cells, nerves, and neuropeptides; concurrent infections; and the mucosa-shielding effect of SIgA provided by breast milk or produced in the infant’s gut. Further, the integrity of the intestinal epithelium depends on homeostatic mechanisms, such as the induction of T(REG) cells (Figure 8).

The incidence of food immune reactivities is suggested to increase development of the IgA system. An underlying deficiency of antigen-specific SIgA has been proposed in a mouse model of food allergy. This finding implies that secretory antibodies are involved in the induction of oral tolerance. Another experimental model investigated the relationship between oral tolerance and hypersensitivity in the presence of a defective intestinal-surface barrier due to a deficiency of SIgA/SIgM. This deficiency can lead to systemic hyperreactivity and production of both IgG and IgA antibodies against various undigested food proteins and peptides that manage to penetrate the barriers.

**THE IMPORTANCE OF REESTABLISHING ORAL TOLERANCE**

The induction of IgG and IgA antibodies to the actual food antigen, and even cross-priming against a bystander antigen, may be of biological significance. Experimental studies both in vitro and in vivo have demonstrated that IgG antibodies, when they are not balanced by a mucosal IgA response, can enhance the epithelial penetration of bystander proteins. Penetration of epithelial cells by bacterial toxins and various food antigens can result in many immune disorders, including autoimmunities. For this reason, significant progress has been made with oral immunotherapy or oral desensitization toward an active therapy for food immune reactivities. The reestablishment of
oral tolerance by oral administration of a food antigen, together with strategies for repairing the gut barriers, are novel therapeutic approaches for targeting mucosal immune responses in patients with food immune reactivities and autoimmunities.

The method of restoring tolerance or adjusting the immune response can be oral immunotherapy or sublingual immunotherapy (SLIT). Itoh et al conducted a study using a rush program of specific oral tolerance induction (SOTI) on 5 children, 7 to 12 years of age, who were confirmed to be suffering from severe, IgE-mediated egg allergy. Rather than following the usual allergen avoidance strategy, the study by Itoh et al had the children ingest increasing doses of egg several times every day. In an average of only 12 days, with a range of 9 to 18 days, all children acquired tolerance to 60 g of egg, or more than 1 whole egg. After the rush SOTI, the patients ingested a maintenance dose of more than 1 heated whole egg at least twice a week. The study lasted for 1 year, and all children had been able to ingest more than 1 whole egg at that point. The researchers concluded that a rush SOTI was a safe and effective method that could replace allergen avoidance as treatment for severe food allergy.

Abbreviations: IgA, immunoglobulin A; SlgA, secretory IgA; LPS, lipopolysaccharide.
The introduction of very low doses of the triggering antigens by SLIT can also modulate the GI mucosal immune response, with the goal of promoting oral tolerance, as shown in Figure 9. SLIT involves the use of a liquid concentrate administered under the tongue. In a study of SLIT for hazelnut allergy, 23 patients received either hazelnut extract or a placebo for 8 to 12 weeks. In the subsequent food challenge, the mean quantity of hazelnut that provoked symptoms increased from baseline by 9 g in the hazelnut group versus 0.6 g in the placebo group. Participants in the hazelnut group also experienced increases in IgG4 and IL-10, although none in the placebo group did. After further analysis, it appeared that the majority of the patients who benefited from SLIT did not have type 1, IgE-mediated allergy but rather had the oral allergy syndrome/pollen food syndrome. This syndrome is characterized by oral itching, tingling, and occasional swelling of the oropharynx in pollen-allergic individuals, after consumption of fresh fruits and vegetables containing cross-reactive proteins. SLIT has also been used in a single case for treatment of life-threatening kiwi allergy.

In fact, evidence exists that this tolerance can also be developed by introducing the triggering antigen nasally rather than sublingually. Induction of long-term tolerance to β-cell autoantigens has been investigated both in animal models and in human type 1 diabetes to prevent the disease. Funda et al investigated whether intranasal administration of gliadin, an environmental antigen with possible etiological influence in type 1 diabetes, or gluten could arrest the diabetogenic
process. Intranasal administration of gliadin to 4-week-old, nonobese, diabetic (NOD) mice significantly reduced the diabetes incidence and lowered the insulins. In comparison with OVA-treated controls, intranasal gliadin also rescued a fraction of prediabetic, 13-week-old NOD mice from progressing to clinical onset of diabetes. Vaccination with intranasal gliadin led to an induction of CD4+FOXP3+ T cells and, even more significantly, induction of γδ T cells in mucosal lymphoid compartments, but not in nonmucosal ones. This prevention strategy was characterized by an increased proportion of IL-10 and a decreased proportion of IL-2, interleukin 4 (IL-4) and interferon-gamma-positive (IFN-γ-positive) CD4+FOXP3+ T cells, and IFN-γ-positive γδ T cells, preferentially in mucosal lymphoid organs.69 Intranasal vaccination with gliadin may represent a novel, safer strategy for prevention or even early cure of type 1 diabetes.

Likewise, Tian et al50 demonstrated that intranasal administration of glutamic acid decarboxylase 65 (GAD-65) could prevent murine, insulin-dependent diabetes in NOD mice. A single intranasal administration of GAD peptides to NOD mice that were 2 to 3 weeks old induced high levels of IgG1 antibodies to GAD-65. Mice treated with GAD-65 displayed greatly reduced IFN-γ responses and increased interleukin 5 (IL-5) responses to GAD-65, confirming the diversion of the response toward a T\(_{H1}\)2 phenotype. Consistent with the induction of an active tolerance mechanism, splenic CD4+ T cells, but not CD8+ cells, from mice treated with the GAD-65 peptide inhibited the adoptive transfer of insulin-dependent diabetes mellitus to NOD-severe combined immunodeficiency/severe combined immunodeficiency mice. This transfer not only inhibited the development of proliferative T-cell responses to GAD-65, but it also limited the expansion of autoreactive T-cell responses to other β-cell antigens. Finally, the treatment reduced insulitis and long-term incidence of insulin-dependent diabetes mellitus. These data suggest that the nasal administration of GAD-65 peptides can induce a T\(_{H1}\)2-cell response that inhibits the spontaneous development of autoreactive T\(_{H1}\)1 responses and the progression of β-cell autoimmunity in NOD mice.50

It is interesting to note that gliadin has been shown to be cross-reactive to GAD-65\(^{12\text{,}51}\) and that the information collected in this article shows that intranasal administration of either substance can be effective in reestablishing oral tolerance and either preventing or curing type 1 diabetes and other autoimmune diseases in which food antigens play a role.

CONCLUSION

The gut’s mucosal immune system has to maintain an intricate immune homeostasis by maintaining tolerance to harmless or even beneficial molecules in the gut while mounting an effective immune defense against pathogens.52 Unresponsiveness to food antigens with subsequent down-regulation of the systemic immune response is characterized as oral tolerance. The failure of this system results in immune reactivities to the foods that humans eat, sometimes with life-threatening consequences, such as allergies and autoimmunities.52 Revolutionary developments in the fields of mucosal immunology and microbiology of the gut in the last few years are the best indication of the importance of commensal flora, gut barriers, and oral tolerance to human health and disease. Exact identification of the different mechanisms of action that separate tolerance from effective immunity against various food and bacterial antigens is the subject of ongoing research at many academic institutions.

When these different mechanisms of action fail to control ingested antigens, the result can be a breakdown in tolerance to soluble antigens, triggering active secretory and systemic immune responses against food antigens. Indeed, individuals in whom the immune exclusion mechanism does not function may experience chronic hyperabsorption of macromolecules and the tendency to develop autoantibodies and even autoimmune disease.53

The current challenge is to further understand the mechanisms responsible for restoration of natural or induced tolerance so that interventions can be developed to induce tolerance more successfully in the majority of patients with food immune reactivity and autoimmunity.54 Indeed, people have already successfully lowered antibody titers and cell-mediated immunity with oral tolerance therapy in animal models of autoimmune and inflammatory diseases. These successes include therapies for experimental autoimmune encephalitis, uveitis, thyroiditis, myasthenia gravis, arthritis, diabetes, experimental colitis, as well as for graft-versus host disease, allergy, antiphospholipid syndrome, asthma, stroke, and atherosclerosis.55 Several clinical trials have been conducted in multiple sclerosis, uveitis, thyroid disease, Crohn’s disease, rheumatoid arthritis, hepatitis, and diabetes.56-58 Based on this research, the reestablishment of oral tolerance in human autoimmune disease by the administration of self or cross-reactive food antigens, either nasally or orally, can help to improve the quality of life of patients with autoimmune disease.

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Molecular Mimicry as a Mechanism for Food Immune Reactivities and Autoimmunity

Aristo Vojdani, PhD, MSc, CLS

ABSTRACT

The mucosal immune system is constantly exposed to challenges from the antigenic substances found in food and released from the body's own microbial flora. The body's normal tolerance to friendly antigenic substances can be disrupted by a number of factors, such as disease, injury, shock, trauma, surgery, drugs, blood transfusion, environmental triggers, etc. When this disruption happens, the ingestion of foods containing antigenic substances that have compositions similar to those of the body's autoantigens can result in the production of antibodies that react not only against the food antigens but also the body's own tissues. This response is known as food autoimmune reactivity. Between 7% and 10% of the world's population suffers from some form of autoimmune disease. Each patient's antibodies, both immunoglobulin A (IgA) + immunoglobulin M (IgM) in the saliva and immunoglobulin G (IgG) and IgA in the blood must be examined to give a complete picture of food immune reactivity. A host of health problems and autoimmune disorders have increasingly become associated with some of the most commonly consumed foods in the world, such as wheat and milk. Many of these problems can be traced to molecular mimicry. The peptide sequences of foods such as milk and wheat are similar to those of human molecules, such as myelin oligodendrocyte glycoprotein, human islet cell tissue, and human aquaporin 4 (AQP4). This similarity can result in cross-reactivity that leads to food autoimmunity and even autoimmune disorders, such as multiple sclerosis (MS), celiac disease (CD), and neuromyelitis optica. Further research is needed to determine what other foods have dangerous sequence similarities to human tissues and what methods are available to test for the autoantibodies resulting from these molecular, mimicry-induced misfires of the immune system. The identification and removal of corresponding food triggers can then be used as the basis of therapy. (Altern Ther Health Med. 2015;21(suppl 1):34-45.)

Aristo Vojdani, PhD, MSc, CLS, is the CEO of Immunosciences Labs, Inc, in Los Angeles, California, and a faculty member in the Department of Preventive Medicine at Loma Linda University in Loma Linda, California.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com

The mucosal immune system is different from the systemic immune system in that mucosal cells are constantly exposed to antigenic substances found in food or released from endogenous microbial flora. Therefore, the mucosal system must have a means of regulating responses to these substances to avoid harmful reactions to common mucosal antigens. This disregard of antigenic stimuli is maintained through the induction by the mucosal immune system of immunologic ignorance (oral tolerance) with respect to dietary proteins and commensal bacteria.1,2

Many studies have indicated that site-restricted, secretory, immunoglobulin A (IgA) and immunoglobulin M (IgM) responses can be induced either by the application of antigens to mucosal surfaces or ingestion of antigens. This fact means that in the absence of oral tolerance, ingestion of antigens results in the production of antibodies that react not only against the food antigens but also the body's own tissues. This response is known as food autoimmune reactivity. Between 7% and 10% of the world's population suffers from some form of autoimmune disease. Each patient's antibodies, both immunoglobulin A (IgA) + immunoglobulin M (IgM) in the saliva and immunoglobulin G (IgG) and IgA in the blood must be examined to give a complete picture of food immune reactivity. A host of health problems and autoimmune disorders have increasingly become associated with some of the most commonly consumed foods in the world, such as wheat and milk. Many of these problems can be traced to molecular mimicry. The peptide sequences of foods such as milk and wheat are similar to those of human molecules, such as myelin oligodendrocyte glycoprotein, human islet cell tissue, and human aquaporin 4 (AQP4). This similarity can result in cross-reactivity that leads to food autoimmunity and even autoimmune disorders, such as multiple sclerosis (MS), celiac disease (CD), and neuromyelitis optica. Further research is needed to determine what other foods have dangerous sequence similarities to human tissues and what methods are available to test for the autoantibodies resulting from these molecular, mimicry-induced misfires of the immune system. The identification and removal of corresponding food triggers can then be used as the basis of therapy. (Altern Ther Health Med. 2015;21(suppl 1):34-45.)
A different mechanism for the production of IgA antibodies in blood is the spillover from increased mucosal IgA production. This effect is well established in patients with celiac disease (CD), where the number of jejunal IgA immunocytes and the level of IgA gliadin antibodies in saliva correlate with circulating IgA gliadin antibodies. Conceivably, an intestinal immune reaction involving IgA immune complexes and proinflammatory cytokines may lead to enhanced intestinal permeability, increased antigen exposition, and intensified production of IgA and IgG activities in serum.8-10

After repeated exposure of mucosal immune cells to dietary proteins and the production of IgA + IgM in the mucosal secretions, these mucosal antibodies then interact with many dietary protein antigens, resulting in immune complex formation that further contributes to the inflammatory reaction in the gastrointestinal (GI) tract, enhanced intestinal permeability, and further production of IgG and IgA in the blood.9

Because the mucosal immune system is a central component of host defense, any dysregulation and inflammatory reaction in the GI tissue as a whole results in intestinal barrier dysfunction and the entry of undigested dietary proteins into the circulation.9,10 The proposed mechanism by which various factors induce intestinal permeability and antigen entry into the circulation is shown in Figure 1.

The entry of dietary proteins into the circulation results in a systemic immune response and the production of very high levels of IgG and IgA against dietary proteins and peptides. This systemic immune reaction depends on the antigenic structure of the protein antigen, particulate antigens, polysaccharides, lipoproteins, or enzymes; molecular size of the protein; and genetic makeup of the exposed individual. One individual may produce IgG antibodies against dietary proteins and peptides, whereas another may produce IgA or IgM antibodies.

This breach of the intestinal barrier by dietary proteins11,12 due to loss of tolerance can lead not only to IgG and IgA production in the blood but also to an immune response to different target organs and the induction of autoimmune diseases.13,14

Therefore, to obtain a complete picture of food immune reactivity, the author proposes that both IgA + IgM antibodies in saliva and IgG and IgA in blood should be examined in patients before therapeutic interventions begin. This comprehensive approach was developed as a result of the author’s 25 years of research experience. He determined that diseases of the GI tract
and autoimmune diseases cannot be fully understood and treated without examination of the coordination of the mucosal and systemic immune responses against dietary proteins and peptides (ie, IgA + IgM in the saliva) and IgG and IgA in the blood against different raw and modified food antigens.

Progress in the field of mucosal immunology has provided intriguing clues to the role of diet and the microbiota in creating risk factors for the development of food immune reactivities and autoimmunity. Detection of very high levels of IgG and IgA in blood against dietary proteins and peptides can help clinicians evaluate their patients for immune-tolerance inflammation and autoimmunity. They can use this information to design new therapeutic strategies that may include elimination diets, re-establishment of tolerance in the intestinal barrier function, and the use of prebiotics, probiotics, glutamine, glutathione, lipoic acid, and other nutritional supplements or medication with anti-inflammatory characteristics.

FOOD IMMUNE REACTIVITY AND AUTOIMMUNE RESPONSE

According to the American Autoimmune Related Diseases Association, 52 million Americans (17%) and between 490 and 700 million people worldwide (7%-10%) suffer from some form of autoimmune disease. Autoimmunity arises when the host’s immune system is directed against self-tissue antigens. Accumulating evidence has suggested a close interplay between genetic factors (30%) and environmental triggers (70%), such as infections, toxicants, and some dietary components in the pathogenesis of autoimmune diseases. Consequently, since 1997, research and publications devoted to environmental triggers in autoimmune diseases have grown by an average of 7% each year.

For this reason, significant progress has been made during recent decades in the search for peptides in food antigens that share a similarity with autoantigens that are involved in autoimmune diseases. The protein peptide in the gluten-rich cell wall represents an example of an antigenic peptide sequence that can start T- and B-cell immune response causing completely different and unrelated diseases, such as psoriatic arthritis, chronic idiopathic urticaria, and food allergy. This finding of a common peptide epitope—the part of an antigen that is recognized by the immune system—that is able to elicit an immune response in patients with food immune reactivities and different autoimmune disorders gives rise to the question of possible links between food antigens, gut mucosa, and systemic immune response.

This autoimmune response is done by T-cell clones specific to particular food antigen epitopes that may arise in the gut mucosa and be recruited to particular sites, such as joints, where they proliferate in response to homologous peptides derived from synovial proteins. Following local inflammation and upregulation of major histocompatibility complex (MHC) molecules, the release of additional self-antigens and/or epitope spreading can lead to a chronic self-perpetuating process of organ inflammation and destruction resulting in autoimmunity. Considering the variety and abundance of food antigens consumed by individuals, this pathological mechanism is very well-studied only for a few food proteins and peptides.

Autoimmunity Associated With Wheat Proteins

The discussion about food immune reactivity, in particular reactivity to wheat and milk, and associated health problems has grown in the past decades. A number of gluten peptides with a capacity to stimulate intestinal T-helper cells have been identified in patients with CD by many researchers. In a recent study, T cells isolated from CD patients were screened for recognition of 21 different peptides, from α-, γ-, and ω-gliadins to glutenins. It was demonstrated that intestinal T cells from CD patients responded to a wide and heterogeneous array of peptides. In some patients, many peptides from the α-gliadin family were recognized, whereas in others, only 1 peptide caused lymphocyte stimulation and interferon production. Further, in a very recent study, the author showed that patients with nonceliac gluten sensitivity (NCGS) and Crohn’s disease reacted to the repertoire of wheat antigens and produced IgG or IgA against them. These antigens include various families: α-, γ-, and ω-gliadins; glutenins; glutemorphins; and wheat germ agglutinin. Continuous exposure to environmental factors, such as wheat, not only causes NCGS and CD but also can result in inflammation and autoimmunities if left untreated.

The endocrinologist in particular should maintain a high suspicion of and alertness to CD and NCGS, which are found in a significant percentage of patients with insulin-dependent diabetes mellitus or autoimmune thyroid disease. Patients with multiple endocrine disorders, Addison’s disease, alopecia, or hypophysitis may also have concomitant CD or NCGS.

CD has been found in 4% to 8% of women with unexplained infertility. In some case reports, successful treatment of infertility occurred after the diagnosis and dietary treatment of CD; some women were able to successfully become pregnant after implementation of a gluten-free diet. Menarche takes place later and menopause earlier in celiac women (ie, the fertility period is shortened) and celiac women on a normal diet suffer from spontaneous abortions and other complications of pregnancy more often than those maintaining a gluten-free diet. This infertility induction by gluten in CD and NCGS is not unique to women but also can affect the male gonadal function and sex hormones, resulting in infertility in men. This effect could be due to antigenic cross-reactivity between wheat protein or peptide antibodies and the human endocrine-tissue antigen.

Indeed, very recently the author showed that monoclonal and polyclonal antibodies made against only 1 of the gluten peptides (α-gliadin 33-mer) could result in antibody reactivity against hepatocyte cytochrome P450,
glutamic acid decarboxylase (GAD), collagen, asialoganglioside, myelin basic protein (MBP), cerebellar, and synapsin. Another study showed that gliadin peptides carrying the QQPPFP epitope interacted directly with actin or smooth muscle, leading to rearrangement of the actin cytoskeleton, with possible autoimmune reactivity against actin and the gliadin peptide. Osteopenia and osteoporosis in the peripheral skeleton are well-known complications of CD and NCGS. This osteopenia can result from the fact that most CD patients have circulating antibodies against wheat proteins that react against bone structures. The bone transglutaminase (tTG) and other bone antigens may be some of the autoantigens involved in CD or NCGS sera immunoactivity.

CD has been shown to be associated with autoimmune myocarditis. Active CD is accompanied by consistent production of IgA autoantibodies to reticulin, a common constituent of the extracellular matrix. Serum IgA antibodies of patients with untreated CD have been reported to react strongly against human brain-blood-vessel structures, and this mechanism has been hypothesized to be involved in the abnormal nervous system manifestations frequently described in association with CD. Recent studies have demonstrated that antigliadin autoantibodies react with common epitopes on gliadin, calreticulin, and enterocytes and with a nuclear autoantigen expressed in intestinal endothelial cells and in fibroblasts.

On the other hand, tTG, recognized as the targeted antigen of CD-specific autoantibodies, is an intracellular enzyme that is distributed in the cells of all organs. A possible link between tTG and cardiac damage, as well as an upregulation of messenger RNA for tTG in rat models of cardiac failure, have been reported. These findings have led the author to hypothesize that antigenic mimicry could actually be involved in the pathogenesis of CD-associated disorders. In patients with autoimmune myocarditis, the author was able to detect an autoimmune process against cardiac antigens that could play a key role in the pathogenesis of inflammatory heart damage. The evidence that improvement of cardiac function and of ventricular arrhythmias was paralleled by the disappearance of antiendomysial antibodies and tTG in the serum supports this hypothesis.

In 3 patients who were first diagnosed with systemic lupus erythematosus, based on an immunological profile of positive antinuclear antibodies and double-stranded DNA, the correct diagnosis of gluten sensitivity, based on molecular mimicry, was made 6 months after the introduction of a gluten-free diet and the subsequent resolution of lupus symptomatologies.

Dermatitis herpetiformis (DH) is another autoimmune disorder associated with antibodies made against gluten and transglutaminase 3 (tTG-3) (ie, epidermal tTG) attacking the skin. Why does gluten sensitivity present as CD in some patients and as DH in others? Studies indicate that in patients with DH, tTG-3 appears to be the target autoantigen. Antibodies in patients having DH show a markedly higher avidity for epidermal tTG. Further, these patients have an antibody population specific for this enzyme. The IgA precipitates in the papillary dermis of patients with DH have been found to contain tTG-3.

Finally, a range of neurological complications has been described in association with CD and NCGS. Gluten ataxia is one of the most common neurological manifestations of gluten sensitivity. Serological markers for the disease are gliadin IgG and IgA antibodies, tTG-6 antibody, cerebellar antibody, and GAD-65 antibody. Gluten ataxia usually presents in combination with myoclonus, palatal tremor, and opsoclonus, which improve on a gluten-free diet. Gluten or peripheral neuropathy, which appears to be present in approximately 25% of patients with CD, can be induced by the gluten antibody. The common antibodies detected in these patients are gliadin, tTG, and asialoganglioside antibodies. For multiple sclerosis (MS), some evidence has been presented regarding an increase in the prevalence of the antigliadin and anti-MBP antibodies. Neuromyelitis optica is an additional clinical syndrome characterized by acute transverse myelitis plus an acute or subacute optic neuritis. In these patients, antibodies against neural antigens, in particular water channel or aquaporin 4 (AQP4), are detected. In 2 cases of patients with gluten sensitivity, neuromyelitis optica was confirmed based on immunological and histological examinations.

Considering the degree of cross-reactivity shown in the author’s lab between gliadin and at least 5 different neuronal antigens, such as MBP, asialoganglioside, cerebellar, synapsin, and GAD-65, medical practitioners should not be surprised that so many autoimmune reactivities target the nervous system and other tissues. The spectrum of autoimmunity and its association with proteins of only 1 food item, wheat, are shown in Figure 2.

The cross-reaction of gliadin antibodies with a variety of tissue antigens may explain why immune reactivity could occur at a specific site that is distant from the digestive system. This effect may not be unique to gluten but may also occur with many other food antigens that have yet to be thoroughly studied. Untreated patients typically have circulating IgG and, in particular, IgA antibodies to gliadin peptides and tTG-2, tTG-3, or tTG-6. Detection of IgG-class antibodies against gliadin has little clinical value for CD, but it is very important for differentiation between CD and NCGS.

As shown in Figure 2, CD and NCGS exhibit extraintestinal manifestations in almost every organ of the body, particularly in the brain. Therefore, detection of IgG-type antibodies against wheat proteomes and their possible cross-reaction with various human tissue antigens could be crucial for the early detection of autoimmune reactivities associated with consumption of wheat and other cereals. For example, in gluten ataxia, IgG-class antibodies to tTG-2, tTG-6, and cerebellar peptides are more common than are IgA.

These findings are in line with data that have provided evidence for intrathecal antibody production against tTG-2,
tTG-6, and cerebellar in patients with neurological diseases. The high prevalence of IgG-class antibodies to tTG-2 and tTG-6 in these patients is consistent with an immune response in the central nervous system (CNS). Antibodies against tTG-2, tTG-6, or both can be found in 85% of patients with ataxia and antigliadin antibodies. Some patients also test positive for anti-tTG-3 antibodies, although the frequency of such antibodies is low when compared with patients who have dermatitis herpetiformis.90,91

Based on antibody detection, the removal of immunological triggers (ie, food antigens) must be the basis of treatment of all manifestations and should be recommended for all patients.84

**Immune Reactivity and Autoimmunity Associated With Milk Proteins**

Nutritionists through the years have seemed to agree that milk is one of the most basic necessities of the healthy diet. Unfortunately, cow's milk proteins are the most common food allergens affecting young infants and some children and adults.92,93 Major allergenic proteins of milk are α-casein, β-casein, κ-casein, and β-lactoglobulin. In addition to IgE-mediated allergy to cow's milk, awareness is increasing that early consumption of cow's milk may also present a risk for the development of autoimmune diseases, such as CD, Crohn's disease, Behçet's disease, MS, mild rheumatoid arthritis in rabbits, systemic lupus erythematosus, uveitis, and type 1 diabetes in humans,94-104 as shown in Figure 3.

These findings are supported by the detection of significantly higher levels of IgG and IgA antibodies in disease sufferers compared with normal controls.96-104 These studies concluded that active immune responses against cow's milk proteins play a putative role in the pathogenesis of autoimmune disorders.95-107 In relation to type 1 diabetes, substantial evidence has been accumulated on the possible association between the disease and consumption of cow's milk.96-99 Exposure to cow's milk proteins may prime the immune system to recognize and react to islet-cell antigens that possess sequence homology to milk proteins. This
phenomenon of similarity between milk-protein and islet-cell antigens is defined as molecular mimicry. This mimicry between caseins and β-cell antigens was demonstrated by β-casein T cell, live immune reactivity to human insulinoma extracts or β-cell antigens, including glucose transporter 2 (Glut-2) peptide. Indeed, bovine β-casein contains a sequence of 5 consequent amino acids in common with Glut-2. Glut-2 has been proposed as one of the autoantigens in type 1 diabetes since antibodies against this epitope were demonstrated in patients with the disease. This sequence homology between β-casein and islet-cell molecules is shown in Figure 4.

From this sequence homology or antigenic similarity between cow's milk protein and β-cell components, the researchers concluded that autoreactive, CD4+ T H1 lymphocyte clones are responsible for type 1 diabetes. Indeed, β-casein T-cell lines can be isolated from patients with type 1 diabetes, and different epitopes are recognized within this protein. This reactivity of β-casein-specific T cells with β-cell antigens of islet cells provides an explanation for an epidemiological association between consumption of cow's milk and the development of type 1 diabetes. Additional evidence for this association comes from dietary manipulation of autoimmunity against β-cell antigens in infants who were at increased risk for type 1 diabetes. A total of 242 newborn infants with genetic susceptibility and first-degree relatives who had type 1 diabetes were randomized after birth to receive hydrolyzed casein or cow's milk formula until the age of 6 to 8 months. These children were under observation for a period of 5 years, during which their serum levels of antibodies to insulin, GAD-65, and insulinoma-associated antigen 2 and islet-cell antibodies were measured periodically. The cumulative incidence of these autoantibodies, which are considered early biomarkers of islet cell autoimmunity, was significantly lower in the group receiving cow's milk formula than in the group receiving hydrolyzed casein hydrolysate than in the group receiving cow's milk.

In another study, researchers further studied the hypothesis that early exposure to complex dietary proteins may increase the risk of autoimmunity and type 1 diabetes in children with genetic susceptibility. Similar to the earlier study, the researchers tested the hypothesis by supplementing breast milk with cow's milk formula or with highly hydrolyzed milk formula, looking for a decrease in the level of diabetes-associated autoantibodies and for the incidence of type 1 diabetes, until the participants were aged 10 years. It was concluded that dietary intervention during infancy through use of highly hydrolyzed milk proteins had a long-lasting effect on the reduction of autoantibodies that are involved not only in β-cell autoimmunity but also in the induction of type 1 diabetes.

These results indicate that a preventive dietary intervention aimed at decreasing the risk of type 1 diabetes may be feasible. Such an intervention would need to be initiated early in life, since the first signs of β-cell autoimmunity can appear before a child reaches the age of 3 months, when the mucosal immune system is not mature. Nutritional intervention during infancy, such as that provided in this study, may be an attractive strategy, because it could be implemented relatively easily as a public measure.

**Neuroimmune Reactivity Associated With Milk Proteins**

Most neuroautoimmune disorders are believed to be inflammatory disorders in which environmental factors, in particular diet, play a significant role in the autoimmune mechanism. MS is a classic example of such a neuroimmune disease, because the incidence of this disorder may be preserved or changed after migration to another sociocultural environment.

Agranoff and Goldberg compared MS mortality rates from 1949 to 1967 to food consumption data for the United States and found extremely high correlation coefficients in the range of 0.8 to 0.9 with milk consumption. Fish and vegetable fat intake were inversely related to MS mortality. Knox compared international MS mortality rates from sources in the World Health Organization (WHO) to food consumption data from 20 countries that were members of the Organisation for Economic Co-operation and Development (OECD). He reported correlations with per capita intake of meat, eggs, butter, sugar, and milk. Butcher emphasized the global correlation between MS prevalence and milk consumption and studied the differences in the intensity of dairy cow breeding between Nordic and Celtic populations in Scotland; an MS gradient parallel to milk consumption in Norway; similar gradients between Australia and South Africa; and correlating time trends between milk consumption and MS incidence in Japan from 1950 to 1969. His report concluded that milk consumption could be a common etiological factor.

Malosse et al focused on the association between MS and dairy and compared MS data from 29 countries to consumption data provided by an international marketing organization. The correlation between MS and milk consumption was good (P = .79). Latitude also correlated...
with both MS ($P = .69$) and milk consumption ($P = .73$). Hypothetically, the toxic role of butyrate or milk-related viruses was discussed. In the search for the mechanism of milk protein involvement in the induction of MS, the highest level of sequence homology was found between a major protein of the milk-fat globule membrane called butyrophilin (BTN) and myelin oligodendrocyte glycoprotein (MOG).

MOG is a major target for the pathogenic autoimmune response in MS and its animal model, experimental autoimmune encephalomyelitis (EAE). MOG is the only myelin autoantigen known to induce both a demyelinating autoantibody response and an encephalitogenic, CD4+ T cell response in animals with EAE. Although the encephalitogenic, MOG-specific, CD4+ T cell response initiates the recruitment of immune effector cells into the CNS and disrupts the blood-brain barrier (BBB), demyelination in the rat depends on the presence of anti-MOG autoantibodies. These antibodies bind to MOG exposed on the myelin surface and mediate demyelination by a combination of complement and cell-mediated immune effector mechanisms. However, the mechanism that may be involved in the disruption of self-tolerance to MOG in MS was obscure, until it was reported that an encephalitogenic T-cell response to MOG could be induced or alternatively suppressed as a consequence of immunological cross-reactivity, or molecular mimicry of the extracellular, immunoglobulin V (IgV)-like domain of the milk protein BTN. In the dark Agouti rat, active immunization with native BTN triggers an inflammatory response in the CNS that is characterized by the formation of scattered meningeal and perivascular infiltrates of T cells and macrophages.

Further, it was shown that this pathology was mediated by an MHC class II–restricted, T-cell response of BTN that cross-reacts with the MOG peptide sequence 76 to 88 as shown in Figure 5.

Note that of the 13 amino acids in these peptides, 9 (70%) are identical. Therefore, it should not be surprising that a pathology that is similar to that induced by MOG peptides could be maintained when MOG is completely replaced with its BTN homologue. In this and other experiments, BTN from milk was identified as an antigen that can influence the clinical outcome of autoimmune responses to MOG, an important antigenic target in EAE and MS. Modulation of the MOG-specific repertoire as a consequence of molecular mimicry with the dietary antigen BTN may be a significant factor in determining the role that MOG plays as a target antigen in the immunopathogenesis of MS. Therefore, consumption of milk products that modulates the pathogenic autoimmune response to MOG should be restricted for patients with neuroautoimmune disorders that are associated with high levels of antibodies against MOG and other neural antigens.

In a different study, the sequence of 120 amino acids in the MOG peptide of the N-terminal domain was compared to milk BTN. By comparing amino acid residues conserved between the 2 proteins, the researchers detected an approximately 50% similarity in the amino acid sequences or cross-reactivity between 9 different peptides of MOG and BTN (Figure 6).
Further, when antibodies against MOG and BTN peptides were measured in patients with MS, much higher levels were detected in the blood as well as in the cerebrospinal fluid (CSF) of MS patients than in the controls. The demonstration of molecular mimicry between MOG and BTN, together with sequestration of BTN-reactive antibodies in CSF, suggests that exposure to this common dietary antigen may influence the composition and function of the MOG-specific autoimmune repertoire in the course of MS. Further, antibody responses to some of the BTN peptides were preferentially detected in the CNS, suggesting they may be involved in the pathogenesis of MS.125

MOG is localized at the outer surface of the CNS myelin sheath where it can be targeted by demyelinating autoantibody responses.125 Epidemiological studies repeatedly associate prevalence of MS with dietary factors, including the consumption of milk and dairy products,118-119 and this association has led to speculation that molecular mimicry involving BTN may modulate MOG-specific, TH1 CD4+ T cell responses to MOG. The results of this study provided the first demonstration of molecular mimicry involving this common dietary antigen in MS and suggest that the composition and function of the MOG-specific immune repertoire may be influenced in the course of the disease by BTN present in milk and dairy products.125

**Neuroimmune Reactivity and Different Dietary Proteins**

Neuromyelitis optica (NMO) is a severe neuroautoimmune disorder associated with antibodies to AQP4 that affects the gray and white matter in the brain and spinal cord, causing demyelination, axonal damage, and necrosis and resulting in sensory loss and paralysis.126 In 75% of cases, NMO is associated with IgG1 antibody development that binds selectively to AQP4, which is a water channel belonging to the aquaporin family.127-128 AQP4 is expressed in the astrocytic foot processes at the BBB, which are in contact with brain microvessels or the subarachnoid space, affecting solute concentration, electrical activity, and modulation of neuronal transmission and excitability.129 After binding, the AQP4 IgG1 antibody has the capacity first to damage the astrocytes and then to cause demyelination in the spinal cord and optic nerve.130 The binding of IgG1 to AQP4 also induces activation of complement cascade and inflammatory infiltrates, which cause demyelination and tissue destruction after the induction of astrocytic cytotoxicity. Because the IgG1 antibody against AQP4 is first produced in peripheral tissues, it has greater access to the CNS, where environmental triggers can compromise the permeability of the BBB, allowing pathogenic antibodies to reach their target antigens.131

In a very recent study, it was hypothesized that pathogenic antibodies to AQP4 may be triggered by exposure to environmental proteins that have a similarity to or molecularly mimic a specific epitope of AQP4.132 As indicated previously, molecular mimicry is a mechanism by which exogenous agents, including plant, bacterial, and viral proteins, can trigger immune responses against self or nonself antigens.133 This molecular mimicry is not surprising given the fact that protein families with similar structural and functional attributes exist across animal and plant kingdoms. Indeed, 5 different plant aquaporins have been well studied. Spinach leaves express 2 thermally stable aquaporins that constitute 20% of the integral membrane protein.134 Soybean aquaporin occurs in the germinating seeds as well as in the root nodules.135 Further, the human AQP4 also cross-reacts with tomato and corn tonoplast, intrinsic proteins.132 This amino acid similarity between human AQP4 and AQP4 from various plants is shown in Figure 7.
In addition, a sequence similar to a primary T-cell epitope in NMO occurs in the potentially immunogenic coat protein of the parsnip yellow fleck virus that infects parsnips, celery, carrots, parsley, cilantro, chervil, and dill. This epitope also showed a similarity to a sequence present in a serine-protease inhibitor in the legume *Medicago truncatula*, for which the complete genome has been sequenced.\(^{133}\)

Considering the aforementioned sequence correlations, it is of particular interest that Asians are major consumers of both soybeans and spinach,\(^{136}\) and neuromyelitis optica constitutes as much as one-half of the demyelinating autoimmune disorders among Asians, confirming the molecular mimicry between AQP4 and various plant peptides as well as their pathogenic role in NMO.\(^{135,137}\)

Aquaporins from different food sources are highly stable in digestion and, hence, may survive as intact proteins or peptides. Due to a breakdown in immunological tolerance, these proteins may become antigenic, and the immune reaction against them could result in antibody production. If these cross-reactive antibodies cross the barrier in susceptible individuals, the immune response could result in NMO. Indeed, when the sera of NMO patients were applied to both AQP4 and various plant peptides, a significant reactivity was observed against both human and plant AQP4.\(^{132}\) These results further delineate the role of the environment in NMO etiology. These naturally expressed proteins should be exploited in therapeutic interventions, such as the sublingual low-dose introduction of dietary antigens\(^ {16}\) as well as the development of guidelines for dietary modification in NMO and other neuroimmune disorders. Figure 8 shows the association between various dietary proteins, peptides, and environmental AQP4 and the development of NMO and other neuroautoimmune disorders.

**CONCLUSIONS**

The author has shown that the components of wheat share sequence similarities to several neuronal antigens. Likewise, milk proteins also share chain correspondence with human tissue molecules such as islet cells. More recently, water channel or aquaporin cells from common food plants, such as spinach, tomato, soy, and corn, have been discovered to have a great structural similarity to

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**Figure 8.** Contribution of various dietary proteins/peptides/environmental AQP4 in the development of neuroautoimmune disorders.

**Abbreviation:** AQP4, aquaporin 4.

![Diagram showing the contribution of various dietary proteins/peptides/environmental AQP4 in the development of neuroautoimmune disorders.](image-url)
human aquaporin. The molecular mimicry of wheat, milk, and plant aquaporins with various human tissues presented here, coupled with genetic and additional environmental triggers, can lead to food immune reactivity first, followed by the immune system's attack on the body's own tissues, resulting in autoimmune diseases such as MS, neumyelitis optica, and other autoimmune disorders. What other foods do we innocently consume without realizing that they bear the potential to strike us down with a debilitating disease? Further research is needed to discover these other sensitive food substances. Medical practitioners must realize that these molecular mimics can be detected by the autoantibodies left behind as the detritus resulting from the body's immune system cannibalizing its own tissues. Based on the detection of these antibodies, the removal of the responsible immunological food triggers can then be the basis of the development of proper treatment and the prevention of autoimmune diseases.

REFERENCES


Vojdani—Molecular Mimicry, Food Immune Reactivities, and Autoimmunity


Lectins, Agglutinins, and Their Roles in Autoimmune Reactivities

Aristo Vojdani, PhD, MSc, CLS

ABSTRACT

Lectins are carbohydrate-binding proteins present throughout nature that act as agglutinins. Approximately 30% of our food contains lectins, some of which may be resistant enough to digestion to enter the circulation. Because of their binding properties, lectins can cause nutrient deficiencies, disrupt digestion, and cause severe intestinal damage when consumed in excess by an individual with dysfunctional enzymes. These effects are followed by disruption of intestinal barrier integrity, which is the gateway to various autoimmunities. Shared amino acid motifs between dietary lectins, exogenous peptides, and various body tissues may lead to cross-reactivity, resulting in the production of antibodies against lectin and bacterial antigens, followed by autoimmunity. The detection of immunoglobulin G (IgG) or immunoglobulin A (IgA) antibodies against specific lectins may serve as a guide for the elimination of these lectins from the diet. It is proposed that this process can reduce the peripheral antigenic stimulus and, thereby, result in a diminution of disease symptoms in some—but not all—patients with autoimmune disorders. (Altern Ther Health Med. 2015;21(suppl 1):46-51.)

Aristo Vojdani, PhD, MSc, CLS, is the CEO of Immunosciences Labs, Inc, in Los Angeles, California, and a faculty member in the Department of Preventive Medicine at Loma Linda University in Loma Linda, California.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com

An agglutinin is a substance that causes particles to coagulate and form a thickened mass. Agglutinins can be antibodies that cause antigens to aggregate by binding to the antigen-binding sites of antibodies. They also can be substances other than antibodies, such as sugar-binding protein lectins. Lectins are carbohydrate-binding proteins that were first discovered in plants and are now generally known to be present throughout nature. Plants produce toxic lectins as a survival mechanism against insects, molds, fungi, and diseases. It is estimated that approximately 30% of our food contains lectins, some of which may enter our circulation because of their resistance to digestive enzymes.1 Because of the ubiquity of glycoconjugates—such as glycoproteins and glycolipids at the epithelial, lymphocyte, erythrocyte, and other cell surfaces—incompletely digested lectins may affect our health.2-4 When consumed in excess by an individual with dysfunctional enzymes, lectins can cause nutrient deficiencies, disrupting digestion and causing severe intestinal damage, followed by disruption of intestinal barrier integrity, which is the gateway to various autoimmunities.1,5-8 After repeated consumption, many foods not only interact with the delicate lining of the small intestine and lead to leaky gut, but can penetrate the barriers and enter the blood stream through the regional lymph nodes.9,10 Once in the blood, due to interaction with the cell surface glycoproteins, the undigested lectins can bind to various tissue cells and antigens, such as collagen tissue, thyroid tissue, pancreas tissue, and adrenal nerve tissue. This binding can cause an immune attack against the lectins and the tissue to which the lectins are bound.11-13 The immune attack against lectins bound to various tissue antigens first results in autoimmune reactivity, followed by autoimmune disease. Common dietary lectins and the body cells and tissues to which they can bind are shown in Table 1.
Lectins are widely recognized as antinutrients within food because they can bind to virtually all cell types and cause damage to several organs. Most lectins are resistant to heat and the effects of digestive enzymes and can bind to several tissues and organs, both in vivo and in vitro. Although lectin activity has been demonstrated in wheat, rye, barley, oats, corn, and rice, the best studied of the cereal grain lectins is wheat germ agglutinin (WGA). The administration of WGA to experimental animals caused hyperplastic and hypertrophic growth of the small intestine, hypertrophic growth of the pancreas, and thymus atrophy.

WGA is able to adhere to cell surfaces such as the epithelial layer of the gut because it binds to N-glycolylineuraminic acid (Neu5Ac), the sialic acid predominantly found in humans. The surface of many prokaryotic and eukaryotic cells are covered with a dense coating of glycoconjugates, also called glycocalyx. At the terminal positions of many surface-exposed glycoconjugates are a wide family of 9-carbon sugars called sialic acids. These acids are used for self-recognition in the vertebrate immune system, but they can also be used as a binding target for pathogenic, extrinsic receptors, and molecular toxins.

When WGA binds to Neu5Ac of the glycocalyx of human cells and pathogens expressing Neu5Ac, it allows for cell entry and could evoke a proinflammatory immune response, thereby disturbing immune tolerance.

These proinflammatory responses are induced by WGA through immune cells. Studies have shown that WGA can (1) trigger histamine secretion and granule extrusion from nonstimulated, peritoneal mast cells in rats; (2) induce NADP-oxidase activity in human neutrophils; (3) stimulate the release of the cytokines interleukin (IL) 4 and IL-13 from human basophils; (4) induce the production of IL-2 in human peripheral blood mononuclear cells (PBMC), while simultaneously inhibiting the proliferation of activated lymphocytes; (5) induce the secretion of IL-12 in a T- and B-cell–independent manner in murine spleen cells; (6) induce the production of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-1 beta

### Table 1. Common Dietary Lectins and Agglutinins That Bind to Different Body Tissues and Cells

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<th>Cells With Affinity to Lectins</th>
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<th>PNA</th>
<th>LA</th>
<th>MA</th>
<th>TA</th>
<th>PA</th>
<th>POT.A</th>
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Abbreviations: WGA, wheat germ agglutinin; SBA, soy bean agglutinin; PNA, peanut agglutinin; LA, lentil agglutinin; MA, mushroom agglutinin; TA, tomato agglutinin; PA, pea agglutinin; POT.A, potato agglutinin; KBA, kidney bean agglutinin; JBA, jack bean agglutinin.
(IL-1β), IL-12, and interferon gamma (IFN-γ) in murine peritoneal macrophages; and (7) induce a significant increase in the intracellular accumulation of IL-1β measured in monocytes after WGA exposure. These results indicate that WGA, when delivered in vitro, is capable of directly stimulating cells that have the ability to initiate and maintain inflammatory responses. Monocytic cells have been shown to engulf WGA by binding to nonreceptor glycoproteins or by fostering receptor-mediated endocytosis.

Human data on the influence of WGA intake on inflammatory markers need more research, but antibodies to WGA have been detected in the serum of healthy individuals. Significantly higher antibody levels to WGA were measured in patients with celiac disease (CD) when compared with patients with other intestinal disorders. These antibodies did not cross-react with gluten antigens and, therefore, could play an important role in the pathogenesis of this disease.

WGA is capable of crossing the intestinal barrier after ingestion. It has been shown to (1) reach the basolateral membrane and walls of the small blood vessels in the subepithelium of the small intestine in animal models; (2) be phagocytosed by binding to membrane, nonreceptor glycoproteins, a process that has been observed in Caco-2 cells; and (3) be endocytosed by antigen-sampling M cells or by enterocytes via binding to epidermal, growth-actor receptors. Another possible route for lectin entry into the periphery is by paracellular transport, a process that can be further aggravated by the binding of gliadin to the chemokine receptor CXR3 on enterocytes.

WGA has been found to affect enterocyte permeability. In vitro, one study showed that exposure to micromolar concentrations of WGA impaired the integrity of the intestinal epithelial layer, allowing passage of small molecules such as lectins. At the basolateral side of the epithelium, WGA concentrations in the nanomolar range induced the secretion of proinflammatory cytokines by immune cells. This secretion may further affect the integrity of the epithelial layer, heightening the potential for a positive feedback loop among WGA, epithelial cells, and immune cells. When combined, these mechanisms are likely able to significantly increase the amount of consumed WGA that can cross the epithelial layer as compared with the low amount of WGA crossing by means of transcytosis (0.1%) alone. This suggests that WGA, together with gliadin, can increase intestinal permeability, resulting in an increase of translocating microbial and dietary antigens that interact with cells of the immune system.

INTERACTION OF DIETARY LECTINS WITH IMMUNE FUNCTION AND INDUCTION OF AUTOIMMUNE DISEASES

It is apparent that dietary lectins that come from cereal grains, legumes, and vegetables increase gut permeability, allowing increased passage of gut-derived bacterial and dietary antigens, including lectins themselves, to the periphery. In addition, lectins have the ability to interact with various components of the immune system, stimulate T-cell proliferation, and induce production of inflammatory cytokines, such as IL-1, TNF-α, and IFN-γ that may facilitate the autoimmune process.

This autoimmune reactivity depends on the interaction of the dietary lectins with the gut microbiota and the facilitation of bacterial growth, such as Escherichia coli and other enterobacters. Release of bacterial toxins, such as lipopolysaccharides, increases the permeability of the gut, allowing increased passage of dietary lectins, other food antigens, and bacterial toxins to the periphery.

The entry of dietary lectins, proteins, and bacterial toxins into the circulation can result in the binding of various lectins to almost every single target-tissue antigen shown in Table 1; these tissues include connective tissue and that of the liver, pancreas, cardiac muscle, prostate, breast, and even brain. The binding can also result in the activation of the immune system to react to these antigens and to produce antibodies against the lectins, other food antigens, and bacterial toxins because of cross-reaction between different food and bacterial antigens with human tissue.

Cellular and antibody attack against lectin-bound tissue antigens or tissue antigens that share a significant amino acid sequence with food and bacterial antigens have a significant role in the development of autoimmunity via molecular mimicry. A diagrammatic illustration of how dietary lectins interact with the gut and immune system to influence the induction of autoimmunity is shown in Figure 1.

IMPLICATION OF LECTINS IN AUTOIMMUNE DISEASE

Understanding the mechanism responsible for the induction of autoimmunities, as above, can enable clinicians to design proper treatments for the prevention of many lectin-induced autoimmune diseases (see Figure 2). For example, in the cases of islet cell autoimmunity or thyroid autoimmunity, lectins stimulate class II human leukocyte antigens (HLAs) of these cells, which normally do not display them. Islet cells carry a very specific disaccharide determinant called N-acetyllactosamine, to which wheat, peanuts, soy, potato, and tomato lectins love to bind. This binding can result in islet cells expressing the class II HLAs and foreign antigens together, creating a situation in which an individual is a sitting duck for autoimmune attack. Therefore, the binding of cytotoxic antibodies to islet cells plus lectins results in the destruction of β-islet cells. The mechanism may explain why wheat and soy, in addition to milk, are known to be diabetogenic.

Another suspected lectin disease is rheumatoid arthritis (RA). The normal IgG molecule possesses a carbohydrate side chain that ends with galactose. In RA, much of the galactose is missing, so that the subterminal sugar N-acetylgalcosamine is exposed instead. For this reason, WGA, which is specific to N-acetylgalcosamine, is one of the triggers of RA. That fact suggests that supplementation with N-acetylgalcosamine oligomers, such as chitotetraose derived from the chitin that forms crustacean shells, might be an effective treatment for diet-associated RA. Interestingly, the health food trade has already seized on N-acetylgalcosamine as an antiarthritic supplement.
Figure 1. The interaction of dietary lectins with the gut and immune system and their contribution to inflammation and autoimmunity.

Figure 2. Contribution of lectins in the development of various disorders.

Abbreviations: NCGS, nonceliac gluten sensitivity; GI, gastrointestinal; IgE, immunoglobulin E.
phenotype that is unique to both Th17 and Th1 cells. It has also been shown that both Th17 (RORC2) and Th1 (T-bet), lineage-specific transcription factors, are expressed on these Th17/Th1 intermediate cells in the inflamed joints. The binding of lectins to the CD161 on Th17/Th1 intermediate cells seems to play a role in the conversion of these cells to the Th17 and Th1 phenotypes that drive the inflammatory environment in the joints. This mechanism by which lectins contribute to the conversion of Th17/Th1 intermediate cells to Th17 and Th1 phenotypes in arthritis is shown in Figure 3. This mechanism of lectin induction of Th17 and Th1 conversion from Th17/Th1 provides unique insights into the biology and regulation of Th17/Th1 cells in the joints of patients with arthritis.

CONCLUSIONS

The author has provided extensive evidence linking dietary substances to the development of autoimmunities. Dietary glycoproteins, and other elements, can influence intestinal structure and function to allow increased translocation of both pathogenic and dietary antigens to the periphery, causing persistent immunological stimulation. Because of shared amino acid motifs among exogenous peptides, HLA-derived peptides, and self-tissue, cross-reactivity may occur, thereby breaking immunological tolerance and resulting in the production of antibodies against lectin and bacterial antigens, followed by autoimmunity. In genetically susceptible individuals, antigenic stimulation by lectins and other food antigens may result in the expression of RA by direct binding to the joint tissue, molecular mimicry, or by affecting Th17 plasticity. This process may cause the activation of autoreactive lymphocytes and antibody production against both the food antigens and the endogenous peptides, thereby causing a malfunction in the immunological tolerance against self-antigens. Detection of IgG or IgA antibodies against specific lectins may serve as a guide to clinicians for the elimination of lectins from their patients’ diets.

It is proposed that the elimination of certain dietary elements, including lectins, that adversely influence both enterocyte and lymphocyte structure and function can reduce the peripheral antigenic stimulus and, thereby, result in a diminution of disease symptoms in some, but not all, patients with autoimmune disorders. Therefore, if individuals have an autoimmune condition, they owe it to themselves to explore the link between lectins and autoimmunity in their journeys toward health and recovery.

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REFERENCES


Immune Reactivity to Food Coloring

Aristo Vojdani, PhD, MSc, CLS; Charlene Vojdani, MA

ABSTRACT
Artificial food dyes are made from petroleum and have been approved by the US Food and Drug Administration (FDA) for the enhancement of the color of processed foods. They are widely used in the food and pharmaceutical industries to increase the appeal and acceptability of their products. Synthetic food colorants can achieve hues not possible for natural colorants and are cheaper, more easily available, and last longer. However, since the use of artificial food coloring has become widespread, many allergic and other immune reactive disorders have increasingly been reported. During the past 50 y, the amount of synthetic dye used in foods has increased by 500%. Simultaneously, an alarming rise has occurred in behavioral problems in children, such as aggression, attention deficit disorder (ADD), and attention-deficit/hyperactivity disorder (ADHD). The ingestion of food delivers the greatest foreign antigenic load that challenges the immune system. Artificial colors can also be absorbed via the skin through cosmetic and pharmaceutical products. The molecules of synthetic colorants are small, and the immune system finds it difficult to defend the body against them. They can also bond to food or body proteins and, thus, are able to act in stealth mode to circumvent and disrupt the immune system. The consumption of synthetic food colors, and their ability to bind with body proteins, can have significant immunological consequences. This consumption can activate the inflammatory cascade, can result in the induction of intestinal permeability to large antigenic molecules, and could lead to cross-reactivities, autoimmunities, and even neurobehavioral disorders. The Centers for Disease Control (CDC) recently found a 41% increase in diagnoses of ADHD in boys of high-school age during the past decade. More shocking is the legal amount of artificial colorants allowed by the FDA in the foods, drugs, and cosmetics that we consume and use every day. The consuming public is largely unaware of the perilous truth behind the deceptive allure of artificial color. (Altern Ther Health Med. 2015;21(suppl 1):52-62.)

Aristo Vojdani, PhD, MSc, CLS, is the CEO of Immunosciences Labs, Inc, in Los Angeles, California, and a faculty member in the Department of Preventive Medicine at Loma Linda University in Loma Linda, California. Charlene Vojdani, MA, is a laboratory assistant at Immunosciences Lab, Inc.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com
family in general. They are heat-labile but fade during processing. To overcome such losses and, ironically, to give a natural, appealing look to foods, synthetic colors are added.4

**Patent Blue V and Brilliant Blue**

Also called Food Blue 5 or Sulphan Blue, Patent Blue V (PB-V) is a dark bluish, synthetic dye used as a food coloring. The E number for PB-V is E131. This color is used in medicine in lymph node biopsy, in lymphangiography, and in dental staining to show dental plaque on teeth. Together with Brilliant Blue (BB), this substance is used as a dye for textiles, leathers, plastics, and papers and as a colorant in printing inks, paints, and other household products. European regulatory agencies have not established quantitative limits for an acceptable daily intake of PB-V as a cosmetic coloring. As a food-coloring additive, the recommended daily intake is 15 mg/kg of body weight in Europe and many other countries. However, in the United States and a few other countries, PB-V is not authorized for use in food.5

Unlike PB-V, BB (E133 or FD&C Blue No. 1) is approved by the US Food and Drug Administration (FDA) for use in food, drugs, and cosmetics products, with an established and acceptable daily intake of 12.5 mg/kg of body weight.5 The molecular formulas for both are shown in Figure 1.

**Tartrazine**

Tartrazine is an artificially synthesized, azo pigment that is also known as FD&C Yellow No. 5 and E102 (Figure 2). Its use is permitted by the Food and Drug Regulations in Canada, where Health Canada, the country's health body, requires the identification of specific colors on food labels.6 The European Food Safety Authority allows for tartrazine to be used in processed cheese, canned or bottled fruit or vegetables, processed fish or fishery products, and wines and wine-based drinks.7,8 Tartrazine was banned in Norway and was also banned in Austria, Sweden, and Germany until the ban was overturned by a European Union directive.9 In the United States, the FDA requires that the presence of tartrazine be declared in food and drug products and that a warning regarding the colorant's possible allergenic effects on sensitive individuals be displayed.10,11 In France, following the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), tartrazine is approved as a colorant in food products, cosmetics, and pharmaceuticals, with a recommended daily intake of 7.5 mg/kg of body weight. It is a color commonly used throughout the world, mainly for yellow, but it can also be used with BB or Green S to produce various shades of green.2,12,13 Products containing tartrazine commonly include processed commercial foods that have an artificial yellow or green color or that consumers expect to be brown or creamy looking.

The following is a list of foods that may contain tartrazine: (1) desserts and sweets—ice cream, ice pops, and popsicles; confectionery products and hard candy, such as gummy bears and marshmallow treats; cotton candy; instant puddings and gelatins; cake mixes; processed pastries; custard powder; marzipan; biscuits; and cookies;
beverages—soft drinks; energy and sports drinks; powdered drink mixes; fruit cordials; and flavored or mixed alcoholic beverages; (3) snacks—flavored corn chips; chewing gum; popcorn, both microwaveable and cinema-popped; and potato chips; (4) condiments and spreads—jam; jelly, including mint jelly; marmalade; mustard and horseradish; pickles and other products containing pickles, such as tartar sauce and dill pickle dip; and processed sauces; (5) other processed foods—cereal, such as corn flakes and muesli; instant or cube soup; rice, such as paella and risotto; noodles; and puréed fruit.

A number of personal care and cosmetics products may contain tartrazine, including (1) liquid and bar soaps; green hand sanitizer; moisturizers and lotions; mouth washes; perfumes; toothpastes; shampoos, conditioners, and other hair products; (2) cosmetics, such as eye shadow, blush, face powders and foundations, and lipstick, even those that are primarily pink or purple (usually the makeup manufacturers use 1 label for all shades in a product line, placing the phrase “may contain” ahead of all colors that are used in that line but not necessarily in the specific shade); and (3) nail polish, nail polish remover, temporary tattoos, and tanning lotions.

Various types of medications also may include tartrazine, primarily for easy identification, to give a yellow, orange, or green hue to a liquid, capsule, pill, lotion, or gel. Types of pharmaceutical products that may contain tartrazine include vitamins; antacids; cold medications, including cough drops and throat lozenges; lotions; and prescription drugs. Most, if not all, data sheets of medications are required to contain a list of all ingredients, including tartrazine. Some include tartrazine in the allergens alert section.

Other products such as household cleaning products, paper plates, pet foods, crayons, inks for writing instruments, stamp dyes, face paints, and envelope glues may also contain tartrazine.

Allura Red

Allura Red or red azo dye is also known as Food Red 40, FD&C Red No. 40, Food Red 17, E129, or Curry Red (Figure 3). Many products, including medications, hair dyes, and a variety of foods contain Allura Red. The extensive use of Allura Red as a color additive in foods, drugs, and cosmetics indicates that the general population may be exposed to this dye via ingestion of food, beverages, and drugs that use this compound. This use may result in its release into the environment through various waste streams.

In one study, rats were fed a diet containing 5.19% of Allura Red. The researchers observed that 0.1% and 29% of the intact dye was excreted in the urine and feces, respectively. In later studies, rats and dogs were pretreated daily with nonradioactive Allura Red. Subsequently, the animals were dosed with the 35S labeled compound and studied for up to 72 hours for excretion and distribution patterns of the color. Both species showed limited absorption of the compound, with the major route of excretion being via the feces. In the dogs, 92% to 95% of the recovered radioactivity appeared in the feces within 72 hours, whereas in the rats, 76% to 92% of the recovered radioactivity appeared in the feces within that period. Urinary recoveries of the color in rats and dogs varied between 5.7% and 19.8% and between 2.7% and 3.6%, respectively. After sacrifice, significant retention of radioactivity was found in the intestinal contents of both species and in the washed intestines of the rats. This result was thought to be due to adhesion of the compound to the intestinal wall, because the total carcass and viscera of these animals contained <0.4% of the administered dose.

These findings indicate that a significant amount of dyes or their metabolites can bind to human tissue and form neoantigens. Repeated use of foods, cosmetics, or drugs containing colorant can result in bioaccumulation in the tissue; the food coloring plus tissue antigens can then be attacked by the immune system, resulting in autoimmune reactivity.

Erythrosine

Erythrosine, which is also referred to as FD&C Red No. 3, E127, or Food Red 14, is an iodine-containing, artificial coloring made from coal tar (Figure 4). Erythrosine is also a xanthene dye, which are a group of brilliant fluorescent dyes ranging in color from yellow to pink to bluish red. They are called xanthene dyes because they all contain a xanthene molecule as their base.

The chemical formula for erythrosine is C_{20}H_{18}I_{2}O_{5}. Figure 5 shows the structure of the erythrosine molecule. The shared part of the formula (highlighted) shows why erythrosine is categorized as a xanthene dye.

Erythrosine is primarily used as a food dye. Some of the more common applications include (1) cocktail and candied...
immune reactivities have been reported. The ingestion of food itself provides the greatest load of foreign antigenic substances that confront the human immune system.

Failure in protective, mucosal immune tolerance against food antigens is the major factor in the induction of immune reactivities and allergies against food antigens. Food coloring and other additives are made of very small molecules, and our bodies do not know how to develop a tolerance against them. If these chemicals manage to bind to food proteins during the manufacturing of foods or to body proteins after metabolization, then these food additives become the major reason for immune tolerance breakdown, and they are able to do so in a stealthy or hidden manner. For this reason, calculating the prevalence of food immune reactivity and allergy is very difficult. Food allergy or hypersensitivity alone is estimated to be between 3% and 35% of the US population.

It is believed that food additives play a significant but hidden role in these immune reactivities. In addition, food coloring, in particular tartrazine, has been suggested as a trigger for asthma and urticarial attacks, particularly in aspirin-intolerant patients. These reactions have occurred following ingestion of meals or products containing tartrazine and other food colorings. For example, one study investigated 25 patients with clinical symptoms of immunoglobulin E (IgE)-mediated food allergy. This study's participants were challenged orally with various food additives, including tartrazine. In 5 of 25 patients (20%), a positive reaction to tartrazine was obtained by an oral provocation test. However, the study did not assess non-IgE immune reactivities by measuring IgG or immunoglobulin A (IgA) antibodies against tartrazine or other food additives that were bound to human serum albumin (HSA).

An even higher percentage of children with severe atopic dermatitis reacted to tartrazine. Tartrazine and other colorants have been reported for their involvement in multiple chemical sensitivities that are not IgE-mediated. Many other studies and case reports have associated the use of many food additives other than tartrazine, including Allura Red and erythrosine, to allergic, immunologic abnormalities such as chronic urticaria, angioedema, and rhinitis, which are normally associated with food allergies.

For example, when 25 patients with clinical symptoms suggestive of allergy to food antigens were examined by oral provocation test with various food additives, almost 50% of the patients positively responded to 2 additives—sodium benzoate and Sunset Yellow. That study and many others associated with food-coloring immune reactivities were reviewed extensively in a review article, which concluded that allergic and immune reactivities to food additives can be considered as triggers or aggravating factors in sensitive individuals. Therefore, clinicians and, particularly, sensitive consumers should be aware of the allergic properties of food additives; the removal of these triggers can help to improve a patient's symptomatologies immensely.

**HEALTH ISSUES AND USE OF COLORING**

Immune and allergic reactions to food additives are a part of food immune reactivities and allergies. Since the use of food additives has become widespread and extensive throughout the world during the past 20 years, many allergic and other

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**Figure 4.** Molecular formula for xanthene: $\text{C}_{13}\text{H}_{10}\text{O}$ (molecular weight: 182.2).

**Figure 5.** Molecular formula for erythrosine: $\text{C}_{20}\text{H}_{8}\text{I}_4\text{O}_5$ (molecular weight: 879.9). The shared part of the formula (highlighted) shows why erythrosine is categorized as a xanthene dye.
Whether it is through licking lollipops repeatedly, eating ice cream, using medications, or applying cosmetics, shaving cream, or skin treatments, a significant amount of food additives manage to enter the bloodstream through the gastrointestinal (GI) tract. Many food additives can penetrate into the blood either via skin absorption after application of cosmetics or skin creams or via the GI tract through food intake. Because most food additives carry very active chemical groups, their entry into the human system results in food-coloring interactions with human proteins.

Almost all proteins are made of polar and nonpolar amino acids along polypeptide backbones. Protein molecules also provide a combination of electrostatic and stereochemical interactions. Because food colors are generally ionic in nature, they interact with proteins strongly and form covalent bonds. In fact, one of the reasons food additives are used extensively throughout the world is because colors form stable complexes with proteins and give uniform color distribution in all common food systems. This covalent binding of colors to human proteins is a major mechanism for the induction of the immune reactivity and hypersensitivity that is associated with various colorants.
In a very recent study, male and female mice were given 0.45% or 1% of tartrazine through drinking water for a period of 13 weeks, together with a standard food-pellet diet. After 13 weeks of administration and 18 hours of fasting, the animals were sacrificed and histological examinations of the intestine, thymus, and spleen were performed. The histological section of jejunum showed significant lymphocyte infiltration and inflammation in all treated male and female groups. In addition, partial villous atrophy was observed in tartrazine-treated groups. This effect of tartrazine was very pronounced on villus length. For example, in comparison with a villus length of 60 mm for the control group, the length of villi for mice treated with tartrazine was approximately 30 mm, a decrease of 50%.

Based on these results and many other findings that are not discussed here, the researchers concluded that subchronic ingestion of tartrazine at a dose of 1% caused structural alterations of the intestine and spleen, thymus-cell damage, and a depressing effect on the humoral immune response in male mice. It is advisable to limit the usage of synthetic food colorants, particularly for products consumed or applied by children and immunologically vulnerable people.

EFFECTS ON PROTEIN DIGESTIBILITY
After ingestion, many food proteins are digested by various enzymes and are broken down, first into peptides and then into amino acids, which are absorbed through the gut barriers. However, a main question in use of food colorings involves the fate of the food-coloring molecules that bind to food proteins during processing versus molecules that are not bound to proteins. Many studies have established that food coloring binds covalently to different body proteins, including HSA and hemoglobin. These publications show that carmoisine, Allura Red, Sunset Yellow, erythrosine, amaranth, tartrazine, Quinoline Yellow, and BB bind with proteins in a variety of food environments and that the protein color complexes are assumed to be digested by proteolytic enzymes. Because different functional groups of colors bind to the active sites of food proteins, that binding can modify and decrease the tryptic digestibility of the different proteins.

This formation of stable complexes between food colors and edible or inedible proteins is shown in Figure 7. Almost all food colors carry very active groups; proteins are made of many amino acids, each containing both amino (NH₂) and carboxylic (COOH) groups. These amino or carboxylic groups are perfect for binding with the colorants’ active groups, forming covalent bonds. In Figure 8, the decrease of the digestibility of albumin by trypsin through cleavage of amino acids is shown using an image of a scissors (lysine = K and arginine = R).

Because the active groups of colorants prefer to bind to the lysine (K), arginine (R), and histidine (H) sites of food proteins to form covalent complexes, the digestibility of
proteins decreases significantly. This covalent binding of colors to the amino acid sequences of albumin blocks the process of digestion, the cutting capacity of the scissors, as shown in Figure 9.

Indeed, one study showed that 156 of 607 amino acids of bovine serum albumin (BSA) bind with the dyes.\textsuperscript{32} To show that binding of colors to food proteins interferes with their digestibility, the researchers performed the following experiment. Preparing each solution separately, a solution of BSA proteins was combined with food dye in a concentration of 10 mg/mL of food dye to 0.1 mg/mL of protein. Equal volumes of BSA protein and color solution were mixed in test tubes separately and incubated at 37°C for 2 hours. After combining them, the incubated mixtures of color-bound proteins were digested separately by trypsin, at the enzyme concentration of 1 mg of trypsin to 50 mg of substrate, for various periods of time. After completion of the different periods, the reactions were terminated by adding 1 mL of 10% tricarboxillic acid (TCA) to the respective enzyme, and the undigested proteins were precipitated. The extent of proteolytic activity of the supernatant was measured spectrophotometrically at 280 mm.

As Figure 10 shows, with the addition of the enzyme only to the albumin, the digestion of the protein increased proportionally to the incubation time, but colors binding to the proteins and the formation of color-protein complexes significantly inhibited the ability of the digestive enzyme to digest the albumin.\textsuperscript{32} It was concluded that the consumption of food colors, particularly synthetic colors, and their binding with body proteins, have significant immunological consequences in the modern lifestyle. If this binding occurs in the digestive tract, the result can be an accumulation of undigested immunogenic peptides in the gut, which can activate the inflammatory cascade. This effect of food colors on tryptic digestibility of dietary proteins, combined with their effects on the villi structure,\textsuperscript{31} can result in the induction of intestinal permeability to large antigenic molecules. If the entry of these molecules through submucosa to the circulation continues, the result could possibly be cross-reactivities and autoimmunities.\textsuperscript{36,37} But if free colors or their metabolites form color-protein complexes or neoantigens formations throughout the body, the result could be autoimmunity against the targeted protein. For example, if colors bind to liver enzymes, the result can be liver autoimmunity, and if colors form neoantigens with hemoglobin, the result can be the destruction of red blood cells (RBCs), and hence low RBC count.

In a very recent study,\textsuperscript{35} this toxic interaction of the food-coloring tartrazine with hemoglobin was investigated at the molecular level. The results showed that tartrazine can bind to the active site or the central cavity of hemoglobin to form a tartrazine-hemoglobin complex. The study concluded that tartrazine had an obvious toxic effect on hemoglobin.\textsuperscript{36} Similarly, the mechanism of the interaction between food dye and HSA was investigated in a physiological buffer\textsuperscript{38} and the results showed that binding of food coloring to HSA induced conformational changes in the molecular structure of the HSA.

HSA is a major soluble-protein constituent of the circulatory system; it has many physiological and pharmacological functions. For instance, it contributes to colloid osmotic blood pressure and is mainly responsible for the maintenance of blood pH. Further, it can bind and transport a large number of the ligands that are present in blood, such as drugs, bilirubin, bile acids, and metabolites.\textsuperscript{39} The binding of chemicals to proteins can change macromolecular conformation and, thus, affect the physiological function of proteins. In addition, the binding of coloring to HSA can induce autoimmune reactivity against the protein, which further interferes with its physiological functions. For example, bilirubin, a neurotoxic product of heme catabolism, is detoxified after binding to albumin, which transports it to the liver for further conjugation and excretion in the form of bile.\textsuperscript{34} Bilirubin-albumin binding is of physiological relevance, and any interference with this interaction may have severe consequences. Unfortunately, several drugs and dyes, including erythrosine, can bind competitively at or near the site of albumin, where bilirubin binds. That binding can displace bilirubin from albumin, resulting in increased blood levels of bilirubin and other serious physiological consequences. In view of these results, the researchers concluded that individuals consuming colored foods and drinks may be at greater risk in developing hyperbilirubinemia.

**EFFECTS ON BEHAVIOR**

Attention-deficit/hyperactivity disorder (ADHD) affects from 5% to 7% of children worldwide. ADHD is characterized by excessive and impairing inattentive, hyperactive, and
impulsive behavior. It is believed that genetics and environmental factors are involved in ADHD and associated disorders. Foods and additives are 2 major environmental triggers that have been studied and that have been found to be associated with such behaviors.42-44

The effect of food antigens, preservatives, and artificial colors as factors involved in ADHD has been a controversial subject. The effect of foods and additives on learning and behavior was postulated as early as 1922, but only in 1973 was it hypothesized that foods containing preservatives and salicylates could cause hyperactivity in children. This theory led to anecdotal reports; only a few appropriately designed, controlled studies have shown any benefits for elimination of artificial colors and foods containing salicylates in the amelioration of hyperactivity in children.

In a test of Feingold's hypothesis that food additives trigger a hyperactive response, 26 hyperactive children were randomly assigned to treatment conditions in which they were given active or placebo medications in combination with challenge cookies with artificial colors or control cookies without the additives. The assessment of the children's behaviors was done by the teachers in a blind fashion. The results, summarized in Figure 11, first of all showed that medication and cookies without colorants resulted in a hyperactivity score of 6. When cookies containing colorant were eaten while the participants were using medication, the hyperactivity score went up to 7.4. However, when the drug was replaced with a placebo and cookies with no colorants or cookies with artificial color were given to participants, the hyperactivity score went up to 11 for the cookies without colorants and to 15 for the cookies with artificial colorants. The rating of hyperactivity clearly indicated that the effects of diet were greatest when children did not take medication and that significant reductions in hyperactivity were related to diet in approximately 25% of the children.

Another investigation evaluated 26 children who met the criteria for ADHD. In an open challenge, 19 (73%) of the children reacted to many foods, dyes, and/or preservatives. Those 19 children responded favorably to treatment with a multiple-item elimination diet (P < .001). A double-blind, placebo-controlled food challenge was completed with 16 of the 19 children, where a significant improvement occurred on placebo days when compared with challenge days (P = .003). Atopic children with ADHD had a significantly higher response rate to food challenges in the study than the nonatopic group. This study demonstrated a beneficial effect when eliminating reactive foods and artificial colors in the diets of children with ADHD, showing that atopic children—who were known to have sensitivity to food antigens, such as wheat, milk, eggs, and oranges—had a significantly more beneficial response to an elimination diet. Therefore, dietary factors may play a significant role in the etiology of a majority of children with ADHD. The study's findings would seem to indicate that a combination of dietary proteins and additives, or a complex formation between them, plays a significant role in ADHD.

The occurrence of adverse skin, gut, and other physical reactions to foods, such as milk and wheat, has stimulated speculation that such foods could also produce adverse behavioral effects. A double-blind, controlled study has suggested that foods and additives can affect hyperactive behavior adversely. In this study, 78 children, referred to a diet clinic because of hyperactive behavior, were placed on an elimination diet, removing foods containing additives, chocolate, dairy products, wheat, oranges, tomatoes, and eggs.

The behavior of 59 of the children improved during this open trial. For 19 of these children, it was possible to disguise foods, additives, or both that reliably provoked behavioral problems. The researchers did so by mixing them with other tolerated foods and then tested their effects in a placebo-controlled, double-blind challenge protocol. The results of a crossover trial on these 19 children showed a significant effect for the provoking foods in worsening ratings on behavior and impairing performance on psychological tests.
Reintroduction of 1 or more additive-containing foods resulted in the reoccurrence of symptomatologies in 70% of participants. This and earlier studies concluded that clinicians should give weight to the accounts of parents and consider treatment with an elimination diet for children with a suggestive medical history of food immune reactivities, particularly food made with additives.43,50

Similar findings were reported in a recent randomized, controlled trial dealing with the effect of an elimination diet on the behavior of children with ADHD.51 The impact of nutrition was measured in 100 children with ADHD, of whom 50 were randomly assigned to 5 weeks of a restricted diet (diet group) and the other 50 to a normal diet (control group). Children in the diet group were restricted to a few foods, such as rice, meat, vegetables, pears, and water. All major triggers such as wheat, dairy, food coloring, eggs, and other allergenic food components were eliminated from their diets. After 5 weeks, 78% of those in the diet group had responded to the diet. The assessment of improvement in the children's hyperactivity scores was done both by pediatricians and teachers (Figure 12). The difference in hyperactivity scores between the diet group and the control group was highly significant ($P < .0001$).

The study showed considerable beneficial effects from a restricted, elimination diet in an unselected group of children with ADHD, with equal effects on ADHD and oppositional defiant disorder. Therefore, the researchers recommended that a dietary intervention should be considered for all children with ADHD, provided that parents are willing to follow a diagnostically restricted elimination diet for a 5-week period and that expert supervision is available. Children who react favorably to this diet should be diagnosed with food-induced ADHD and should enter a challenge procedure to define the foods to which each child reacts and to increase the feasibility and minimize the burden of the diet. In children who do not show a behavioral improvement after following the diet, standard treatments such as drugs, behavioral treatments, or both should be considered.51

Finally, one study elegantly presented a systemic review and meta-analysis of published works from 1970 to 2013 that had performed randomized, controlled trials of dietary components.52 This study has provided much evidence on the harmfulness of food additives for individuals with ADHD. Collectively, exclusion of artificial food colors from the diet of individuals with ADHD had statistically significant effects. As part of clinical guidance about nonpharmacological treatment of ADHD, the researchers concluded that the only dietary or psychological treatments that improved core symptoms of ADHD were supplementation with omega-3/omega-6 free fatty acids and the elimination of artificial food colorings.

**SUMMARY AND CONCLUSIONS**

Artificial food dyes are made from petroleum and are generally approved by the FDA and similar organizations worldwide for the enhancement of the color of processed foods. They are found in everything from potato chips, soft drinks, and puddings to baked goods and even tandoori chicken. During the past 50 years, the amount of synthetic dye used in foods has increased by 500%. Simultaneously, an alarming rise has occurred in children's behavioral problems, such as aggression, ADD, and ADHD.

The Centers for Disease Control recently found that 1 in 5 boys of high-school age and 11% of children overall have been diagnosed with ADHD, which represents a 41% increase in the past decade. In this article, the author has presented data extracted from different scientific articles that show that some association exists between the increased use of petroleum-based food colorings and behavioral problems in children.

After reviewing more than 250 journal articles to write the current review, as an immunologist the author is astounded that these kinds of products remain approved by regulatory officials for human consumption. Further, the author is even more dumbfounded at the amount allowed for human use on a daily basis. For example, officially it is perfectly fine to consume up to 500 mg or 0.5 g of BB or FD&C Yellow No. 5 (tartrazine) on a daily basis. Did you know that if you put 0.5 g of BB or tartrazine in a swimming pool, you can stain the water in the whole swimming pool blue or yellow? Therefore, no one should be surprised by the titles of the following articles, because the author has already shown earlier in the current article that colorants bind covalently to human tissue: (1) "Blue Colon at Autopsy",53 (2) "Green Colon: An Unusual Appearance at Autopsy",54 (3) "Systemic Absorption of Food Dye in Patients With Sepsis",55 (4) "Toxicity of Food Drug and Cosmetic Blue No. 1 Dye in critically Ill Patients",56 (5) "Reports of Blue Discoloration and Death in Patients Receiving Enteral Feeding Tinted With Dye Blue No. 1",57 (6) "Differential Colon DNA Damage Induced by Azo Food Additives",58 (7) "Reproductive and Neurobehavioral Toxicity of Tartrazine Administered to Mice in the Diet",59 (8) "Reproductive and Neurobehavioral Toxicity of Erythrosine Administered to Mice in the Diet",60 (9) "Effects of Organic Synthetic Food Colours on Mitochondrial Respiration",61 (10) "Neurotransmitter Release..."
from a Vertebrate Neuromuscular Synapse Affected by a Food Dye,”11 "Erythrosine B Inhibits Dopamine Transport in Rat Caudate Synaptosomes,”12 and (12) “Immunological Aspects of the Common Food Colorants, Amaranth and Tartrazine.”13

Despite all these articles published in scientific journals, the public unfortunately is largely unaware of the extent of the use of food additives. The author has been equally guilty of being fooled by an attraction to the colors used in various foods. One of his favorite food types is Indian food—particularly the taste, aroma, and color of tandoori chicken. So he enjoyed eating it for many years, thinking that the pink or reddish color on the chicken was from spices such as curcumin, paprika, or cayenne pepper. However, to his surprise, he found that this color is due to the addition of red food coloring. It is a simple fact; if you want your tandoori chicken to be a bright red shade, food coloring is the only way to do it. Only food coloring and not cayenne pepper or paprika can bind covalently to the meat proteins and stain them strongly.

The author is now aware that food coloring can cause the following issues, as shown in Figure 6: (1) a failure in oral tolerance; (2) interference with digestive enzymes; (3) enhanced intestinal permeability; (4) liver toxicity; (5) mitochondrial dysfunction; (6) food immune reactivity; (7) hypersensitivity; (8) atop dermatitis; (9) allergic rhinitis, asthma, and angioedema; (10) neurobehavioral disorders; (11) interference with neurotransmission; and (12) reproductive abnormalities. Therefore, from now on, he will no longer fall prey to the deceptive lure of the color of his lollipop, medications, sorbet, and soft drinks, or—to his sadness—even his tandoori chicken.

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Immune Reactivities Against Gums

Aristo Vojdani, PhD, MSc, CLS; Charlene Vojdani, MA

ABSTRACT

Context • Different kinds of gums from various sources enjoy an extremely broad range of commercial and industrial use, from food and pharmaceuticals to printing and adhesives. Although generally recognized as safe by the US Food and Drug Administration (FDA), gums have a history of association with sensitive or allergic reactions. In addition, studies have shown that gums have a structural, molecular similarity to a number of common foods. A possibility exists for cross-reactivity.

Objective • Due to the widespread use of gums in almost every aspect of modern life, the overall goal of the current investigation was to determine the degree of immune reactivity to various gum antigens in the sera of individuals representing the general population.

Design • The study was a randomized, controlled trial.

Participants • 288 sera purchased from a commercial source.

Outcome Measures • The sera was screened for immunoglobulin G (IgG) and immunoglobulin E (IgE) antibodies against extracts of mastic gum, carrageenan, xantham gum, guar gum, gum tragacanth, locust bean gum, and β-glucan, using indirect enzyme-linked immunosorbent assay (ELISA) testing. For each gum antigen, inhibition testing was performed on the 4 sera that showed the highest IgG and IgE immune reactivity against the different gums used in the study. Inhibition testing on these same sera for sesame albumin, lentil, corn, rice, pineapple, peanut, pea protein, shrimp, or kidney bean was used to determine the cross-reactivity of these foods with the gum.

Results • Of the 288 samples, 4.2%-27% of the specimens showed a significant elevation in IgG antibodies against various gums. Only 4 of 288, or 1.4%, showed a simultaneous elevation of the IgG antibody against all 7 gum extracts. For the IgE antibody, 15.6%-29.1% of the specimens showed an elevation against the various gums. A significant percentage of the specimens, 12.8%, simultaneously produced IgE antibodies against all 7 tested extracts.

Conclusions • Overall, the percentage of elevation in IgE antibodies against different gum extracts, with the exception of carrageenan, was much higher than for the IgG antibody. The results of the current study showed that a subgroup of healthy individuals who produced not only IgG but also IgE antibodies against various gums may suffer from hidden food immune reactivities and sensitivities. Further study is needed to examine the clinical importance of gums and cross-reactive food antibodies in symptomatic individuals. (Altern Ther Health Med. 2015;21(suppl 1):64-72.)

Aristo Vojdani, PhD, MSc, CLS, is the CEO of Immunosciences Labs, Inc, in Los Angeles, California, and a faculty member in the Department of Preventive Medicine at Loma Linda University in Loma Linda, California. Charlene Vojdani, MA, is a laboratory assistant at Immunosciences Lab, Inc.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com

Humans have been using gum since the first caveman discovered that he derived pleasure from chewing the hardened sap that came out of trees and other plants. Since then, gum use has evolved into far more than only exercising jaws, blowing bubbles, or sticking shoes to the pavement. Today, gums have very wide industrial uses, including being employed in the food industry as stabilizers, thickening agents, gelling agents, emulsifiers, and fixing agents in foods and soft drinks and in the printing, textile, pottery, lithography, cosmetics, and candy manufacturing industries as adhesives and binding agents. Together with natural resins and latexes, gums form a group of perhaps the most widely used and traded category of nonwood forest products that are not consumed directly as foods, fodders, or medicines. In 1993 alone, the world market for gums as food additives was approximately $10 billion. This amount does not include nonfood uses for gums.

As early as 1982, it had already been demonstrated that various gums are highly antigenic, meaning that these substances are capable of eliciting an immune response comparable with those of protein antigens. Despite this fact, no serious attempt has been made to measure immunoglobulin G (IgG) and immunoglobulin E (IgE) antibodies in the blood of healthy subjects. Due to the extensive use of gums in almost every single product made, the overall goal of this
investigation was to determine the degree of immune reactivity to various gum antigens in 288 healthy individuals representing the general population.

Currently, gums that are generally recognized as safe by the US Food and Drug Administration (FDA) are labeled as such by the industries that submit materials to that agency. However, in conjunction with the extensive use of gums in almost every single product made, many work-related illnesses have been reported in the literature. For example, as early as 1933, a case of asthma caused by acacia gum was described. Later, occupational asthma among printers exposed to gum dust was repeatedly reported in more than 50 patients, some of whom exhibited positive skin tests to acacia, tragacanth, and gum arabic but not to gum karaya. Airborne exposure to these gums causes sensitization leading to allergic rhinitis, asthma, and urticaria.

The first thoroughly authenticated case report of clinical allergy caused by sensitivity to gum tragacanth was released in 1941, detailing how in 1939 a perfectly healthy 26-year-old female went to work in a New York gum factory where gum tragacanth dust freely proliferated in the air. Starting with what at first appeared to be a persistent head cold, the woman’s health grew increasingly worse until, in 1941, she was suffering severe attacks of asthma that sometimes lasted for hours. Direct skin tests and passive transfer tests proved strongly positive for gum tragacanth and gum arabic but not gum karaya.

A case report from 2007 featured a 52-year-old male who took a slimming aid that contained guar gum. He first experienced generalized urticaria, then severe anaphylactic reaction, including circulatory collapse. Skin prick tests pinpointed guar and/or carob gum as the likely culprits. This variation in skin-testing results was described to be associated with antigenic variability between the gums.

To document the prevalence of immune reactivity and allergic symptoms in the work environment, 162 workers of a carpet manufacturing plant were tested; results demonstrated a 5% prevalence of skin reactivity to guar gum. Despite this IgE-mediated immune reactivity, no serious attempt was made to measure the IgG, IgA, or IgM isotype antibodies to discover the degree of immune reactivities against various gums with which the human body is in contact on a daily basis. One study, however, described 3 individuals, one who worked for a pharmaceutical company and a second and third who were employed at a carpet manufacturing plant. For the 3 individuals, occupational asthma caused by exposure to guar gum was demonstrated, together with IgE and IgG specific to the product.

Various types of natural gums are used in industry. Gum tragacanth is widely used in the printing industry, whereas guar gum is used extensively in numerous pharmaceutical and food products, such as lotions and creams, soups, salad dressings, ice cream, cheese, baby foods, and preserved foods. The pharmaceutical industry also uses gums as excipients or drug delivery agents and focuses particularly on natural gums because they are nontoxic, stable, easily available, less expensive, and less subject to regulatory issues than their synthetic polymer counterparts.

**THE STRUCTURE OF GUMS**

From a chemical, structural point of view, most gums are composed of complex and variable mixtures of oligosaccharides, polysaccharides, and glycoproteins, with a polysaccharide that has an extremely high molecular weight that is attached to a hydroxyproline-rich, polypeptide backbone that accounts for approximately 2% of the molecular size. Together, these substances form a huge molecular structure with the size of 200 to 2000 kDa, consisting of 4000 to 40 000 amino acids. For comparison, insulin is composed of 110 amino acids; β-casein, the major protein found in human milk, is composed of 199 amino acids; human serum albumin (HSA) consists of 609 amino acids; and α-gliadin ranges from >200 to >300 amino acids. Therefore, if the partially digested molecules of certain gums manage to get into the circulatory system, they would induce a very strong immune response that would result in very high levels of IgG, IgA, or IgE antibodies against the gum molecules.

**TYPES OF GUMS**

Natural gums, such as mastic gum, gum arabic, carrageenan, guar gum, locust bean gum, and gum tragacanth are mainly derived from the woody elements or seed coatings of various plants. These gums may be grouped into seed gums, exudate gums, marine gums, and microbial gums. Synthetic gums are basically the products of chemistry and laboratories.

**Seed Gums**

Seed galactomannans, generally known as seed gums, are vegetable, heterogeneous, storage polysaccharides composed of galactopyranosyl (Gal) and mannopyranosyl (Man) residues. Galactomannans have attracted considerable academic attention as well as industrial interest due to their property of forming viscous solutions or gels in aqueous media. They can thus be used in different forms for human consumption. The different chemical properties of these gums make them versatile materials for many different applications. Some of these important properties include holding H₂O, thickening, gelling, binding, suspending, and emulsifying as well as forming films. These characteristics have opened avenues for gums to be used in various industries, such as paper, textiles, petroleum, drilling, pharmaceutics, food, cosmeceuticals, and explosives.

The 2 galactomannans of major commercial importance are guar gum from *C. tetragonolobus* and carob or locust bean...
gum from Ceratonia siliqua. Carob gum forms gels at higher concentrations, the interaction of carob with a little xanthan produces a gelling that is used in commercial applications. The scope of the varied applications using seed gums that are obtained from different sources is huge.16

Guar Gum. Guar gum, also called guaran, is primarily the ground endosperm of guar beans. The guar seeds are dehusked, milled, and screened to obtain the gum.1 It is typically produced as a free-flowing, off-white powder and is not self-gelling, although it forms viscous solutions.1 However, it can be cross-linked with either borax or calcium to cause it to gel.18 Its chemical structure consists of repeating units of guar galactomannan.

Carob Gum. Carob or locust bean gum is the whitish powder obtained from grinding the endosperm of the seeds of C. siliqua, a tree widely cultivated in the Mediterranean region.1 It consists mainly of galactomannan-type polysaccharides, with a galactose:mannose ratio of approximately 1:4.19 It is employed in a wide range of products, among the most important of which are ice cream, baby foods, and pet foods. It is also used in soups, sausage products, soft cheeses, bakery products, pie fillings, powdered desserts, sauces and salad creams, and dairy products other than ice cream. It can be added during the paper-making process to improve the physical characteristics of the paper. In the textile industry, locust bean is used either alone or in combination with starch and synthetics as a sizing agent for cotton and other natural fibers. It is also used as a print-paste thickener in both roller and screen-printing to help provide greater purity and uniformity of shades and deeper penetration of dyes. Other minor uses include incorporation in oil-drilling fluids and some pharmaceutical and cosmetics applications.1

Exudate Gums

The previous reference in this article to early humans deriving pleasure from chewing the hardened sap that oozed out of trees referred to exudate gums. Exudate gums are obtained as the natural secretions of different tree species; when the bark of certain trees and shrubs is injured, an aqueous gum solution is exuded to seal the wound, preventing infection and dehydration of the plant.20 The exudates exhibit unique properties in a wide variety of applications. Five thousand years ago, they were already being used as thickening and stabilizing agents. Exudate gums have been important items of international trade in the food, pharmaceutical, adhesive, paper, textile, and other industries for centuries.20

Gum Arabic. The exudate known as gum arabic is the oldest and most well known of all natural gums, deriving its name from its place of origin. As far back as the 3000 BC, the ancient Egyptians were shipping it as an article of commerce. It was used as a binder and adhesive for pigments, paints, inks, and cosmetics and even for flaxen wrappings for mummies.20 Strictly speaking, gum arabic is defined by the Food and Agriculture Organization and World Health Organization's Joint Expert Committee for Food Additives as "a dried exudate obtained from the stems and branches of Acacia Senegal or Acacia seyal." The term is now applied more loosely to other gums produced by other Acacia species and, thus, the substance is sometimes also referred to as gum acacia or acacia gum.

Gum Tragacanth. Tragacanth gum is the dried exudate produced by tapping the taproot and branches of certain shrubby species of Astragalus, particularly those that occur wild in Iran and Turkey. The gum is exported from the country of origin in ribbon or flake form and has a rather horny texture. Chemically, it is a complex, highly branched, heterogeneous polysaccharide that occurs naturally as a slightly acidic, calcium, magnesium, and potassium salt. It has a molecular mass of approximately 8.4 × 106 Da.20 The most important applications of tragacanth are now in foods and pharmaceuticals. Gum tragacanth consists of 2 fractions. Tragacanthic acid or bassorin is insoluble in water but has the capacity to swell and form a gel. The other fraction is called tragacanthin and is water soluble. Both fractions contain small amounts of proteinaceous material. The tragacanthic acid fraction that swells in water has a high molecular weight and a rod-like molecular shape. The watersoluble tragacanthin is a neutral, highly branched arabinogalactan with a spherical molecular shape.

Mastic. Although usually termed a gum, mastic is a hard resin, produced by tapping the stem bark of the small tree Pistacia lentiscus, which is cultivated on the Greek island of Chios. Mastic is produced in the form of small tears that are pale yellow in color, clear and glassy in nature, and liable to fracture. Its age-long use in Arab countries has been for chewing, where it sweetens the breath and helps preserve the teeth and gums. Its aromatic properties also make it suitable as a flavoring agent for alcoholic beverages. In the past, it was also used in the manufacture of high-grade varnishes for paintings and in medicinal applications. An essential oil can be distilled from the gum and finds some use for fragrance and flavoring purposes.1

Marine Gums

Marine gums are extracted from seaweeds. The cell walls of many seaweeds contain phycocolloids (algal colloids) that can be extracted by hot water. The 3 major phycocolloids are alginates, agars, and carrageenans. These phycocolloids are polymers of chemically modified sugar molecules, such as galactose in agars and carrageenans, or organic acids, such as mannuronic acid and glucuronic acid in alginates. Most phycocolloids can be safely consumed by humans and other animals, and many are used in a wide variety of prepared foods, such as ready-mix cakes, instant puddings and pie fillings, and artificial dairy toppings.21

In the general market for polysaccharides, phycocolloids compete with seed gums, such as guar gum and locust bean gum; plant exudates, such as gum arabic, pectin, and other plant extracts; starch and cellulose derivatives; and various biosynthetic gums. They frequently offer distinct chemical and economic advantages.21

Carrageenans. Carrageenans are extracted from various red algae, including Eucheuma in the Philippines; Chondrus, also called Irish moss, in the United States and the Canadian
maritime provinces; and *Iridaea* in Chile. They are used for thickening and stabilizing dairy products, imitation creams, puddings, syrups, and canned pet foods. They are also used in the manufacture of shampoos, cosmetics, and medicines.

**Microbial Gums**

Microbial polysaccharide gums are as natural as seed, exudate, and marine gums. They are the product of the natural process of fermentation, involving bacteria, fungi, and some plants. However, this natural process is generally induced, augmented, and highly controlled in a laboratory, so that some refer to microbial gums, such as xanthan gum, as synthetic or at least semisynthetic. For example, xanthan gum is a polysaccharide produced by a pure culture fermentation of a carbohydrate with *Xanthomonas campestris*, and it is composed of glucose, glucuronic acid, 6-acetylmannose, and 4,6-pyruvylated mannose residues.

**Xanthan Gum.** Xanthan gum is a natural polysaccharide and an important industrial biopolymer. It was discovered in the 1950s at the Northern Regional Research Laboratories of the United States Department of Agriculture. The polysaccharide B-1459, or xanthan gum, produced by the bacterium *X campestris*, was extensively studied because of its properties that allow it to supplement other known natural and synthetic water-soluble gums. Extensive research was carried out in several industrial laboratories during the 1960s, culminating in semicommercial production as Kelzan by Kelco. Substantial commercial production began in early 1964.

**β-Glucan.** β-Glucans are produced by fungi, yeasts, grains, and seaweed. β-Glucans are the constituents of the cell wall of certain pathogenic bacteria—*Pneumocystis carinii* and *Cryptococcus neoformans*—and fungi—*Aspergillus fumigatus*, *Histoplasma capsulatum*, *Candida albicans*, and *Saccharomyces cerevisiae*. The main components of the fungal cell wall are polysaccharides and glycoproteins. It is used both in the formulation of cosmetics and as a direct food additive.

**CROSS-REACTIVITY**

Because sensitization to gums, such as gum arabic, occurs through a carbohydrate-rich structure and a repetitive polysaccharide sequence, cross-reaction to other carbohydrate-containing molecules has been reported. For example, when preliminary investigation about the structure of gum arabic was made by high-performance liquid chromatography, it was shown that the gum arabic glycoprotein had an 8% to 15% similarity to oligosaccharides of horseradish peroxidase that is found in the roots of horse radish and pineapple bromelain. Further, these cross-reactive carbohydrate determinants (CCDs) that contain fucose and xylose exist in almost all plant extracts.

The existence of the CCDs containing fucose and/or xylose in the pollen of the olive tree, Japanese cedar, and Bermuda grass and in glycoproteins of celery, potato, tomato, bean, soybean, and pea have been shown in numerous studies. Based on these and more recent studies, it was concluded that immune reactivity to gum carbohydrate structures occurs in patients allergic to pollen, without obvious exposure to gums. This cross-reactivity between the repetitive polysaccharide sequences of gums with plant enzymes, such as horseradish peroxidase or bromelain, pollens, trees, celery, potato, tomato, beans, and pea led the research team to believe that individuals may produce IgG or IgE antibodies against the CCD of gums that cross-react with many other food and environmental antigens.

**MATERIALS AND METHODS**

Sera from 288 healthy individuals of different ethnicities, aged 18 to 65 years, were obtained from Innovative Research, Inc (Southfield, MI, USA). Of that total, 144 were males with a median age of 35.5 years, and 144 were females with a median age of 36.2 years. These individuals were qualified to donate blood based on a health questionnaire provided by the FDA. Each individual at the time of the blood draw did not exhibit any health complaints. Prior to shipping, each blood sample was tested according to FDA guidelines for the detection of hepatitis B surface antigen, antibodies to HIV, antibodies to hepatitis C, HIV-1 ribonucleic acid (RNA), hepatitis C RNA, and syphilis.

**Process**

Mastic Gum, carrageenan, xantham gum, guar gum, gum tragacanth, locust bean gum, and β-glucan were purchased from Sigma Aldrich (Saint Louis, MO, USA). Ten grams of each gum were extracted in 500 mL of buffer pH 4.6 by mixing it for 8 hours at 25°C on a magnetic stirrer. The solution was centrifuged at 20,000 × g, and supernatant was removed and concentrated by a factor of 10 using an Amicon filter. The protein concentration was measured using a kit provided by Bio-Rad (Hercules, CA, USA). All extracts were aliquoted and stored frozen at -20°C until used.

Different gum extracts were dissolved in 0.1M phosphate buffered saline (PBS). These antigens were diluted 1:50 in 0.1M carbonate buffer pH 9.2, and 100 µL of each gum antigen was added to different wells of the microtiter plate coated with gum-specific antibodies and incubated overnight at 4°C. After washing, the unoccupied sites in the wells were saturated by adding 200 µL of 2% bovine serum albumin (BSA) and incubated overnight at 4°C. Plates were washed again, and 100 µL of the 288 sera, with a dilution of 1:4 for determination of the level of IgE and 1:400 for level of IgG, were added to duplicate wells of each microtiter plate and incubated for 4 hours at room temperature.

This procedure was followed by washing and addition of optimal dilution of alkaline phosphatase-labeled antihuman IgE to 1 set and antihuman IgG to a different set of plates, followed by incubation for 1 hour at room temperature. After another washing and the addition of the substrate paranitrophenyl phosphate to the wells, the color development was measured at 405 nM. Four different sera from patients with known allergy to gums and 4 individuals with no known allergy to gums were used as positive and negative controls. Several wells coated with unrelated proteins such as HSA, rabbit serum albumin, and BSA were used only for the
determination of background in the enzyme-linked immunosorbent assay (ELISA).

Inhibition Study

ELISA inhibition was performed with 4 different sera that showed the highest IgG and IgE immune reactivity against different gums used in the study. A total of 2 mL of each serum pre-diluted 1:4 for IgE and 1:100 for IgG determination was pre-incubated with 100 µg of either HSA or 100 µg extracts of mastic gum, carrageenan, xanthan gum, guar gum, gum tragacanth, locust bean gum, β-glucan, sesame albumin, lentil, corn, rice, pineapple, peanut, pea protein, shrimp, or kidney bean for 2 hours at 37°C and 2 hours at 24°C. After centrifugation at 3000 × g, 100 µL of each supernatant was transferred to the microtiter plate coated with the specific gum extract. After 4 hours incubation at 24°C the ELISA procedure was continued according to the standard procedure. Results were expressed as percent inhibition after subtraction of optical density (OD) from HSA.

Statistical Analysis

Coefficients of intra-assay variation were calculated by running 5 samples 8 times within a single assay. Coefficients of interassay variation were determined by measuring the same samples in 6 consecutive assays. This replicate testing established the validity of the ELISA assays, determined the appropriate dilution with minimal background, and detected serum IgG and IgE against different gums. Coefficients of intra- and interassay variations for IgG and IgE against all tested antigens and peptides were lower than 15%.

RESULTS

ELISA Testing

Using indirect ELISA testing, the 288 samples of sera were screened for IgG and IgE antibodies against extracts of mastic gum, carrageenan, xanthan gum, guar gum, gum tragacanth, locust bean gum, and β-glucan.

IgG Antibody Response. For 60 of the 288 tested specimens, the data for the IgG antibody with the 7 groups of antigens is summarized in Figures 1 through 5. These figures show a significant variation in IgG antibody response against various gum extracts. At 2 standard deviations above the mean of the 288 specimens, 4.2% to 27.1% of them showed a significant elevation in IgG antibodies against various gums (Table 1). The lowest percentage of IgG elevation was observed against guar gum in 12 of the 288 individuals (4.2%). The highest percentage of elevation of IgG was detected against carrageenan. Seventy-eight of 288 specimens (27.1%) reacted against carrageenan. Interestingly, although some individuals reacted against 1 or more gum antigens, only 4 of 288 (1.4%) showed simultaneous elevation in IgG antibody against all 7 gum extracts.

IgE Antibody Response. IgE-specific antibody reactivity against various gum extracts for the same 60 individuals is presented in Figures 6 through 10. At the cutoff of 2 standard deviations above the mean, 29.1%, 19.8%, 16.6%, 17.7%,
Optical density at 405 nM for samples from patients 1 through 12, tested for IgE against chewing gum + mastic gum + gum arabic, carrageenan, xanthan gum, gum guar, gum tragacanth, locust bean gum (carob bean gum), and β-glucan.

Abbreviation: IgE, immunoglobulin E.

Optical density at 405 nM for samples from patients 25 through 36, tested for IgE against chewing gum + mastic gum + gum arabic, carrageenan, xanthan gum, gum guar, gum tragacanth, locust bean gum (carob bean gum), and β-glucan.

Abbreviation: IgE, immunoglobulin E.

Optical density at 405 nM for samples from patients 49 through 60, tested for IgG against chewing gum + mastic gum + gum arabic, carrageenan, xanthan gum, gum guar, gum tragacanth, locust bean gum (carob bean gum), and β-glucan.

Abbreviation: IgG, immunoglobulin G.

Optical density at 405 nM for samples from patients 13 through 24, tested for IgE against chewing gum + mastic gum + gum arabic, carrageenan, xanthan gum, gum guar, gum tragacanth, locust bean gum (carob bean gum), and β-glucan.

Abbreviation: IgE, immunoglobulin E.

Optical density at 405 nM for samples from patients 25 through 36, tested for IgE against chewing gum + mastic gum + gum arabic, carrageenan, xanthan gum, gum guar, gum tragacanth, locust bean gum (carob bean gum), and β-glucan.

Abbreviation: IgE, immunoglobulin E.
we performed similar inhibition studies with these gum extracts as well. We found that addition of different gum extracts also caused a significant inhibition in IgE reactivity against specific gum extracts. For example, addition of mastic gum to a highly-reactive sera with mastic gum, resulted in 82.3% inhibition when the same sera was absorbed with mastic gum and reacted with mastic gum coated plate. Repeating similar experiments by replacing mastic gum with carrageenan during absorption and then reacting the sera against mastic gum coated plate resulted in 56.7% inhibition of IgE reactivity of mastic gum antibody against mastic gum antigens (Table 2). Similarly, mastic gum caused 48.5% inhibition in carrageenan-anticarrageenan antibody reactivity. This is in comparison with 85.2% inhibition when carrageenan was added to sera with highly IgE-reactive against carrageenan. Similar to inhibition study with different gums, different food antigens were used for absorption of gum-specific IgE antibodies. Addition of these food antigens to the liquid phase and testing gum IgE reactive sera against gum-antigen-coated plates resulted in a significant inhibition of antigen-antibody reaction. This inhibition of gum IgE antibody by different food antigens was the highest when sera were absorbed with pineapple. As is shown in Table 2, pineapple caused between 39.7% and 53.7%; sesame, 28.6% and 41.2%; and kidney bean, 19.5% and 26.8% in inhibition of IgE reactivity against various gum antigens.

**Inhibition Study for IgG Antibodies**

Inhibition studies were performed with specific, non-specific gum extracts and other possible cross-reactive food antigens such as sesame albumin, lentil, corn, rice, pineapple, peanut, pea protein, shrimp and kidney bean, to demonstrate the specificity of the IgG and IgE antibodies detected against different gum extracts. In comparison with HSA, which caused only between 1.3% and 7.2% inhibition of IgE antibody reactivity, the addition of specific gum antigens to the sera resulted in 76.9% to 86.5% inhibition in antigen-antibody reaction (see Table 1). A significant percentage (12.8%) simultaneously produced IgE antibodies against all 7 tested extracts. Overall, this percentage elevation in IgE antibodies against different gum extracts was much higher than elevation in IgG antibodies ($P < .011$), with the exception of carrageenan.

**DISCUSSION**

Inspired by the detection of IgG against guar gum by Lagier et al., the current study measured IgG and IgE antibodies against different gum extract preparations in 288 healthy individuals.

### Table 1. Percentage Elevation in IgG and IgE Antibodies Against Various Gum Antigens in Sera of 288 Healthy Individuals, at 2 SDs Above the Mean

<table>
<thead>
<tr>
<th></th>
<th>Mastic Gum (%)</th>
<th>Carrageenan (%)</th>
<th>Xanthan Gum (%)</th>
<th>Guar Gum (%)</th>
<th>Gum Tragacanth (%)</th>
<th>Locust Bean Gum (%)</th>
<th>β-Glucan (%)</th>
<th>Simultaneous Elevation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgG</strong></td>
<td>42/288 (14.6)</td>
<td>78/288 (27.1)</td>
<td>30/288 (10.4)</td>
<td>12/288 (4.2)</td>
<td>15/288 (5.2)</td>
<td>33/288 (11.5)</td>
<td>18/288 (6.2)</td>
<td>4/288 (1.4)</td>
</tr>
<tr>
<td><strong>IgE</strong></td>
<td>84/288 (29.1)</td>
<td>57/288 (19.8)</td>
<td>48/288 (16.6)</td>
<td>51/288 (17.7)</td>
<td>48/288 (16.6)</td>
<td>45/288 (15.6)</td>
<td>66/288 (22.9)</td>
<td>37/288 (12.8)</td>
</tr>
</tbody>
</table>

Note: $P = .011$.

Abbreviations: IgG, immunoglobulin G; IgE, immunoglobulin E; SD, standard deviation.
Depending on the gum's structure, the research team found significant immune reactivity against various gum extracts. For example, an IgG response against gum tragacanth was found in 15.6% of tested individuals, indicating that not only is a significant percentage of the healthy population exposed to various gum products, but also that these individuals immunologically react against them and produce non-IgE-mediated immune reactivity. In relation to IgE antibodies, a much higher percentage of the sera tested not only reacted immunologically against various gum antigens but also produced a significant level of IgE antibodies. Elevations in levels were detected against locust-bean gum in 15.6% of tested individuals and against mastic gum in 29.1% (Table 1). Although the research team did not have information about hypersensitivity to various allergens and antigens for the individuals whose sera was tested, it seems that these IgE antibodies could have contributed to allergy and sensitivity in them.

Indeed, when the team conducted inhibition studies to demonstrate the specificity of these antigen-antibody reactions, the addition of specific gums to sera with high titers of IgG antibody caused more than 69% inhibition in the antibody titers, whereas the addition of nonspecific food antigens caused only 5% to 14% inhibition in the antibody titers. Inhibition studies conducted with gum-specific, IgE-containing sera, in addition to inhibition of IgG antibody caused more than 69% inhibition in the antibody titers, indicated that not only is a significant percentage of the healthy population exposed to various gum products, but also that these individuals immunologically react against them and produce non-IgE-mediated immune reactivity. In relation to IgE antibodies, a much higher percentage of the sera tested not only reacted immunologically against various gum antigens but also produced a significant level of IgE antibodies. Elevations in levels were detected against locust-bean gum in 15.6% of tested individuals and against mastic gum in 29.1% (Table 1). Although the research team did not have information about hypersensitivity to various allergens and antigens for the individuals whose sera was tested, it seems that these IgE antibodies could have contributed to allergy and sensitivity in them.

### Table 2. Demonstration of specificity through percentage of ELISA inhibition of antigen-antibody reaction by the addition of specific and nonspecific inhibitors to the serum prior to testing.

<table>
<thead>
<tr>
<th>INHIBITORS</th>
<th>Mastic Gum</th>
<th>Carrageenan</th>
<th>Xanthan Gum</th>
<th>Guar Gum</th>
<th>Tragacanth Gum</th>
<th>β-Glucan</th>
<th>Sesame Albumin</th>
<th>Lentil</th>
<th>Corn</th>
<th>Rice</th>
<th>Pineapple</th>
<th>Peanut</th>
<th>Pea Protein</th>
<th>Shrimp</th>
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Abbreviation: ELISA, enzyme-linked immunosorbent assay.
REFERENCES

ACKNOWLEDGMENTS

CONCLUSIONS

The research team concluded that the subgroup of individuals who appear to be symptom free but have IgG- and IgE-mediated immune reactivities against gums should be put on diets that remove these gums, and that serious consideration should also be given to removing foods that cross-react with these gums. Otherwise, patients may continue to suffer from hidden allergies and food immune reactivities, even after the elimination of the specific offending food antigens.38-41

The team strongly encourages further clinical research with symptomatic individuals to examine whether gum reactivity contributes to multiple food and chemical reactivities and strongly urges that the correlation between inhalation-induced reactivity and ingestion-induced reactivity be given serious study.

ACKNOWLEDGMENTS

The research team would like to acknowledge Joel Bautista for his wonderful figures and for his extensive efforts toward making this manuscript suitable for publication.

REFERENCES

ABSTRACT

Context • Certain individuals are sensitive enough to react to peanuts and peanut oil, sometimes with deadly effect. It is thus crucial to have an accurate testing methodology for the assessment of allergies and immune reactivities to peanuts and their components, such as agglutinins and oleosins. Currently, skin-prick testing is performed only with the water-soluble components of peanut proteins and can produce false negatives. Testing with all possible food antigens and with both immunoglobulin G (IgG) and immunoglobulin E (IgE) antibodies may offer a more accurate alternative.

Objective • The research team intended to measure IgG and IgE antibodies against peanut proteins, agglutinins, and oleosins to identify variations in IgG and IgE immune reactivities to these antigens among the general population.

Design • Sera from 288 healthy individuals—144 males of different ethnicities, aged 18-65 y with a median age of 35.5 y, and 144 females of different ethnicities, aged 18-65 y with a median age of 36.2 y—were obtained from Innovative Research, Inc. Four sera from patients with a known allergy to peanuts and 4 sera from individuals with no known allergy to peanuts were used as positive and negative controls. Several wells in the microtiter plate were coated with unrelated proteins, such as human serum albumin, rabbit serum albumin, and bovine serum albumin and used only for the determination of any background in the enzyme-linked immunosorbent assay (ELISA).

Setting: Immunosciences Lab, Inc, Los Angeles, CA, USA.

Outcome Measures • The sera were screened for peanut-specific IgG and IgE antibodies against water-soluble proteins of peanut, peanut agglutinins, and peanut oleosins, using the ELISA. Color development was measured at 405 nM. For demonstration of the specificity of the antibodies, inhibition ELISA was performed with 4 sera that had very high levels of IgG and IgE antibodies.

Results • Using mean values as the cutoff, 19%, 17%, and 22% of the specimens tested for IgG antibodies and 14%, 11%, and 14% of the specimens tested for IgE antibodies produced high levels of antibodies against peanut proteins, agglutinins, and oleosins, respectively.

Conclusions • The study’s findings support the proposition that IgE sensitization to foods may not necessarily coincide with positive prick tests to commercial extracts. Falsely negative skin testing or IgG, IgA, or IgE antibody testing is often linked to the nature of the preparation of the food antigens and their use in in-vivo and in-vitro testing. The study’s results support the need to improve the quality of food extracts used in the diagnosis of allergies and immune reactivity to nuts and seeds. Testing should use all possible food antigens and measure both IgG and IgE antibodies. (Altern Ther Health Med. 2015;21(suppl 1):73-79.)

Immune Reactivities to Peanut Proteins, Agglutinins, and Oleosins

Aristo Vojdani, PhD, MSc, CLS

Aristo Vojdani, PhD, MSc, CLS, is the CEO of Immunosciences Labs, Inc, in Los Angeles, California, and a faculty member in the Department of Preventive Medicine at Loma Linda University in Loma Linda, California.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com

Peanuts are among the most popular of many types of plant seeds that are consumed worldwide as snacks, toppings, garnishes, flavorings, and ingredients. Peanuts can be found in movie-house concessions, on cakes, and in candy and ice cream. They are also a source of highly useful plant oil. Unfortunately, this widespread use means that peanuts will inevitably fall into the hands of the small percentage of the population who are sensitive enough to react to them, sometimes with deadly effect. It is thus crucial, even vital, to have an accurate testing methodology for the assessment of allergies and immune reactivities to the peanut
and its allergenic components, such as proteins, oleosins, and agglutinins.

The energy in plant tissues is preserved in the form of proteins, carbohydrates, and lipids. These substances are stored in their own, small, subcellular particles that are called protein bodies, starch granules, and oil bodies, respectively.

Oil bodies are also called lipid bodies or oleosomes. They are stably packed in aqueous environments (ie, the cystolic compartment of seed cells). Seeds and nuts are widely used in the food industry, mostly because they contain 20% to 30% proteins as well as 30% to 50% oils in the form of oleosomes. These oil bodies are composed of a core of oil molecules or triacylglycerol (TAG), surrounded by a phospholipid monolayer containing densely packed proteins called oleosins. These oleosins are relatively small proteins of 15 to 25 kDa.

Oleosins play a key role in the stability of seed-oil bodies through electrostatic repulsion and steric hindrance (ie, the prevention or retardation of inter- or intramolecular interactions as a result of the spatial structure of a molecule). This structural function prevents freeze- or thaw-induced damage and the coalescence (increased temperature) of oleosomes during seed dessication.

Further, oleosins are suggested to be bifunctional enzymes that have both monoacylglycerol-acyltransferase and phospholipase activities, which play a role in seed maturation.

The oleosins are composed of different domains; the hydrophobic domain anchors the oleosins to the TAG core, whereas the hydrophilic termini reside on the surface. Vegetable cooking oils commonly extracted from various oily seeds are made out of the TAG molecules that tend to separate from the aqueous solution and form a transparent layer on the surface of the liquid phase.

An agglutinin is a substance that causes particles to coagulate to form a thickened mass. Agglutinins act like antibodies that cause antigen aggregation. They can also be substances other than antibodies, such as sugar-binding protein lectins. Lectins are carbohydrate-binding proteins first discovered in plants and now generally known to be present throughout nature. It is estimated that approximately 30% of our foods contain lectins, some of which may enter our circulation because of their resistance to digestive enzymes. Incompletely digested lectins may affect our health.

Food immune reactivity involves mainly the protein components of foods or food antigens. Even minute quantities of these proteins, when ingested by sensitive individuals, are capable of provoking severe reactions. Vegetable oils produced from a diverse range of plant species contain proteins that can be highly allergenic. Production involves the pressing of plant seeds, followed by a series of purification steps to refine the oils to the desired degree. Although this refining process results in an almost complete removal of proteins, sometimes enough proteins remain in an oil to provoke a reaction in a sensitive individual. Therefore, ascertaining the protein content of various oils is important for the following reasons:

**Risk Assessment.** The identity of the protein component that is responsible for the potentially allergenic properties of a food and the amount of that component present in an oil are crucial for risk assessment.

**Safety of Oil Products.** Information about the protein content of oils, detected by refined methodologies, can help to establish thresholds of immune reactivities and, therefore, can contribute to the safety of the oil products.

Currently, not much data are available regarding the safe levels of allergens in oils such as soy, maize, sunflower, sesame, and palm oils. Peanut oil, however, has been most thoroughly studied as a major allergen, and in its unrefined form, it can provoke severe reactions in some individuals. For example, when allergic individuals were exposed to 10 to 50 mg of peanut flour, an allergic response was not observed. But when these highly sensitive individuals were exposed to 100 mg of peanut flour, a significant allergic response was observed. Because oleosins in the oleosomes make up approximately 10% of the total seed protein, the threshold of safety for these proteins is only approximately 1.4 mg.

Refined vegetable oils are used in a wide variety of food products. The use of these oils may change from time to time and from product to product. However, several oils derived from plants, such as peanuts, soy beans, and sesame seeds, have been recognized as potent antigens and allergens. As a result, a serious ongoing discussion is occurring about labeling each oil individually based on its allergenic risk.

Due to their protein structure and strong antigenicity, oleosins potentially are hidden allergens in refined oils and oil-derived products, capable of eliciting immunoglobulin E (IgE) and non-IgE-mediated immune reactivity. In fact, sera from patients who suffer from allergies to seeds and nuts have reacted with oleosin proteins from different nuts and seeds in different oils. Because oleosins are hidden and their epitopes are buried in the inner molecules, the preparation of most commercial nut and seed extracts actually does not remove the oleosins from the oils.

However, because all oleosins are fat soluble, not water soluble, an allergy testing method that uses nut and seed extracts prepared in aqueous solution would be using a preparation that actually does not contain oleosins. Therefore, for testing immune reactivities to oleosins, they must either be synthesized or purified through sophisticated biochemical steps.

Peanut oleosins have been studied extensively compared with other oleosins. For this reason, the current study measured immunoglobulin G (IgG) and IgE antibodies against peanut proteins, agglutinins, and oleosins to demonstrate variations in IgG and IgE immune reactivities to these antigens among the general population.

**METHODS**

Sera from 288 healthy individuals who were qualified to donate blood—144 males of different ethnicities, aged 18 to 65 years with a median age of 35.5 years, and 144 females of different ethnicities, aged 18 to 65 years with a median age of...
Preparation of Peanut Antigens

Peanut agglutinin was purchased from Sigma Chemicals (St Louis, MO, USA). Peanut antigens were prepared from products purchased from the supermarket in both raw and roasted form. For that preparation, 10 g of mixed raw and roasted peanuts were put in a food processor using 0.1 M of phosphate buffer saline (PBS) at pH 7.4. The mixer was turned on and off for 1 hour and then kept on the stirrer overnight at 4°C. After centrifugation at 20 000 × g for 15 minutes, the top layer, which contained oil bodies, was discarded. The liquid phase was removed and dialyzed against 0.01 M of PBS using dialysis bags, with a cutoff of 6000 kDa. Dialysis was repeated 3 times to make sure that all small molecules were removed. After dialysis, protein concentrations were measured using a kit provided by Bio-Rad (Hercules, CA, USA).

Purification of Peanut Oleosin

To purify the oleosin from the peanuts, the peanuts were prepared according to the method described above and elsewhere. A total of 100 mL of chloroform/methanol (2/1, v/v) was then added and blended for 2 minutes using a food processor. The mixture was put in a 50-mL tube and centrifuged at 14 000 RPM for 5 minutes. The liquid in the upper phase was filtered through 2 layers of filter paper. The resultant filtrate was collected in multiple glass bottles and dried under a stream of air, with strong continuous agitation. The chloroform/methanol extraction step was repeated twice.

A total of 20 mL of diethyl ether was then added, and the white, solid material stuck on the surface of the glass bottles was detached and resuspended in diethyl ether. At this point, 10 mL of water was added to each bottle, which was centrifuged at 20 000 × g for 5 minutes. The upper diethyl ether layer that contained lipids was removed, and the white, solid, interface material containing the oleosins was collected and transferred to microtubes with a minimum volume of water and diethyl ether.

The microtubes were centrifuged at 20 000 × g for 5 minutes. The interfacial material was exposed to a stream of nitrogen to evaporate the remaining diethyl ether. One mL of chloroform/ethanol (95/5, v/v) was added to the interfacial material in each tube. The contents of each tube were quickly vortexed and transferred to a glass flask.

To separate any protein contaminants from the oleosins, 10 mL of chloroform/methanol (95/5, v/v) was added, and the mixture was filtered through filter paper that was previously rinsed with chloroform/methanol. The filtrate was collected in a flask and dried under a stream of nitrogen. The dried oleosins were dissolved in chloroform/methanol and applied to a Sephadex LH-60 column (Bio-Rad, Hercules, CA) using chloroform/methanol as the solvent. The collected fractions of oleosins were checked by sodium dodecyl sulfate (SDS)-gel electrophoresis.

RESULTS

Sera from 288 individuals were screened for peanut-specific IgG and IgE antibodies against water-soluble proteins of peanut, peanut agglutinin, and peanut oleosin. For the 3 antigens, Figures 1 through 4 summarize the data for IgG antibodies for 98 of the 288 tested specimens. The cutoffs for all testing were at the mean ODs for all 288 specimens. At a cutoff of 0.62 OD for peanut proteins, 55 of the tested specimens (19%) had a significant elevation in those antibodies. At a cutoff of 0.45 OD for peanut agglutinins, 49 of the specimens (17%) showed an elevation in IgG.
antibodies. For peanut oleosin, 63 of the specimens (22\%) exhibited an elevation in IgG antibodies at a cutoff of 0.54 OD. Whereas some individuals reacted only against peanut proteins, and others reacted against only agglutinin or oleosin, only 3 specimens were reactive against combinations of 2 or of all 3 peanut allergens simultaneously.

Data related to IgE-specific antibodies against peanut proteins, agglutinins, and oleosins are presented in Figures 5 through 8. At a cutoff of 0.65 OD, 41 of the tested specimens (14\%), showed an elevation in the levels of IgE antibodies against peanut proteins. Of this 14\%, 4\% had moderate elevations, and the other 10\% had very significant elevations in IgE antibodies. At a cutoff of 0.46 OD, 15 of the tested specimens (5\%) had very significant elevations of IgE antibodies against peanut agglutinins, and an additional 18 (6\%) showed moderate elevations. Finally, at a cutoff of 0.42, 16 (6\%) exhibited very high levels of IgE antibodies for peanut oleosins, and an additional 24 (8\%) showed moderate elevations.

Further analysis of the data showed that almost 50\% of the specimens showed only IgE-specific antibodies against water-soluble components of peanut proteins, whereas the
other 50% showed simultaneous elevations in IgE antibodies against peanut proteins and peanut agglutinin; peanut proteins and peanut oleosin; or peanut proteins, peanut agglutinin, and peanut oleosin. In only 11 of the 288 tested specimens (4%) were the elevations for oleosin antibodies much greater than those for agglutinin or peanut proteins. To demonstrate the specificity of this immune reaction and possible cross-reactivity between peanut proteins, agglutinin, and oleosin, inhibition with the specific and nonspecific antigens was performed. Although the addition of roasted-peanut proteins to 2 different sera with high levels of IgG antibodies against this protein resulted in a 70% to 73% inhibition of IgG reactivity with plates coated with peanut proteins, the addition of peanut agglutinin and oleosin resulted only in 8% to 20% inhibition of this assay (Figure 9).

Although a similar inhibition with specific antigens was observed for inhibition of IgE antibodies, the addition of peanut agglutinin and oleosin resulted in more significant inhibitions of IgE antibodies. For example, the percentage inhibitions for agglutinin and oleosin for one serum were 56% and 33%, respectively, and for a second serum were 43% and 22%, respectively.

Figure 5. Optical density at 405 nM for specimen Nos. 1–24 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.

Figure 6. Optical density at 405 nM for specimen Nos. 25–48 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.

Figure 7. Optical density at 405 nM for specimen Nos. 49–72 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.

Figure 8. Optical density at 405 nM for specimen Nos. 73–96 tested for IgE antibodies against peanut roasted, peanut agglutinin, and peanut oleosin.

Abbreviation: IgE, immunoglobulin E.
DISCUSSION

Food immune reactivities represent an increasingly prevalent problem that has significant adverse effects on an individual's quality of life. Among more than 200 foods that cause immune reactivity, peanut proteins represent a particularly well-known and well-documented threat for sensitive individuals worldwide. Peanuts and peanut butter are favorite foods in homes in the United States as well as in many other countries. Peanuts contain a high percentage of protein (25%-30%), oil (40%-50%), and a lot of valuable nutrients. Because of their high fat content, peanuts are used as an excellent source of oil.

So far, more than 32 peanut allergens that can bind to IgE and IgG have been identified. The majority of these allergens, including peanut agglutinin, are water-soluble glycoproteins and are stable to heat, stomach acid, and enzymatic digestion. However, the hydrophobic proteins called oleosins found in the membranes of oil bodies stored in nuts and seeds are not water soluble. Almost all commercially available peanut antigens are prepared from water-soluble proteins. This fact could be the reason why many cases of anaphylaxis have been reported despite negative results for both in vitro, IgE-specific test and in vivo, skin-prick tests using commercially available antigens.

Because of this issue, the current study put a major emphasis on measuring both IgG and IgE against 3 different groups of peanut antigens: water-soluble proteins, pure peanut agglutinin, and peanut oleosin. First, the current author wanted to find the percentage of healthy individuals who produce such a significant level of IgE antibodies against these antigens that their exposure to them may indicate a risk of exhibiting type 1 allergic reaction. The data summarized in Figures 5 through 8 show that 14%, 11%, and 14% of 288 tested sera exhibited elevation of IgE antibodies against water-soluble proteins, agglutinin, and oleosin in peanuts, respectively. This finding indicates that both peanut oleosin and agglutinin, as peanut allergens, are equally as important as water-soluble peanut proteins and, therefore, in vitro or in vivo testing with water-soluble peanut proteins alone can result in many false-negative results.

Further, researchers have suggested that severe allergic reactions in the face of a lack of detection of IgE antibodies may mean that an anaphylaxis was IgG mediated. Other data have confirmed that the potent immunogenicity of oleosin induces a polyspecific—meaning an IgG, an IgA, and, in some cases, an IgE response—supporting the assumption of anaphylactic reaction by non-IgE antibodies. Precisely for that reason, the current study measured not only IgE but also IgG antibodies against all of the aforementioned peanut antigens. Results presented in Figures 1 through 8 showed that approximately 30% of the antibody-reactive individuals produced both IgG and IgE simultaneously. For the other 70%, some produced only IgG antibodies, whereas others produced only IgE antibodies.
ELISA inhibition proved that these antibodies were specific and that epitope similarity exists between peanut proteins, peanut agglutinin, and peanut oleosin to some extent regarding the production of IgG antibodies and, to a greater extent, of IgE antibodies.

In addition, in a study of 32 patients displaying immediate symptoms of allergic reaction, such as asthma, urticaria, and angioedema, sesame allergy was diagnosed by a convincing clinical history and double-blind, placebo-controlled food challenges. However, of the 32 patients, 10 had negative prick and IgE-specific test results using available saline-extracted antigens. When the oil-soluble component of sesame was prick-tested and the patients were tested against sesame oleosin, the sera from all patients reacted with this fraction of the sesame protein.16 The researchers concluded that oleosins are major allergens of sesame seeds and can be relevant to severe anaphylaxis. False negative prick tests could be due to the lack of oleosin in presently available extracts or to the fact that the epitopes were buried in the inner molecule. Detection tests currently used to identify sesame allergens based on sesame vicillins or other storage proteins may be insufficient for the detection of sesame immune reactivities.16

The current study's findings support the proposition that IgE-sensitization to foods may not necessarily coincide with positive prick tests to commercial extracts, because a maximum of diagnostic sensitivity is difficult to achieve. However, some studies point to the possibility that false-negative skin testing or IgG-, IgA-, or IgE-antibody testing is often linked to the nature of the preparations of the food antigens.24-26 Those studies and the results presented for the current study support the obvious need to improve the quality of food extracts used in the diagnosis of allergies and immune reactivity to nuts and seeds, in which oleosins correspond to 1% to 2% of total seed and nut proteins. This fact means that a few milliliters of oil is enough to cause hidden food immune reactivity while masking the source.15,27 The fact that oleosins are major allergens supports the adoption of oleosins as new markers for testing of food immune reactivity, because otherwise many false-negative results may be reported, bringing many years of suffering that could have been avoided.

Because many oleosins cross-react with each other, and even with other allergens, individuals with immune reactivity to oleosins should be aware of this cross-reactivity and consider the possible elimination of such cross-reactive foods from their diets. For example, recently it was found that peanut oleosin cross-reacts with buckwheat.28 Therefore, if patients react to peanut oleosin, both peanut and buckwheat should be removed from their diets.

CONCLUSIONS

The author concludes that a more accurate detection of allergies and immune reactivity in vitro and in vivo requires that testing be performed with all possible antigens of the suspected, specific foods and that both IgG and IgE antibodies be measured.

REFERENCES

For the Assessment of Intestinal Permeability, Size Matters
Aristo Vojdani, PhD

ABSTRACT
The purpose of this review is to demonstrate that an intestine leaky to small molecules can be impermeable to large antigenic molecules. The author proposes that the permeability of the epithelium to very small sugar molecules such as lactulose/mannitol—used for the past 50 years to gauge intestinal permeability—does not necessarily correlate with epithelial permeability to macromolecules. This article begins with the history and science behind the use of small sugars to measure permeability, a method developed in 1899. The lactulose/mannitol test may give useful information regarding the overall condition of the digestive tract; however, the author suggests that the test is not indicative of the transport of macromolecules such as bacterial toxins and food antigens, which have the capacity to damage the structure of the intestinal barrier and/or challenge the immune system. This article describes the various mechanisms and physiological transport pathways through which increased antigen uptake may result in immunological reactions to food antigens and bacterial lipopolysaccharides, resulting in the pathogenesis of disease. Finally, the article presents evidence indicating that increased intestinal, antigenic permeability plays a key role in the development of various inflammatory and autoimmune disorders. Therefore, more knowledge about the epithelium’s permeability to large molecules undoubtedly contributes not only to early detection but also to secondary prevention of many inflammatory autoimmune, neuroimmune, and neurodegenerative disorders. (Altern Ther Health Med. 2013;19(1):12-24.)

Aristo Vojdani, PhD, MSc, CLS, is CEO at Immunosciences Lab, Inc, Los Angeles, CA, and Cyrex Laboratories, LLC, Phoenix, AZ, USA.

Corresponding author: Aristo Vojdani, PhD, MSc, CLS
E-mail address: drari@msn.com

The understanding of the science behind gut permeability is said to have started in 1899 with Hober, who studied the absorption rates of several sugars and salts and found that dogs absorbed galactose slightly faster than glucose.1 In 1900, Hedon published a study comparing the rates of absorption of glucose, fructose, galactose, and arabinose in a loop of rabbit’s gut.2 Hewitt later compared the rates of absorption of dilute solutions of glucose, fructose, and galactose from loops of gut.3 The results were inconclusive in rabbits, but Hewitt observed that cats absorbed glucose much more rapidly than fructose, with galactose registering at an intermediate rate. Killing the intestinal mucosa with infusions of hot liquids or sodium fluoride resulted in equal absorption rates for all of the sugars. None of these methods were applicable, however, to humans. For this reason, McCance and Madders designed a method by which it was possible to compare the absorption rates of rhamnose, arabinose, and xylose.4 In humans, these three sugars are all excreted readily by the kidney and are destroyed in the tissues comparatively slowly. The researchers compared the rate and amount of each sugar excreted (a) when injected intravenously and (b) when taken orally. They concluded that:

1. Arabinose, rhamnose, and xylose are readily excreted when intravenously injected in humans. Their curves of excretion are of identical shape and may all be superimposable.
2. The relative rates of absorption of arabinose, rhamnose, and xylose are the same in rats and humans. If the rate of absorption of rhamnose = 1, then those of arabinose and xylose are 2.33 and 3.6, respectively.
3. All of these sugars are absorbed at the same location high in the small intestine. Little or no absorption
occurs further down the intestine.

4. In a normal person, the absorption of these sugars proceeds rapidly and linearly for 1.5 hours. After that point, absorption almost ceases, even when a large excess still remains in the intestine.

5. It is reasonable to assume that the relative rates of absorption of glucose, galactose, and fructose found in rats also hold true in humans; galactose is absorbed slightly faster than glucose, and glucose is absorbed twice as fast as fructose.

In the 1970s, the introduction of nonmetabolizable oligosaccharides as test substances made it possible to develop feasible methods for assessing intestinal barrier function. The intestinal permeability test consists of the oral administration of sugars and the subsequent measurement of these substances in the urine; it is a noninvasive method that has been used to assess the integrity of the epithelial barrier to small sugar molecules. Some evidence supports the role of gut-barrier dysfunction as a primary disease mechanism in intestinal disorders. As a result, the intestinal permeability test is used in both clinical practice and research.

Two different sugars, mannitol—a monosaccharide with a molecular weight (MW) of 182 Da and a molecular radius of less than or equal to 0.4 nm—and lactulose—a disaccharide with an MW of 342 Da and a molecular radius of 0.42 nm, have been used in this noninvasive, functional, intestinal-permeability test. The different sizes (MWs) of these molecules allow the evaluation of the relative importance of the two separate entrance routes that are postulated. Molecules up to the size of monosaccharides, such as mannitol, are believed to use the transcellular route, and disaccharides or larger molecules are believed to be transported through the paracellular route across the intestinal wall, as shown in Figure 1.

This sugar permeability test has been used in the determination of health and disease, including celiac disease (CD) and Crohn's disease. In the case of Crohn's disease, Blomquist et al and Bjarnason et al all suggested that a defect in the intestinal barrier function might be an etiological factor in the pathogenesis of the disease. Many factors, however, can influence the uptake of these sugars by epithelial cells, including (1) GI motility; (2) the body's distribution of the tracers; (3) the use of medications such as nonsteroidal anti-inflammatory drugs like meclofenamate; (4) smoking; (5) the use of alcohol; (6) variations in gastric emptying; (7) intestinal transit time and surface area; (8) mucosal blood flow; and (9) renal clearance. These factors can change the permeability of the epithelial cells, and hence, cause the tests to yield false-positive results.

Furthermore, normal ranges vary from laboratory to laboratory and country to country, with the ranges being higher in most tropical areas because of eating habits and the presence of tropical enteropathy. In addition, intestinal permeability to very small molecules (182-342 Da) is not necessarily related to structural damage in the tight-junction barrier that permits increased penetration of large molecules. Only molecules 5000 Da or larger can challenge the immunological system of the bowel, resulting in a T-cell response and the production of cytokines and antibodies. The alteration of the gut barrier to antigenic molecules leads to the absorption of endotoxin and lipopolysaccharides from the lumen. Both of these substances potentiate acute-phase reactions and the secretion of IL-1β, IL-6, and tumor necrosis factor (TNF), which have been shown to be important mediators of inflammation in many GI disorders.

For this reason, many attempts have been made to measure intestinal permeability to large, 12 000- to 15 000-Da polysugars in humans. The size of these sugars is similar to that of many food proteins, suggesting that these sugars may be suitable markers for intestinal permeability to macromolecules such as bacterial toxins and food antigens.

This conclusion is based on the fact that the human gastrointestinal tract allows a certain degree of physiological absorption of undegraded, macromolecular dietary antigen, whether free or antibody bound. This antigen uptake is influenced by the permeability of the gut and the local and systemic immune responses. Hobbie et al studied the passage of dietary antigens into the blood of children with celiac disease and other children who were suspected of celiac disease but exhibited normal jejunal biopsies (silent celiac).

For 7 hours after a test meal, researchers investigated the uptake of egg ovalbumin (OA) and beta-lactoglobulin (BLG) from cow's milk into the blood of five children with confirmed celiac disease, both when on a gluten-free diet and after gluten challenge, and the blood of five children suspected of silent celiac disease with normal jejunal mucosas. An ELISA detected OA in three of the five confirmed celiac children (maximal concentrations 8-178 ng/mL in the serum) and in all of the five children suspected of silent celiac disease (maximal concentrations 4-91 ng/mL in the serum). BLG was detected in three of the five confirmed celiac children (maximal concentrations 0.6-6 ng/mL in the serum).

**Figure 1.** The Transcellular and Paracellular Pathways Permit the Transfer of Differently Sized Molecules

![Image of Figure 1](image-url)
serum) and in two of the five children suspected of silent celiac disease (maximal concentrations 0.5 and 50 ng/mL in the serum).

HPLC fractionation in combination with ELISA detected OA and BLG in the serum of all the confirmed celiac children and suspected silent celiac children. The serum concentrations of OA and BLG were increased after gluten challenge in four of the five confirmed celiac children, indicating increased macromolecular passage through the gut mucosa in untreated celiac disease.20

It was concluded that challenge with gluten and the subsequent development of villous atrophy in celiac children leads to the increased uptake of macromolecular dietary antigens. Therefore, intestinal permeability should be measured against molecules that are representative of dietary antigens and bacterial toxins and not against very small sugar molecules.

Menard discussed the issue of gut permeability to large macromolecules in a very elegant 2010 review, “Multiple Facets of Intestinal Permeability and Epithelial Handling of Dietary Antigens.”21 Menard found that the intestinal epithelium is not fully impermeable to macromolecules. In the steady state, the transepithelial passage of small amounts of food-derived antigens and microorganisms contributes to the induction of a homeostatic immune response that is dominated by immune tolerance to dietary antigens and the local production of secretory immunoglobulin A (SIgA), thus preventing pathogenic and commensal microbes from entering internal compartments.

Obviously, no universal marker can provide a definitive answer on the capacity of the intestinal mucosa to sense the intestinal content and deliver antigens or bacteria to the underlying immune system. It is important to keep in mind that beyond the controversies of paracellular versus transcellular permeability, one important feature in intestinal disease is the failure of the intestinal barrier to contain the macromolecular luminal content, a phenomenon likely to exacerbate unwanted immune responses.

Whether, and the degree to which, the entrance of antigenic macromolecules across the gut epithelium initiates and/or perpetuates chronic inflammation remains a matter of debate, as do the respective contributions of paracellular and transcellular permeability. Thus, the experimental studies that use small inert molecules to assess intestinal permeability in vivo do not necessarily correlate with the uptake of larger dietary antigens.

MACROMOLECULES AND INERT SUGARS DO NOT CORRELATE

Currently, the gold standard for measuring intestinal permeability to small molecules is the lactulose/mannitol test. Although it is a useful test in clinical studies, providing information on the overall condition of the digestive tract (villous atrophy, inflammation), it does not indicate the transport of macromolecules such as food antigens and bacterial lipopolysaccharides (LPS). The transport of large intestinal molecules does not correlate with intestinal electrical resistance or the lactulose/mannitol permeability test.

Indeed, studies have shown the lack of correlation between the permeation of inert sugars and macromolecules. In neonatal pigs, intestinal closure to β-lactoglobulin (molecular weight 18 000 Da), a major allergen in cow's milk, occurs within 6 days of birth. The permeation of lactulose, a marker of paracellular permeability, persists, however, throughout the suckling period. This finding means that the body develops tight-junction structures such as occludin/zonulin, claudin, and JAM family proteins between paracellular spaces and prevents the movement of large antigen molecules into the submucosa within 6 days of birth.22,23 These spaces, however, are not tight enough to prevent the permeation of very small molecules such as lactulose, which continues to move throughout the first 6 to 12 months of life.23,24 This result indicates that inert soluble markers do not represent macromolecular absorption and do not reflect antigen handling by the gut.23

In addition, the lack of direct correlation between lactulose/mannitol IPT and the absorption of beta-lactoglobulin can also be observed in children with rotavirus diarrhea.25 Finally, in a mouse model of a celiac-like disease, mice challenged with gluten exhibited increased fluxes of horseradish peroxidase (HRP, a molecular tracer) in the absence of increased ionic conductance, whereas the addition of indomethacin to gluten promoted an increase in ionic conductance (paracellular pathway) and a further increase in HRP transcytosis. Thus, one should remember that electrical resistance (or its reverse, ionic conductance) is mainly related to the permeation of ions, and at best, small molecules but not always food-type antigens.25,26 Thus, studies that use very small, inert molecules to assess intestinal permeability in vivo do not necessarily correlate with the uptake of larger dietary antigens and bacterial toxins.21

It is accepted that intestinal permeability is a generic term related to the absorption of variously sized molecules ranging from small, inert solutes (mannitol) to large macromolecules. In intestinal diseases, the increased permeability of large molecules (food antigens, microbial fragments) can have a detrimental effect by facilitating or magnifying inappropriate immune responses. Whether the transport pathway is paracellular or transcellular, it is mandatory to use appropriate probes (proteins, bacteria) to delineate which materials can cross the epithelial barrier. In this regard, small inert markers cannot mimic large molecules because of the size selectivity of tight junctions.

MICROBIAL TRANSLOCATION AND IMMUNE ACTIVATION

A recent study showed that compromised gastrointestinal integrity in pigtail macaques (PTMs) was associated with microbial translocation (increased levels of LPS in the submucosa), immune activation, and IL-17 production by Th17 cells.27
The study of nonhuman primates is essential in understanding how and to what extent dysfunction and damage to the mucosal immune system can affect systemic immune activation in vivo. Infecting Asian rhesus macaques (RMs; *Macaca mulatta*) with pathogenic simian immunodeficiency virus (SIV) is the most widely studied nonhuman primate model for the pathogenesis of the human immunodeficiency virus (HIV) to date.

A comparison of the pigtail and rhesus macaques is interesting in that PTMs typically progress to AIDS more rapidly than RMs. After infection with SIVsmE543, the majority of PTMs progress to AIDS within 6 months of infection, as opposed to approximately 2 years for RMs. The rapid disease progression observed in PTMs is most likely not associated with viral inoculation but is instead due to host factors. Interestingly, uninfected PTMs in captivity have an increased incidence of diarrhea and GI diseases, and older animals frequently present with systemic amyloidosis. Indeed, a 5-year study of uninfected monkeys revealed that the majority of them had at least two bouts of diarrhea requiring treatment. Therefore, these animals are more susceptible to death resulting from intestinal permeability and the deposition of LPS in the mucosal tissue.

To determine the mechanisms underlying the permeability of the GI tract and consequent microbial translocation, the GI tract tissues were stained with antibodies against the tight-junction protein claudin-3 to measure the continuity or observed damage of the structural barrier of the gut epithelium. Significant damage to the tight-junction proteins was observed both through immunohistochemical studies and through calculations that measured the breach/intact ratio by comparing the length of the tight epithelial barrier that was not stained for claudin to the length of the colon barrier that was stained for claudin. In comparison to controls, which exhibited a breach/intact ratio of 0.017, monkeys with diarrhea showed a breach/intact ratio of 0.303; hence, a putative mechanism for the increased diarrhea, intestinal permeability, and microbial translocation in these monkeys may be associated with increased pre-existing damage to the structural barrier of the GI tract.

To determine whether these breaches in the epithelial tight junctions correlated with the increase in microbial translocation, the researchers studied the colon sections with an antibody against the LPS core antigen to directly measure the bacterial products present within the lamina propria (LP). They found that the monkeys with diarrhea had increased levels of LPS in the LP of the colon compared to the controls. Using quantitative image analysis, they determined the percent of the colonic LP area that contained LPS and found that, on average, LPS accounted for 13.00% of the LP area in PTM monkeys, whereas only 0.274% of the LP was occupied by LPS in the RMs (controls). These data strongly suggest that the mechanism underlying the increased microbial translocation involves structural damage to the gut epithelium in monkeys with diarrhea.

Furthermore, the authors demonstrated a significant positive correlation between damaged tight-junction proteins and the level of LPS in the tissue and blood. In addition, a positive correlation was found between the extent of LPS staining in the colon and mesenteric lymph nodes. Both the degree of damage to the tight junctions and the level of LPS staining in the colon and lymph nodes correlated with the level of LPS in the plasma. The level of LPS in the plasma of pigtail macaques with diarrhea averaged 45.3 pg/mL, a level that was much higher than that observed in the control monkeys, which averaged 19.2 ± 13 pg/mL. The researchers hypothesized that the rapid disease progression observed in the PTMs after SIV infection may in part be due to pre-existing conditions that cause the dysfunction of and damage to the mucosal immune system and lead to increased microbial translocation and consequent immune activation.

Based on these findings, it can be concluded that an assessment of intestinal permeability to large antigenic molecules can use the bacterial toxins that first play a significant role in damaging tight-junction and structural proteins (occludin/zonulin) and actomyosin and then open the paracellular pathway, thus facilitating the entry of tight-junction proteins, actomyosin, and bacterial LPS into the submucosa, the regional lymph nodes, and the circulation. This entry of tight-junction proteins and bacterial LPS into the circulation can challenge the immune system, resulting in the production of significantly elevated levels of occludin/zonulin-, actomyosin- and LPS-specific IgG, IgM and IgA in the blood.

### MECHANISM RESPONSIBLE FOR UPTAKE OF IMMUNOGENIC MOLECULES

The uptake of immunogenic molecules (antigens) from the lumen plays a significant role in the pathogenesis of gastrointestinal disease. The excessive uptake of these antigens, in addition to a breakdown in immunological tolerance or the suppression of immune responsiveness, can induce immunological activity both within the intestine and beyond. This activity seems to be a prerequisite for disease development. For example, serum immunoglobulins to the food antigens beta-lactoglobulin, wheat, and maize have been found in inflammatory bowel disease (IBD). It is probable that protein macromolecules permeate in increased amounts, causing systemic immune responsiveness.

Because an increase in the uptake of antigens is involved in this immunological reaction to food antigens and bacterial toxins, an understanding of the physiology of this uptake is central to an appreciation of the pathogenesis of disease. This antigen uptake process is divided into physiological transport and pathological transport.

The physiological-transport pathways include (1) ligand-receptor uptake, (2) antibody uptake, and (3) microfold or M cell transport.

Pathological transport can be antigen-nonspecific or antigen-specific. Antigen-nonspecific transport occurs through transcellular (intracellular) or paracellular pathways when the tight junction becomes more permeable or dam-
aged by environmental factors. This excessive uptake of antigens may occur as a result of an immature gut, postenteritis, allergic enteropathy, gut dysbiosis, and other environmental factors that activate inflammatory cascades.34-36

Antigen-specific transport via the transcellular or paracellular pathways can induce a specific disease. For example, gliadin has been linked to celiac disease; casein and beta-lactoglobulin cause allergic enteropathies; gut dysbiosis, and other environmental factors that activate inflammatory cascades.34-36

From all the above information, the authors conclude that increased antigen uptake in the intestine precedes the onset of many immunologically mediated, gastrointestinal diseases.

**LIGAND-RECEPTOR TRANSPORT OF ANTIGENS**

Macromolecules cross intestinal epithelial cells in two ways of which we can be certain. They can be shuttled through absorptive cells using specific receptors—in which case, only those macromolecules that bind to a receptor will pass—or they can pass through specialized epithelial cells (ie, the M cells previously mentioned).

Macromolecules are transferred by a mechanism that is altogether different from those that transport nutrients such as glucose and amino acids. Nutrient molecules enter the intestinal-cell cytoplasm at the apical membrane and exit through the basolateral membrane. Macromolecules, however, transverse the cell in membrane-bound compartments that invaginate from the apical membrane. The first step in this process is attachment of macromolecules to receptors on the apical surface of enterocytes, where they are endocytosed and processed by lysosomal enzymes, which degrade the antigen and transport it to the basolateral pole.35 This process of transcellular transport is shown in Figure 2.

**ANTIBODY-MEDIATED UPTAKE OF ANTIGENS**

Humoral immunity as mediated by secretory IgA (SIgA) and IgG plays an important role as a first line of defense against microorganisms in mucosal tissues. SIgA is transported from tissue spaces and across epithelial cells into the lumen through an active, unidirectional process involving the polymeric Ig receptor pIgR. IgG can also be detected within the intestinal lumen of the adult human; in certain tissue locations, it may reach levels approximating those observed for SIgA.

The passive administration of neutralizing IgGs has also been reported to prevent mucosal transmission of human immunodeficiency virus in rhesus macaques or neonatal macaques. Together, these observations support the concept that the presence of IgG along the mucosal surfaces can serve an important role in mucosal protection.38-40 The mechanism(s) by which IgG accesses the lumen and biological functions once in place, however, remain to be defined.

Evidence has recently indicated a role for the neonatal Fc receptor (FcRn) in these processes. Interestingly, FcRn has been functionally linked not only to the passive acquisition of immunity in neonatal rodents through the transport of maternal IgG but also to IgG-mediated immune surveillance. This finding is based upon the indirect morphological observation that FcRn is also capable of transporting antigen-antibody complexes across the intestinal epithelium from the lumen during neonatal rodent life. From this finding, one can hypothesize that a major function of FcRn in adult human life is to transport IgG into the apical region of the epithelium for the retrieval of antigens so that FcRn can recycle these complexes for transport back into the lamina propria. Such a pathway could contribute significantly to the regulation of immune responses by providing a mechanism for luminal antigen(s) to gain access to professional antigen-presenting cells such as dendritic cells (DCs). These cells are known to be present at this location and are capable of interacting with regulatory T cells to induce immunological tolerance.41

Under steady-state conditions, epithelial cells sample molecules with molecular weights greater than 600 Da (such as food antigens and peptides) by endocytosis at the apical membrane and transcytosis toward the lamina propria. During transcytosis, the full-length peptides or proteins are partly degraded in acidic and lysosomal compartments and released in the form of amino acids (total degradation) or breakdown products (partial degradation) at the basolateral pole of the enterocytes.

**Figure 2. Transcellular Transport Pathways**

Under steady-state conditions, epithelial cells sample molecules with molecular weights greater than 600 Da (such as food antigens and peptides) by endocytosis at the apical membrane and transcytosis toward the lamina propria. During transcytosis, the full-length peptides or proteins are partly degraded in acidic and lysosomal compartments and released in the form of amino acids (total degradation) or breakdown products (partial degradation) at the basolateral pole of the enterocytes.
ANTIBODY-MEDIATED TRANSPORT OF ANTIGENS

The most representative Ig isotype at the mucosal surface is IgA. The basal-to-apical secretion of dimeric IgA, in the form of SIgA, through the polymeric Ig receptor is a common receptor-mediated IgA transport mechanism in the intestines. SIgA retains potentially noxious antigens in the intestinal lumen, thus performing a vital role in intestinal immunity. While restricting the passage of exogenous antigens into the intestinal mucosa seems to be the main function of SIgAs, apical-to-basal retrotransport, can occur, with either deleterious or beneficial effects on the intestinal mucosa.

In animal models, *Shigella flexneri* alone or as an SlgA immune complex (ICs) was administered into ligated intestinal loops containing Peyer’s Patches (PP); this process allowed ICs, but not free bacteria, to enter the PP and be captured by DCs, thereby contributing to the induction of protective immunity and preserving the integrity of the intestinal barrier.

In some pathological situations, the abnormal retrotransport of SlgA ICs can allow bacterial or food antigens to enter the intestinal mucosa, with various outcomes. Indeed, SlgA can mediate the intestinal entry of SlgA/*S. flexneri* ICs through M cells and interactions with dendritic cells, inducing an inflammatory response aimed at improving bacterial clearance and the restoration of intestinal homeostasis. In healthy individuals, undigested gliadin peptides are taken up by nonspecific endocytosis in enterocytes and entirely degraded/detoxified during transepithelial transport. In celiac disease, however, the ectopic expression of the transferrin receptor CD71 (also known as the IgA receptor) at the apical membrane of epithelial cells favors the retrotransport of IgA ICs and inappropriate immune responses. SlgA allows the protected transcytosis of gliadin peptides. Because of the constant flow of gluten in the gut, this process is likely to trigger exacerbated adaptive and immune responses and precipitate mucosal lesions. This IgA-mediated transport of antigens is shown in Figure 3.

Whereas the retrotransport of SlgA/bacterial ICs aids in the development of immune responses to clear pathogenic microbes, this retrotransport might turn deleterious to the host when food antigens are concerned. This deleterious effect occurs in CD, an enteropathy induced by the abnormal activation of T cells by gluten-derived gliadin peptides. In CD, gliadin peptides are transported intact across the intestine by IgA/gliadin ICs.

Studies suggest that dietary antigens, including gluten peptides, are complexed to antigen-specific, intraluminal SlgA. The gliadin peptides now complexed with secretory IgA bind to the IgA receptor, which then transports and protects them from lysosomal degradation through a specific transcytosis pathway. This IgA receptor has been recently identified as CD71.

Normally, CD71 is only expressed on the basolateral enterocyte membrane in the normal intestine and in patients on a gluten-free diet. This receptor efficiently binds polymeric and secretory IgA but not monomeric IgA. In contrast, in active CD, CD71 expression is greatly increased and CD71 is found at the apical enterocyte membrane, where it colocalizes with IgA. The gliadin peptides that complex with SlgA can then bind to CD71, which mediates their protected endocytosis and translocation from the intestinal lumen into the lamina propria.

In healthy individuals, gliadin peptides are taken up nonspecifically by enterocytes and degraded by lysosomal proteases during fluid-phase transcytosis. Very few toxic peptides are delivered into the intestinal lamina propria. In patients with active CD, the abnormal expression of CD71 at the apical pole of enterocytes allows the receptor-mediated uptake of SlgA-gliadin peptide complexes and their protected transport toward the lamina propria and, thus, the local immune system. The exact domain of the SlgA molecule involved in CD71 binding is not known. Blocking gliadin peptide entry into the intestinal mucosa might serve as the basis for a novel therapeutic strategy in CD. The CD71-mediated transport of IgA food complexes is also shown in Figure 3.

IgA-mediated gliadin transport is involved in the overstimulation of the local immune system. While the IgA-mediated transport of pathogenic bacteria might be benefi-
cial for improving bacterial clearance and restoring intestinal homeostasis, the application of the same mechanism to a normally nonpathogenic antigen such as gliadin may cause the effects to be deleterious rather than protective.\textsuperscript{47} Additionally, the presence of large aggregates of gliadin-specific IgA in duodenal secretions, the lamina propria, and the serum of celiac patients could provide a danger signal that promotes the rupture of oral tolerance and/or triggers tissue damage. The damaging effects of IgA-complex deposition in tissues have been exemplified in IgA nephropathy.\textsuperscript{48}

THE IGG-MEDIATED TRANSPORT OF ANTIGENS

It is now accepted that gastrointestinal secretions contain significant amounts of IgG. IgG-mediated intestinal transport primarily seems to be implicated in protective immunity. The role of intestinal FcRn was initially reported in suckling rats that receive passive immunity from their mother through the intestinal absorption of IgG from maternal milk. The polarized absorption of IgG is explained by the binding properties of IgG to FcRn at the acidic pH (less than 6.5) recorded close to the apical membrane of the intestinal epithelial cell. The dissociation of IgG from FcRn at neutral pH leads to the release of IgG on the basolateral side of the enterocyte. In contrast, while the human neonatal intestine is not a major site for the transfer of passive immunity, FcRn can be found at the apical pole of enterocytes in the fetal and adult intestine, even though the relevance of such expression has not been clearly established.\textsuperscript{49,50} The Fc ligand valency influences the intracellular processing of IgG during transcytosis (protection versus degradation). The Fc fragment displays two binding sites for FcRn, and the presence of both binding sites is required for efficient transcytosis and the protection of IgG from catabolism.\textsuperscript{51,52} The functional role of FcRn in the transfer of IgG ICs has been characterized using polarized, epithelial cell lines and transgenic mice. The cells transfected with hFcRn did not degrade OVA during the apical-to-basal transport of IgG/OVA ICs, and OVA-specific CD4\textsuperscript{+} T cells were activated after IC transport.\textsuperscript{41}

The low-affinity IgE receptor CD23 is abnormally overexpressed in intestinal epithelial cells in allergic humans and murine models of allergy. The overexpression of CD23 at the apical side of enterocytes can drive the transport of intact IgE/allergen ICs from the intestinal lumen to the lamina propria, triggering mast-cell degranulation and an allergic inflammatory cascade.
Moreover, in vivo studies investigating transgenic mice expressing hFcRn and β2-microglobulin, showed the FcRn-mediated transcytosis of IgG ICs and their efficient presentation to OVA-specific, helper T lymphocytes by CD11c+ DCs. While the outcome of this immune response in vivo is not known, it has been reported that IgG ICs might induce immune suppression. In addition to food antigens, bacteria can also be transported as IgG ICs through FcRn, a feature likely to have a role in the defense against intestinal pathogens. These findings thus underline a potential role of FcRn in the maintenance of intestinal homeostasis. The IgG-mediated transport of antigens is shown in Figure 4.

**IGE-MEDIATED TRANSPORT OF ANTIGENS OR ALLERGENS**

The human, low-affinity IgE receptor (Fc-epsilon-RII, CD23) can mediate the transport of IgE ICs in food allergies (Figure 5). CD23 is primarily expressed on hematopoietic cells but is also observed on the apical and basal surfaces of IECs in patients with gastrointestinal diseases such as autoimmune enteropathy, cow’s milk protein enteropathy, CD, ulcerative colitis, and immune activation. High levels of Th2 cytokines, which are involved in allergic disease, enhance expression of the IgE receptor.

Although it can be found in lavage fluids from parasitic infection and in food allergy patients, IgE is not considered a secreted Ig. Rodent models of allergy have unraveled the roles of epithelial CD23 and IgE ICs in the mucosal entry of food allergens. Sensitizing rats to horseradish peroxidase (HRP) led to the increased uptake of HRP into IECs and faster transcellular transport in these rats compared with naïve control rats. This enhanced transport has been shown to involve an IgE-dependent, receptor-mediated process. Immune sensitization enhanced CD23 expression on the IECs and allowed the IgE/allergen complexes to bypass epithelial lysosomal degradation, resulting in the penetration of a large concentration of intact allergens into the mucosa.

Intra-epithelial lymphocytes express high levels of the CD23 receptor, which is involved in the apical-to-basal transport of IgE or IgE ICs.

It is likely that the IgE ICs delivered to the lamina propria after epithelial transport can degranulate mast cells, underlining the ability of the IC to activate local cells. This mechanism could be involved in the rapid onset of intestinal symptoms in IgE-dependent food allergy.

**TRANSPORT OF ANTIGENS BY M CELLS**

The passage of intact macromolecules across the gut is at odds with the role of the gut as a macromolecular barrier. For macromolecules to cross the gut in a controlled manner, specialized epithelial cells have evolved to control the passage of antigens and larger particles through the intestinal epithelium. These cells are called microfold or M cells.

M cells are highly specialized epithelial cells that are joined to their neighbors by tight junctions that restrict the paracellular pathway. Several important immunological and pathophysiological functions, such as the capture of antigens from the gut lumen and their transport to lymphocytes and macrophages, have been recognized for M cells.

The transepithelial, vesicular transport activity of M cells, however, provides functional openings in the epithelial barrier. M cell membranes are equipped with a thick, brush border to promote the sampling of foreign materials from the lumen through the adherence and uptake of food antigens, microbiota, and mucosal pathogens. Because of this unique structure, the SIgA that has been transported into the lumen selectively adheres to the M cells’ apical membranes. Furthermore, IgA/antigen complexes also adhere to the M cells’ membranes and are transported into the intraepithelial pocket. This uptake of specific IgA/antigen complexes by M cells can induce a secretory immune response and the production of antigen-specific IgA in the secretions.

The microorganisms, macromolecules, and particles taken up at the M cell’s apical surface are internalized into endosomal tubules and vesicles and large multivesicular bodies that lie between the apical membrane and the intraepithelial pocket. These macromolecules and particles can be released at the pocket membrane within 10 to 15 minutes. The antigens and pathogens are then captured by immature dendritic cells in the subepithelial region that lies in close proximity to the M cells. This allows for the efficient presentation of the ingested antigens to the immune system, resulting in the activation of immune responses against food antigens and other environmental allergens.
proximity to the organized T cell and B cell zones where antigen presentation may occur. These cellular interactions with antigens and pathogens are likely to be important determinants of the mucosal immune response against these same antigens and pathogens; they may also facilitate the dissemination of pathogenic factors that exploit the M cell transport pathway.68,84

This pathway, however, provides rapid entry into the mucosa and consequently has a vital role in the pathogenesis of certain bacterial and viral diseases. The risk of pathogen invasion at these sites is high because the intestinal M cells are constantly exposed to the lumen of the gut and are relatively accessible to pathogens.

The uptake of microorganisms by M cells may also play a key role in the maintenance of the normal bacterial flora in the intestine. M cells can transport noninvasive, commensal bacteria into Peyer’s Patches, a process that may be crucial in regulating endogenous microbial populations in the lumen or eliminating and inactivating bacteria that have crossed the mucosal epithelium.84 In neonates, the uptake of nonpathogenic bacteria may be vital for the maturation of the mucosal immune system and for the development of tolerance to food antigens.63 The excessive internalization of antigens by M cells, the subsequent capture of these antigens by dendritic cells, and the initiation of the immune response against them, however, can cause the overproduction of IgA antibodies, the formation of immune complexes, the initiation of the inflammatory response, and the breakdown of immunological tolerance to various food antigens. The specialized antigen-transporting feature of the M cell is only one of several mechanisms employed by the epithelia of all mucosal surfaces to provide samples of the external environment to the immune system. Normally presenting a selective barrier because the intercellular tight junctions of the intestinal epithelial barrier control the equilibrium between tolerance and immunity to nonself antigens that originate from dietary proteins and infectious agents.63,68 Thus, when the zonulin/occludin pathway is deregulated in genetically susceptible individuals, intestinal and extraintestinal inflammatory and autoimmune disorders can occur.63 In these cases, the intestinal tight junctions allow the passage of macromolecules from the intestine to the submucosa, and the regional lymph nodes stimulate the immune system to mount cellular and humoral immune responses against various tissues or organs.63 This theory is echoed and strengthened by different studies, lending support to the understanding of the role that the gut-associated lymphoid tissue (GALT) plays in the excessive increase in intestinal permeability during development of autoimmune diseases, such as celiac disease, type 1 diabetes, rheumatoid arthritis and multiple sclerosis.63,68,73,83

This finding was verified by measuring the zonulin levels in the sera of patients with autoimmune diseases. Elevated serum zonulin was detected in 70% of the subjects at a time point of 3.5 plus or minus 0.9 years before the onset of the disease.64 In addition to an increase in the permeability of the blood-brain barrier (BBB) of multiple sclerosis patients, a subgroup of these patients demonstrated increased intestinal permeability.64,84,85 To lend further support to the detection of intestinal permeability abnormalities in MS patients, the serum zonulin levels were measured in different patient subgroups.68 Approximately 30% of the patients with either relapsing-remitting MS (RRMS) or secondary-progressive MS (SPMS) showed elevated serum-zonulin levels that were 2.0-fold higher than the serum-zonulin levels in healthy controls. Interestingly, this percentage was similar in MS patients who had increased intestinal permeability. These findings further support the pivotal role that increased intestinal and BBB permeability plays in the development of severe autoimmune disorders.

As with autoimmune disorders, the pathology of PD is believed to be associated with an interaction between genes and susceptibility to environmental factors.86 The GI tract and its large number of neuronal cells serve as the largest interface between the environment and neural tissue, but it can also serve as a major site of oxidative stress.87 The close proximity of this extensive neuronal network to microbiota permits the creation of a proinflammatory environment and an increase in oxidative stress in the enteric nervous system (ENS) due to autoantibodies against various tissue antigens and the development of autoimmune diseases.65-67 In fact, scientists have often observed that less than 10% of the subjects with a genetic susceptibility to autoimmunity progress to clinical disease in their lifetime.68-71 This suggests that exposure to environmental factors such as toxic chemicals, infection, and dietary proteins is involved in the development of autoimmune disease.68 In addition to the gene-environment interaction, however, GI dysfunction and the trafficking of macromolecules to the submucosa and into the circulation are additional factors in autoimmunity.63 This situation occurs because the intercellular tight junctions of the intestinal epithelial barrier control the equilibrium between tolerance and immunity to nonself antigens that originate from dietary proteins and infectious agents.63,68 Thus, when the zonulin/occludin pathway is deregulated in genetically susceptible individuals, intestinal and extraintestinal inflammatory and autoimmune disorders can occur.63
## Table 1. Why Size Matters

<table>
<thead>
<tr>
<th>Lactulose/Mannitol Test</th>
<th>Antigenic Intestinal Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The test assesses the permeability to small molecules in the upper region of the small intestine.</td>
<td>1. The test assesses not only the entire length of the small intestine but the large intestine as well.</td>
</tr>
<tr>
<td>2. The test measures the permeability of small sugar molecules 342 Da in size.</td>
<td>2. The test measures permeability to large molecules (ie, 10,000 Da or larger).</td>
</tr>
<tr>
<td>3. Small sugars the size of lactulose are not antigenic, and therefore, do not challenge the immune system.</td>
<td>3. The 10,000 Da large molecules are antigenic and challenge the system.</td>
</tr>
<tr>
<td>4. The intestinal permeability to small sugar molecules does not necessarily correlate with the uptake of much larger dietary antigens and bacterial toxins.</td>
<td>4. Intestinal permeability to large molecules does correlate with digestion-resistant fragments of food antigens and bacterial endotoxins.</td>
</tr>
<tr>
<td>5. The interaction between small molecules and the immune system cannot lead to immunologically mediated damage.</td>
<td>5. Interaction between macromolecules and the immune system could lead to immunologically mediated damage.</td>
</tr>
<tr>
<td>6. Measuring permeability to small sugar molecules does not correlate with gut dysbiosis, endotoxin release, microbial translocation, and activation of the mucosal immune system.</td>
<td>6. Measuring permeability to large molecules such as LPS does correlate with gut dysbiosis, microbial translocation, and immune activation.</td>
</tr>
<tr>
<td>7. Epithelial cells permeable to small sugar molecules will not be permeable to large molecules; hence, more false positive results.</td>
<td>7. The epithelial cells that are permeable to large molecules will be permeable to small molecules as well; hence, no false positive results.</td>
</tr>
<tr>
<td>8. Permeability to small sugar molecules does not reflect damage to the structure of tight junctions.</td>
<td>8. Large-molecule permeability indicates damage to the structure of tight junctions.</td>
</tr>
<tr>
<td>9. Due to a repair mechanism, small openings in tight junctions can be repaired within hours, which means more false negative results.</td>
<td>9. Large openings in tight junctions (which are associated with structural damage to tight-junction proteins) cannot be repaired within hours and do not lead to false negative results.</td>
</tr>
<tr>
<td>10. Lactulose/mannitol is inconvenient. It entails the oral administration of a tracer and the collection of urine hours later.</td>
<td>10. Measuring permeability to large molecules is more convenient. It requires neither a tracer nor urine collection.</td>
</tr>
<tr>
<td>11. Lactulose/mannitol can be affected by GI motility, the distribution of the tracer, variations in gastric emptying, renal clearance, the use of medication, smoking, and alcohol consumption, leading to even more false positive results.</td>
<td>11. Permeability to large molecules is not affected by GI motility, the distribution of the used tracer, variations in gastric emptying, renal clearance, the use of medication, or smoking and alcohol consumption, thus producing fewer false positives.</td>
</tr>
<tr>
<td>12. The passage of small inert materials is not an indication of a breakdown in immunological tolerance, which is the root cause of allergies and autoimmunity.</td>
<td>12. Permeability to large antigenic molecules and the immune response against them is an indication of a breakdown in immunological tolerance, which is the root cause of allergies and autoimmunity.</td>
</tr>
</tbody>
</table>
gut dysbiosis and the release of bacterial toxins. This situation may result in the formation of the neuronal inclusions called Lewy bodies, which consist of aggregated and phosphorylated alpha-synuclein. The discovery of these abnormal protein aggregates in the intestinal enteric nerves led to the hypothesis that the GI tract might present the first evidence of PD as a response to pathogens or environmental toxins.

These findings further support the concept that the ENS may be the route by which a toxin or pathogen initiates the production of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, thereby affecting the permeability of the BBB and initiating neuroinflammation and its progression into PD over a period of many years.

A different line of evidence indicates that an endotoxin-induced increase in intestinal permeability also triggers neuroinflammation in PD. For example, the administration of LPS either directly into the CNS or systemically can induce the selective loss of dopaminergic (DA) neurons in the substantia nigra and the development of PD in an animal model. Thus, PD patients appear to have an intestinal epithelium that is hyperpermeable to bacterial toxins, which can induce oxidative stress and the misfolding of alpha-synuclein. This situation may lead to very important biological consequences and even the initial injury of the ENS, which is followed by the induction of neuroinflammatory events, enhanced BBB permeability and the development of PD in genetically susceptible individuals.

CONCLUSION

The lactulose/mannitol test has long been held to be the gold standard for determining the permeability of the intestinal epithelium. The information presented in this article, however, calls for a reassessment as to what may actually be the best methodology for determining intestinal barrier function. Table 1 provides an easy-to-view, side-by-side comparison of the information the authors have reviewed, which can be summarized as follows: The permeability of the epithelium to small sugar molecules does not necessarily correlate with its permeability to larger macromolecules. A misconception may exist that a system sensitive enough to detect and measure the permeability of small sugar molecules makes the measurement of larger molecules superfluous; however, this statement is simply not true.

The table details how small sugars are not antigenic, do not challenge the immune system, do not lead to immunologically mediated damage, do not correlate with the conditions of barrier dysfunction, do not indicate real damage to tight junctions, and in fact, are not an indication of a breakdown in immunological tolerance, thus leading to false negative or false positive results. In comparison, large molecules are antigenic and challenge the immune system, can lead to immunologically mediated damage, correlate with intestinal barrier dysfunction, indicate real damage to the structure of tight junctions, and indicate a breakdown in immunological tolerance without false negative or positive results. All of the transport pathways for the different ligands and antibodies that have been detailed in this review are associated with large antigenic molecules, not small sugar molecules such as lactulose and mannitol.

The loss of the intestinal barrier to antigenic molecules that occurs secondary to the upregulation of occludin/zonulin and environmentally induced inflammation is largely responsible for the switch from tolerance to an immune response against nonself antigens that cross the intestinal barriers. This continuous stimulation of the immune system by nonself antigens and activation of the inflammatory cascade and/or cross-reaction with various self-antigens appears necessary to perpetuate the autoimmune and neurodegenerative processes. Therefore, a hyperpermeable intestinal epithelium may first injure the ENS and then induce neuroinflammatory events, increase BBB permeability and ultimately promote the development of neuroautoimmunity and neurodegenerative disorders.

Despite significant progress in the field of mucosal immunology during the past 2 decades, much still remains to be learned regarding everything that happens to transported antigens and the factors that influence the nature and magnitude of the resulting immune responses; however, it is clear that size does matter.

ACKNOWLEDGEMENTS

Joel Bautista created the figures and prepared this article for publication.

REFERENCES


Increased Intestinal Permeability Is The Gateway For Multiple Autoimmune Disorders

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Size Matters!!!!
Aristo Vojdani, PhD: Environmental Factors and Autoimmune Disease

Interview by Karen Burnett

Aristo Vojdani, PhD, is a respected researcher, scientist, speaker, and author. He has published more than 150 articles in peer-reviewed scientific journals, is a multiple US patent holder for laboratory assays, and has received the Herbert J. Rinkle Award from the American Academy of Environmental Medicine for excellence in teaching the techniques of environmental medicine, the Linus Pauling, PhD Award from the American College for Advancement in Medicine, and the F. R. Carrick Research Institute's extremely distinguished Lifetime Achievement Award. He is the founder, technical director, and CEO of Immunosciences Lab, Inc, in Los Angeles, California, and serves as chief scientific advisor for CYREX Laboratories in Phoenix, Arizona. He sits on the editorial board of four scientific journals and is a guest editor of six journals. (Altern Ther Health Med. 2015;21(suppl 1):80-86.)

Alternative Therapies in Health and Medicine (ATHM): Dr Vojdani, where did you grow up?

Dr Vojdani: I was born and raised in a very small town in Iran up to age 18. After high school, I went to Israel to continue my higher education with a bachelor’s—then master’s and doctoral degrees in the field of immunology.

ATHM: What were your early influences that fostered your interest in health?

Dr Vojdani: My father wasn’t a doctor, but he was very interested in herbal medicine and, indirectly, that probably affected me. On the other hand, I was born in a Jewish family. Living in Iran, as you know, in our struggle for survival, education was the only recourse that could protect us against discrimination.

Therefore, that is why most Jewish families say, “My son should be a doctor.” That’s what my mother did. She named me Aristo with the hope that I would follow in the footsteps of Aristotle.

ATHM: When you went to Israel, what did you study?

Dr Vojdani: I went to Israel to earn my bachelor’s degree in biochemistry and microbiology. After that came a master’s degree in microbiology and immunology. Finally, I finished my PhD in the field of immunology. Again, all these 3 happened in Israel.

ATHM: Did you have a specific experience that made you curious about food sensitivities and the effects of environmental chemicals and autoimmune issues?

Dr Vojdani: Yes. We are all influenced by the experiences we go through. After finishing my PhD, I did 1 postdoctoral study at the Weizmann Institute of Science in Israel, and then I went back to Iran, where I became an assistant professor at Free University and National University in Tehran. When the revolution occurred, we came to the United States, and I started another postdoctoral study at UCLA. That is where everything really started.

When I started my postdoctoral studies at UCLA, I became interested in the effects of toxic environmental chemicals on the immune system. That was in 1979, when a very interesting article about immigrants and disease in the United States was published in a scientific journal. They compared the rate of cancers in Japanese in Japan versus Japanese who migrated to the United States with similar comparisons for Chinese and other immigrants. The conclusion of the article revealed that Japanese in Japan suffer from stomach cancer, but when they come to America, they don’t end up with stomach cancer, but they develop colorectal cancer. Japanese women in Japan do not have breast cancer. However, 20 or 30 years after immigration or migration to the United States, they do develop breast cancer. The question, then, was: Is it genes or the environment?

That was the first thing that caused me to become interested in the role of environmental factors in immune disorders. When I moved from UCLA to Charles R. Drew University of Medicine and Science, I became a faculty member and I started writing my own grant application through the National Institutes of Health in relation to the effect of toxic environmental chemicals and cell-mediated...
immunity. Through this research, I learned more about the role of environmental triggers in immune disorders. In 1986, I was hired as a consultant to a laboratory in Los Angeles that was very interested in testing for food sensitivity. I developed the first ELISA, enzyme-linked immunosorbent assay, for measurements of IgG antibodies against a variety of food antigens. I used this test to look for antibodies against wheat, corn, soy, or milk, and I found that a very high percentage of the population was reacting to wheat gluten. Consequently, I also became interested in the role of diet in the induction of immune disorders.

**ATHM:** Would you say that there are many more undiagnosed celiac cases in our society than we know about?

**Dr Vojdani:** Yes. For clarification, however, let’s divide gluten reactivity into 2 subgroups. One group is celiac disease—or CD—about 1% to 2% of the population, which is a classical disorder initiated by some kind of trigger, such as a bacterial toxin. For example, a child is completely healthy but has a rotavirus infection or small intestinal bacterial overgrowth. The bacterial toxin or rotavirus changes the integrity of the child’s mucosal immune system. Because of that, the child cannot digest wheat proteins properly—in this case, gluten. If that child also has a certain genetic makeup, called HLA-DQ2/DQ8, inherited from the mother and father, a genetic susceptibility exists. Now the food, in this case gliadin peptide, is not digested properly. Some inflammation in the gut occurs, initiating an enzyme called transglutaminase to deamidate the gliadin peptide, which in the process binds to transglutaminase itself. Now the body—the immune system—will react against gliadin as well as its own enzyme, the transglutaminase, which under normal conditions should not occur. This is a classical example of gluten sensitivity resulting in celiac disease in an individual with the genetic makeup for this disorder. Again, this is initiated by a trigger—in this example, a bacterial toxin or a virus, or even medication.

Normally, when a gastroenterologist does a tissue biopsy for CD and finds abnormal tissue, that is considered a positive for CD. However, existing literature suggests that for every single case of celiac disease, there are 8 undiagnosed. Why? We call this silent celiac disease, or atypical celiac disease. Therefore, we need to do laboratory testing—a blood test—in order to be able to detect the celiac disease. We need to do laboratory testing for measuring IgG and IgA antibodies against gliadin and also IgG and IgA antibodies against transglutaminase. If the IgA antibody against transglutaminase is positive and the IgA antibody against gliadin is positive and the biopsy taken from the patient is also positive, that will confirm a diagnosis of celiac disease. That is one category.

The second category is based on an article published during 2012 in a scientific journal. They started using another terminology, which is nonceliac gluten sensitivity, or NCGS. NCGS in this case is the patient who makes IgG or IgA antibodies against gliadin or other wheat proteomes but does not make the antibody against transglutaminase. This makes NCGS very difficult to detect. A biopsy of an NCGS patient would come out absolutely normal. The classic blood test, which confirms diagnosis of celiac disease, also comes out negative because transglutaminase IgA is negative—because no antibody was made against it. The patient is sent home and told that they can have any amount of gluten they would like. Unfortunately, because the body continues to react against gluten IgG and IgA, the transglutaminase can eventually turn around and cause an antibody that can attack tissue after years—5 years, 10 years, or 15 years. This undetected NCGS could eventually become autoimmune reactivity or autoimmune disease.

In my opinion, NCGS is more serious than celiac disease because celiac disease is more easily detected by gastrointestinal specialists. Based on testing, they can tell if a CD patient should go on a gluten-free diet. And when they go gluten-free, the majority of them improve significantly. However, if NCGS patients don’t get the proper testing, they may continue consuming glutsens for years. To me, this is why NCGS is more serious than CD, because undetected NCGS could result in autoimmunity years later.
ATHM: Does this lead you toward recommending that most people avoid gluten?

Dr Vojdani: I’m not saying that most people should avoid gluten. What I am saying is that individuals who have GI symptomatology—even though they go to the gastroenterologist and show a normal biopsy—should still do an array of testing for antibodies against wheat proteomes. It is not enough to measure antibodies against just 1 component of wheat. As I demonstrated in an article that I published in International Scholarly Research Notices Allergy about a year ago, antibodies should be measured against various wheat proteomes because, while one individual could react to component A of wheat, a second individual may react to the component B, the third individual may react to component C, and the next individual may react to A, B, and C at the same time. Therefore, we have to measure antibodies against all wheat proteomes. If the test results are negative, then the patient should not go on a gluten-free diet. That’s my opinion. If they are positive for antibodies, then that justifies putting those individuals on a gluten-free diet.

ATHM: You have said that the 3 environmental factors that cause complex diseases such as autoimmunities and autism are infectious agents, toxic chemicals, and dietary proteins. Could you explain that further, please?

Dr Vojdani: Since we were talking about dietary proteins, let’s continue with that, first, and then we’ll get to toxic chemicals and infection. Dietary proteins such as wheat or milk or dairy—alpha casein, beta casein from dairy products—or other proteins from different foods, if not digested properly, can cause inflammation. When our mucosal immune system is not working properly, inflammation in the gut can result in leaky gut syndrome. That allows the entry of undigested food protein, such as gliadin and casein, into the submucosa, from the submucosa to the regional lymph nodes, and from the regional lymph nodes into the circulation, where our lymphocytes will attack it as a foreign material and produce antibodies against it.

Unfortunately, due to similarity between various food antigens and human tissue, now the antibodies produced against alpha gliadin and casein or other food antigens will attack our own tissue, resulting in autoimmunity. It is well established that wheat antibodies can attack the cerebellum, can attack neurons, can attack thyroid tissue, can attack joints and the heart muscle, and almost every single one of these tissues cross-reacts with various food antigens.

This is a situation where dietary proteins and peptides escape the mechanism involved in protecting us against the entry of macromolecules into the circulation. If that mechanism is broken, the result could be autoimmune attacks against almost every single kind of tissue in the body. That’s why arthritis patients who begin a gluten-free diet or casein-free diet—or avoid any other food reactions—feel significantly better, because those foods can cause inflammation and autoimmunity. In fact, my own research showed that children with autism not only make antibodies against gliadin and casein, they also make an antibody against cerebellar tissue, resulting in gluten ataxia, which is a neurologic disorder.

Another article I recently had published in Food and Nutrition Sciences describes other foods that could cross-react with wheat. Among those are corn, rice, yeast, millet, and milk. If you want to be sure of removing all the triggers, then when you remove gluten from your diet you may also need to remove casein—meaning dairy products—as well as rice, millet, yeast, and other cross-reactive foods from the diet.

Very often, clinicians observe that some patients put on gluten-free diets will not improve. Then, they have to go on casein-free diets. If they don’t improve, then you have to put them on a corn and rice and millet and yeast-free diet. Hopefully at that level, they will see improvement. Therefore, it is a little bit more complicated than what we think. This is the mechanism behind how dietary proteins and peptides can cause autoimmunities.

The second factor involved in autoimmunity is toxic chemicals. Let’s use the example of 2 medications given to patients with ulcerative colitis and inflammation in the gut. Hydralazine is one of them. There are some other medications as well. These medications try to suppress the immune system in the gut in order to prevent inflammation and autoimmunity. Unfortunately, these chemicals have the capacity to bind to human tissue and induce autoimmunity by themselves. In some individuals, medications we take for pain, like some of the painkillers, can bind to human tissue and cause autoimmunity.

I was recently the lead guest editor of a special issue for a journal called Autoimmune Diseases; the special issue dealt with environmental triggers, particularly, toxic chemicals. I have read a lot of articles about bisphenol A and various other chemicals in plastic. Unfortunately, these days everything comes in plastic bottles. We put plastic containers in the microwave. We drink coffee in a paper cup, which is coated with a layer of bisphenol A. We drink soda or eat food from cans which are coated with chemicals. In a study that I published in the Journal of Applied Toxicology, I found that about 20% of supposedly healthy subjects actually have a harmfully high chemical body burden; this is why they react against these chemicals immunologically and produce antibodies against them and self-tissue antigens.

I believe that the American population’s blood and tissue is highly loaded with bisphenol A and other chemicals. Yes, some of those chemicals get secreted by the kidney, but 50% of these chemicals gets metabolized by the liver and binds to human tissue. I was completely amazed by this article showing that bisphenol A can even get into the brain and bind to a protein called bisphenol A binding protein. When bisphenol A binds to myelin basic protein in the brain, isn’t that a mechanism by which chemicals induce autoimmunity—in this case neuroautoimmunity?
There are many, many examples. Pesticides, herbicides, and many, many other chemicals have a similar mechanism of action; bisphenol A is not the only one. So medications and environmental toxins bind to human tissue, resulting in antibody production against the chemicals as well as our own tissue; that’s a mechanism of autoimmunity.

The third factor is infection. Earlier, you asked me if I had a personal experience that motivated my interest in the role of environmental factors in autoimmunities. The answer is absolutely, yes. My mother developed osteoarthritis at age 43. Ten years before that, I used to accompany her to a so-called dentist. Remember, we lived in a very small town in Iran. We had dental technicians, not dentists. My mother had a severe infection of the gum. One day, I remember this technician removing 3 or 4 teeth while she had the infection.

Now imagine what happened. Let’s say she had infection with Porphyromonas gingivalis or Streptococcus sanguis. These are 2 oral bacteria causing infection. Each of these bacteria strains releases a toxin. He removed the teeth, the barriers were broken, and these toxins immediately got into her blood. Her immune system started making antibodies against the bacterial toxins and, because of the similarly between the toxin and the joint tissue, her own antibodies started attacking her joints. After 5 or 10 years, she started having symptoms of rheumatoid arthritis. After another 5 or 10 years, this resulted in complete osteoarthritis, which required total knee replacement. At that time, I was a student in Israel, where my mother came to have the procedure. Because I had just started my master's degree, I wanted to see whether or not the experience she had with the dental technician years before had something to do with her arthritis. I took blood samples from her and from her perfectly healthy friends and made an antigen from the 2 bacterial antigens. When I tested her blood against those 2 bacteria, Porphyromonas gingivalis and Streptococcus sanguis, in comparison to her healthy friends, she had 10 times more antibodies against those 2 bacteria. At that time, actually, I did connect her rheumatoid arthritis to oral infection. Now, after 40 years, there are many articles in scientific journals about the connection between oral infection and autoimmune diseases. One of them from the March 2012 issue of Mucosal Immunology, titled “Periodontitis, Porphyromonas, and the Pathogenesis of Rheumatoid Arthritis,” confirmed this connection and described the mechanism of action thus: “The process of citrullination, a post-transitional protein modification, has been highlighted as a process common to both diseases. The evidence for a relationship between the diseases is explored and its potential mechanisms discussed.” What happens is that the toxin from the bacteria changes the protein of our body, causing our body to react against our own protein. This results in autoimmunity, such as rheumatoid arthritis. Many bacteria, whether it’s chlamydia, mycoplasma, Klebsiella, and many others, can be involved in rheumatoid arthritis, cardiovascular disease, and other autoimmune disorders.

Let me give you another example because this is November and we are getting closer to Thanksgiving. We are going to cook our turkey. There is a bacterium called Campylobacter jejuni. This bacterium causes food poisoning similar to Salmonella. A small percentage of turkeys or chickens carry this bacterial strain. Unfortunately, it infects the skin of turkeys and chickens during processing. If we don’t cook the chicken or turkey properly, this bacteria gets into our GI tract, causing diarrhea—severe diarrhea, which opens the tight junctions of the gut. Bacterial toxins get into the blood. The immune system then reacts against the toxins. In 95% of the cases, the antibodies produced against the bacterial toxin get neutralized and the body gets rid of that infection in a week or so. That’s why 95% have no problem. In the other 5% of the population, due to genetic makeup and being sensitive to this kind of toxin, the antibody attacks their gangliosides in the peripheral nerve as well as the central nervous system, resulting in the disease called Guillain-Barre syndrome. Here we have another example of infection releasing a toxin—our immune system reacting against the toxin—a cross-reaction between the toxin and our nerve cell antigens, and the result is a neuroautoimmune disorder.

ATHM: Considering all of these factors that act as triggers, do vaccines also play a role in these diseases?

Dr Vojdani: Without making this issue political, just being a scientist, what do we have in the vaccine out of those 3 factors? We talked about dietary proteins, infection, and toxic chemicals. Did you know that most probably we have all 3 of these in the vaccine?

Most of the time they grow a vaccine in the egg, so there are components of egg proteins in the vaccine. That’s number 1. Number 2, what is a vaccine? We vaccinate against what? Against measles, mumps, rubella, and others—what are measles, mumps, and rubella? Viruses. That’s an infectious agent. Right? That’s the second item. The third item in the vaccine is a toxic chemical. Can you name a chemical more toxic than mercury or even aluminum?

You have all 3 components, or if they don’t grow in egg, at least we have 2 major components. The virus and the toxic chemical together, in an individual with genetic susceptibility to that infectious agent plus the presence of the toxic chemical, can result in immune disorders such as autism, ADD, ADHD, and also autoimmunities.

Personally, I’m not against vaccination. Let’s make that clear. I’m a father of 3 children. I proudly say that, yes, when they were young we had them vaccinated, but we did not do their vaccinations in a way that is recommended by pediatricians—meaning to do it all right away in the first 3 months. We waited for 6 months and we gave them 1 vaccine at a time. We waited another 3 months; we gave them the second vaccine. We waited 3 more months and then completed all the vaccinations. We were not in a rush to do those vaccinations. We waited and divided them. That way, I
protected my children against possible harm from the vaccines.

**ATHM:** Have you found a connection between autistic children and cerebellar peptides? Is that something you’ve researched?

**Dr Vojdani:** I published an article in *Nutritional Neurosciences* about the cross-reaction between the antibody against gliadin and the cerebellum. In fact, I found the exact amino acid in gliadin as well as in the cerebellum, which are 50% identical. So, children with autism, if their immune systems produce antibodies against alpha gliadin, those antibodies my cross the blood-brain barrier, or BBB, and react with their own cerebellar cells. The normally selective BBB can be opened by many factors, such as infection and disease, so that large gluten molecules can slip through. The antibodies against alpha gliadin will recognize these molecules, but because of that 50% correspondence with cerebellar tissue, the antibodies will mistakenly recognize cerebellar cells as foreign antigens and thus attack the cerebellum as well; that is why more cerebellar-associated abnormalities are seen in children with autism. And that is also why a gluten-free diet is helpful for autistic children.

**ATHM:** You have studied the effects of treating veterans with Gulf War illness with doxycycline. Could you please describe the study and the conclusions you drew from it?

**Dr Vojdani:** My lab was one of the laboratories funded by the Department of Defense; I believe it was around 2002, or 2003. This was a multicenter study. Participating in the study were the University of Texas, the University of Washington, and some others. They took blood samples from patients with Gulf War syndrome, sending them to 4 or 5 different laboratories, including university laboratories and Immunosciences Lab. Then, we used PCR, or polymerase chain reaction, to detect *Mycoplasma fermentans* in their blood. If they were positive with *Mycoplasma fermentans*, they were given doxycycline in order to get rid of that mycoplasma and improve the clinical condition of our soldiers with Gulf War syndrome. Unfortunately, the study was designed in such a way that they didn’t reach a final, final conclusion. The results were not promising, and at the final stage I was out of the loop, and I don’t know what happened in the end. They discontinued the study. Overall, some patients with arthritis and Gulf War syndrome who took doxycycline showed improvement in clinical symptomatology. Why? There are 2 reasons: first, doxycycline is antimycoplasma and antibacterial. Second, it is an anti-inflammatory. Patients took it, showed some benefit, and, therefore, they wanted to take more of it. Again, we should not forget that medication is a chemical and taking chemicals for a long period of time is going to affect our immune system. It is possible that long-term medication can induce immune reactivities and autoimmunities.

**ATHM:** You testified before the US Senate Committee on Veteran Affairs in 1993 regarding immunological studies on blood samples of Persian Gulf War veterans and controls stating that some of the veterans who had been exposed to chemical agents while serving in that war had neuroimmunological disorders. This helped pass a law to provide free medical care to Persian Gulf War veterans. What was that experience like?

**Dr Vojdani:** It was one of the most important experiences in my life because that was where I could put 30 years of my experience into action. At the same time, it was an opportunity for me to give back to America. Remember, in the beginning of this conversation I said the turning point of my life was coming to America. I was attending the American Academy of Environmental Medicine in 1992. A major from the US Army came to the Immunosciences Lab booth. At that time, he knew that I was studying the effects of toxic chemicals on the immune system. He asked me several questions, and at the end of an hour of conversation he said, “Ari, are you ready to help our soldiers from the US Army who are sick?” Of course, my answer was yes. He said, “I’m going to take this back to my command and most probably I’ll get back to you next week, but I would like you to offer free testing for our soldiers.” Remember, these tests were about $1000 per person because it consisted of an immune system evaluation—antibody testing against the nervous system, antibody testing against different tissues, and many more.

My answer was yes but I had 1 condition. He said, “What is your condition?” I said, “The condition is that you cannot conduct research with individuals who are sick if you don't have proper controls.”

“That’s easy to do,” he said. “Write to me exactly what you want.” I said, “If you want to do this study, send me 50 blood samples from patients with Gulf War syndrome and another 50 blood samples from soldiers who were part of the US Army but did not participate in the Gulf War.”

After a week, I received 100 blood samples: 50 controls and 50 soldiers with Gulf War syndrome. I immediately started doing those tests, because it’s important to do them while the blood is fresh, first the immune evaluation, and then the antibodies later on. I was amazed to see that these individuals had abnormality at the cellular level. At the humoral level they were making antibodies against their own myelin sheath. They were making antibodies against their own joints and striated muscle and so forth. After 3 months, I wrote a summary. Based on that, I was invited to go before the US Senate and present this to the committee. At that time, based on my finding, yes, I did testify that our soldiers are not suffering from PTSD. Every soldier who was sick—they called it PTSD, posttraumatic stress syndrome—but I said they were suffering from neuroimmune disorders due to exposure to environmental factors. That was a fantastic experience.

When I came back to LA, I was interviewed by several TV stations. They asked my opinion: What are the environmental factors that you believe are involved in this
There are chapters in medical textbooks about different toxins and infectious agents and dietary proteins and peptides as the triggers of autoimmunities, which affect about 53 million Americans and about 10% of the world population.

As the triggers of autoimmunities, which affect about 53 million Americans and about 10% of the world population.

These conditions, plus the chemicals, can affect your nervous system resulting in neuroautoimmunity, which I described in our soldiers. To summarize, it really was incredible that the US Senate accepted their illness to be war-related and provided them with free medical care. It wasn't just me—there were several other doctors who testified—but I was one of those who contributed to this and I'm very proud of that.

ATHM: As a top immuno-neurologist with an interest in reducing the number of environmental toxins we expose our immune systems to, what kind of support have you found in the medical community for your work?

Dr Vojdani: Thank you for asking that. I had the honor of working with Andrew Campbell, MD, and many other excellent clinicians. In fact, I had many blood samples from patients exposed to various toxic chemicals. In one instance a train, which was loaded with toxic chemicals, derailed and toxic chemicals very similar to the one used in Bhopal, India, were released into the Sacramento River and people living on the river banks became very sick. Other exposures included MTBE, which is a gasoline additive; patients with silicone breast implants; and many, many, many others. I handled thousands of blood samples from patients exposed to various toxic chemicals. I found that these patients had many immune abnormalities very similar to our soldiers with Gulf War syndrome. When we presented this in different medical conventions, the doctors looked at us as if we came from a completely different world.

Therefore, your answer depends upon the definition of medical community. If you are asking about complementary and alternative medicine, absolutely, yes. If you are talking about functional medicine, they absolutely recognize these types of abnormalities. There are thousands and thousands of articles in scientific journals beginning 40 years ago and continuing on through today. Unfortunately, the medical doctors who are practicing medicine do not have time to read these scientific journal articles. Therefore, they are not educated in the field and do not recognize that environmental toxins and infectious agents and dietary proteins and peptides as the triggers of autoimmunities, which affect about 53 million Americans and about 10% of the world population.

They accept that medication can induce autoimmunity. There are chapters in medical textbooks about different medications causing autoimmunities. Three to 6 months after they remove these medications from the environment of the patient, the autoimmunity in the patient is reversed. So, they accept that. When you change the name of that toxic chemical from medication to formaldehyde or isocyanide or bisphenol A, they cannot accept that—even though the mechanism of action is exactly identical. That's very, very unfortunate.

In fact, one day I conducted some informal research for myself. As I sat in the UCLA cafeteria, very close to the cashier, I found myself looking at the drinks chosen by the doctors and surgeons who came to have lunch. I would say 60% to 70% of those hundreds I was observing chose diet sodas. The other 20% to 30% chose either water in a plastic bottle or orange juice in a plastic bottle. Remember, the pH of diet soda is about 3.5—very acidic. The pH of orange juice is about 3.5, maybe 3.0. Imagine that these canned or bottled liquids have been in storage for months until they got to the cafeteria. Don't you think that all that bisphenol A in the bottle is also mixed in with the orange juice and water, as well as in the soft drinks? If our doctors and professors at prestigious universities are drinking diet sodas in cans and orange juice in plastic bottles, what do you expect from the rest of the population?

You are asking me a very interesting question. Unfortunately, no, there is complete denial. Hopefully, they will read the articles in scientific journals and change their minds.

Unfortunately, it isn't until some of these individuals have children with autism or ADD or ADHD that they find time to go to the library and check the evidence. Then, they turn around and say, “Now I believe in all of this.” In fact, some of these individuals then join the autism societies. Again, why must we go through these types of experiences in order to become believers? The evidence is overwhelming. It takes people to read it, understand it, and apply it. I don't know how many years it will take until our leaders will become knowledgeable enough about some of these environmental factors so as not to use plastic and other chemicals in our daily life. These chemicals cause so many problems in our body; just remember that estrogenic compounds in plastic act like estrogen or even testosterones in males and females, which is how you get endocrine disorders.

ATHM: What will you be working on in the near future?

Dr Vojdani: First of all, I am in the process of developing more methods to detect the effects of environmental triggers. Right now, we have a limited number of methods for detection of triggers as a cause of autoimmune disorders. Let me quote a couple of paragraphs from one of the articles I wrote in a journal called Expert Opinion in Medical Diagnostics, in 2008. Molecules called predictive autoantibodies appear in the blood years before people show symptoms of various disorders. Predictive antibodies, for example, are...
antibodies that can detect issues with bisphenol A. If those antibodies are elevated, then the patient should not use plastic. Let's go one step further: I don't think you even need a blood test in order to say you should not drink from plastic—again, at least when one has the antibody against it, meaning one's body is reacting to it. Tests that detect it—these molecules—could warn of the need to take preventive actions.

Here's another quote. Researchers and clinicians should ask the question: Why does the human body react to its own antigens—why do I react to my own joints, my own thyroid, my own cerebellum? The cause may be due to environmental factors such as bacterial or viral infections or haptenic toxic chemicals binding to human tissue and causing modification of cells, antigens, and subsequent production of autoantibodies, which attack and destroy our tissue, causing autoimmunities.

Finally, considering the fact that the evolution of autoimmune response in using new antigens occurs over time, more diverse autoreactive antibodies will be detected. Therefore, only inclusion of antibody assays against a panel of antigens, some of which are tissue-specific and others related to the etiologic agents, may enhance clinical sensitivity, specificity, and predictive value in future studies. My future studies are going to be related to predicting antibodies for early detection of chronic illnesses. I have a few of them right now, but I'm going to expand the list of the predictive antibodies for the detection of chronic illnesses.

When a patient visits a doctor and orders some of the specialized testing developed by me at either Immunosciences Lab or at Cyrex Laboratories in Phoenix, Arizona, these tests will cover antibodies against various wheat proteomes, haptenic chemicals, infections or antibodies against myelin basic protein, or ganglioside, or other tissue antigens. The clinicians can then find the causative triggers, and by removing them from the patient or environment, make a huge difference in their patients' overall health.

Imagine this triangle: You detect, remove, and repair. Detect uses the most accurate biomarkers, which I'm in the process of developing. Remove the triggers. If the triggers are in the body, inflammation will continue. Continuous inflammation in the body can cause autoimmunities. You have to remove environmental toxins. You have to minimize the use of drugs and medication—of course, consulting your doctor. You have to minimize infection, in general, and also pay attention to your gut immune function. Unfortunately, the ratio of good to bad bacteria has changed. You have to restore the balance to more good bacteria and less bad bacteria. Finally, repair. How can we repair the barriers and improve your regular T-cell function? Vitamin A; vitamin D; omega-3 fish oil; EPA; DHA; coconut oil; green tea EGCG; resveratrol; probiotics; prebiotics, such as inulin from artichokes; vegetables such as the Cruciferous family, which contains 3-indole-carbinol; and finally, anti-inflammatories such as curcumin, Boswellia, and do not forget fermented foods, of which our ancestors used to eat a lot—an example is organic kimchi, which is fermented cabbage.

Detect, remove, and repair. That's my final message.
AUTOIMMUNE DISEASE

This new epidemic may be caused by one of man’s oldest enemies.

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AND over-consumed foods on a gluten-free diet

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Testing 8 wheat proteins and peptides, not just one.
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