Cross-Reactivity Between Dietary Proteins and Chemicals Bound to Albumin With Thyroid Axis Target Sites

CROSS-REACTIVITY BETWEEN DIETARY PROTEINS AND CHEMICALS BOUND TO ALBUMIN WITH THYROID AXIS TARGET SITES

By

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I have reviewed this thesis. It represents the work done by the author under my guidance/supervision.

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Chapter 1: Overview of the Thesis Manuscripts

The rate of autoimmune diseases is on the rise worldwide. Autoimmune thyroid disease is the most common autoimmune disease. No known cures have been found for autoimmune diseases; however, environmental factors, including dietary proteins and chemicals, may influence the expression of the disease by mechanisms of cross-reactivity. Immunologic cross-reactivity occurs when adaptive immune response against one antigen also occurs in another antigen that shares the amino acid sequence homology. The first manuscript in this thesis titled, "Immunological reactivity using monoclonal and polyclonal antibodies of autoimmune target sites with dietary proteins," investigates the role of 204 purified dietary proteins for cross-reactivity in autoimmune target sites of the thyroid axis.

Cross-reactivity can also occur when chemicals bind to proteins and induce a conformational change in the macromolecule, thereby creating new peptide structures that act as neoantigens. When epitopes of neoantigens share surface topology similar to other binding sites, there is a potential for development of cross-reactive interactions. The second manuscript in this thesis titled, "Reaction between antibodies to thyroid axis target sites and chemicals bound to human serum albumin," investigates the role of commonly encountered chemicals in autoimmune thyroid disease target sites.

The identification of immunological cross-reactivity in the thyroid axis target sites supports the understanding of how specific environmental triggers may interfere with thyroid hormone replacement, disrupt hormone metabolism, act as a potential triggering agent for thyroid disease, non-thyroid illness syndrome, and thyroid autoimmune diseases

such as Hashimoto's and Grave's. Both manuscripts, followed by a conclusion and discussion of their combined role in the thyroid disease, are listed in this thesis.

Chapter 2: Manuscript 1

Immunological Reactivity Using Monoclonal and Polyclonal Antibodies of Autoimmune Target Sites with Dietary Proteins

Abstract

Many hypothyroid and autoimmune thyroid patients experience reactions to specific foods. Additionally, food interactions may play a role in a subset of individuals who have difficulty finding a suitable thyroid hormone dosage. Our study was designed to investigate the potential role of dietary protein immune reactivity in thyroid hormones and thyroid axis target sites. We identified immune reactivity between dietary proteins and target sites on the thyroid axis that include thyroid hormones, thyroid receptors, enzymes, and transport proteins. We also measured the immune reactivity of either target specific monoclonal or polyclonal antibodies to the thyroid-stimulating hormone (TSH) receptor, 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, and triiodothyronine in 204 purified dietary proteins commonly consumed in cooked and raw forms. Dietary protein determinants included unmodified (raw) and modified (cooked and roasted) foods, herbs, spices, food gums, brewed beverages, and additives. There were no dietary protein immune reactions to the TSH receptor, thyroid peroxidase, and thyroxine-binding globulin. However, specific antigen-antibody immune reactivity was identified to several purified food proteins with triiodothyronine, thyroxine, thyroglobulin, and 5'deiodinase. A laboratory analysis of immunological cross-reactivity between thyroid target sites and dietary proteins is the initial step necessary in determining whether dietary proteins may play a potential immunoreactive role in autoimmune thyroid diseases.

1. Introduction

Immunologic cross-reactivity occurs when adaptive immune response against one antigen also occurs against another antigen with amino acid structural similarity. Immunological cross-reactivity was first identified in 1942 when it was found that individuals sensitized to pollen allergens developed immune reactivity to specific fruits.[1] Further study found that cross-reactivity with pollen could also occur to human tissue target proteins.[2] Exposure to antigens that share amino acid sequence homology with self-tissue proteins in susceptible hosts has been theorized as a trigger for tissue-specific autoimmune diseases.[3] [4]

Various antigens have been shown to specifically cross-react with thyroid tissue and trigger thyroid autoimmunity. Additionally, heat shock protein 60 (Hsp60), a mitochondrial chaperonin involved in stress responses, diabetes, and immunological disorder, has a structural similarity to thyroglobulin and thyroid peroxidase molecules. Enzyme-linked immunosorbent assay (ELISA) evaluations have also shown that immunological cross-reactivity plays a role in Hashimoto's thyroiditis.[5]

Numerous gastrointestinal pathogens have demonstrated molecular mimicry with the thyroid tissue. For example, human monoclonal thyroid-stimulating hormone receptor (TSHr) has been shown to cross-react with *Yersinia enterocolitica* (*Y. enterocolitica)*, thereby providing a mechanistic framework for molecular mimicry in Graves' disease, where *Y. enterocolitica* antibody production promotes a cross-reactive pathogenic response to the TSH receptor.[6] Researchers have discovered that the outer membrane of the porin F protein of *Y. enterocolitica* shares cross-immunogenicity with a leucine-rich

domain of the TSH receptor and plays a role in inducing autoimmunity to the TSH receptor through molecular mimicry.[7]

Immune cross-reactivity between *Helicobacter pylori (H. pylori)* and autoimmune thyroid diseases have been suspected due to correlations between the early onsets of *H. pylori infections*, Hashimoto's hypothyroidism, and Graves' disease.[8] [9] Cross-reactivity with *Candida albicans* and thyroid antigens has been identified and associated with the development of autoimmune thyroid diseases.[10] The protozoa *Toxoplasma gondii* has also been reported to potentially induce autoimmune thyroid cross-reactivity through molecular mimicry mechanisms.[11]

In addition to gastrointestinal pathogens, *Clostridium botulinum* neurotoxin A (Btx) has been reported to induce serum elevations of the TSH. Researchers found that Btx and thyroid autoantigens share an amino acid sequence homology and may play a role in the cross-reactive complication of an autoimmune thyroid disease.[12] *Borrelia burgdorgeri* has been shown to have protein homology with the TSH receptor and, therefore, plays a role as an antigenic trigger in autoimmune thyroid diseases.[13] *Coxsackie* virus antibodies have also demonstrated cross-reactivity with the thyroid and have been reported to be a contributing factor in the pathogenesis of autoimmune thyroid disorder.[14]

The list of pathogenic organisms, such as viral, bacterial, fungal, spirochete, and protozoa antibodies, that may contribute to tissue-specific thyroid autoimmunity via antibody amino acid sequence homology is a growing field of study. However, little research has been done on food protein antibodies and their potential role in thyroid specific cross-reactivity. Published research has looked at gluten and thyroid

autoimmunity, but the immunological reactive role that other dietary proteins may play with the thyroid function has been a limited area of research.[15] [16]

In this study, we evaluated the potential for food cross-reactivity with the thyroid axis by evaluating immune reactivity between purified dietary proteins and thyroid target-specific antibodies. We measured the immune reactivity of either target specific monoclonal or polyclonal antibodies to the TSH receptor, 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, and triiodothyronine in 204 purified dietary proteins that are commonly consumed in raw or cooked forms. Food determinants included unmodified (raw) and modified (cooked and roasted) food proteins, herbs, spices, food gums, brewed beverages, and additives. We included raw versus modified food antigens, since, in our earlier study, we already showed that some individuals may react to raw food antigens but not heat-modified ones, while, conversely, others may react to heat-modified but not raw food antigens.[17]

Specific target proteins along the thyroid axis associated with autoimmune thyroid diseases were evaluated in our study, including the thyroid-stimulating hormone receptor (TSH-R), 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, and triiodothyronine. The TSH-R antibody reactions are the hallmark of the Graves' disease. These antibody reactions not only promote autoimmune reactivity against the target protein, but a homosteric binding to these receptors can also promote an increased production of thyroid hormones, leading to hyperthyroidism.18[17] Thyroid peroxidase (TPO) is the key thyroid enzyme necessary for the synthesis of thyroid hormones and is the target in Hashimoto's autoimmunity disease. TPO antibodies are associated with the destruction of the thyroid and thyroiditis.19[18] Thyroglobulin (Tg) is a

dimeric protein synthesized in the thyroid and used for production of thyroid hormones. Its level is elevated with thyroid tissue breakdown, such as with thyroiditis and differentiated thyroid cancer.[19] Tg is a common target in thyroid autoimmunity diseases.[20] Thyroxine-binding globulin (TBG) is the transport protein in thyroid hormones in circulation and has a high affinity toward thyroxine and triiodothyronine.[21] Levels in the body can change due to metabolic disease, pregnancy, oral contraceptive use, and hormone replacement therapy.[22] Type II iodothyronine deiodinase (DIO_2) is the enzyme that converts the prohormone thyroxine by outer ring deiodination to bioactive triiodthyronine.24[23] Thyroxine (T_4) is a prohormone synthesized by the thyroid gland and composed of four iodine molecules attached to thyroglobulin. Triiodothyronine (T_3) is a bioactive thyroid hormone that is responsible for the key physiological mechanisms of thyroid target tissue function.[24]

The identification of immunological food protein cross-reactivity in the laboratory with thyroid axis target sites is the first step in understanding whether dietary proteins may potentially play an immunological reactive role in autoimmune thyroid diseases. In this study, we attempt to take the first step by determining any potential patterns of immunological cross-reactivity within a diverse list of food proteins and specific thyroid axis autoimmune target sites.

2. Materials and Methods

2.1 Polyclonal and Monoclonal Antibodies

Affinity-purified rabbit polyclonal thyroid stimulating hormone receptor antibody, affinity-purified goat polyclonal thyroxine 5-deiodinase (DIO_2) antibody, monoclonal

antibody to thyroid peroxidase, monoclonal antibody against thyroglobulin, mouse monoclonal thyroxine antibody, mouse monoclonal antibody to triiodothyronine, and monoclonal antibody to thyroxine-binding globulin were purchased from MyBioSource, Inc. (San Diego, CA, USA).

2.2 Preparation of Dietary Antigens

Food antigens were prepared from products purchased from the supermarket in both raw, roasted, or cooked form. For that preparation, 10 g of food product was put in a food processor with 0.1 M of phosphate buffer saline (PBS) at pH 7.4. The mixer was turned on and off for one 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 in 0.01 M of PBS using dialysis bags, with a cutoff of 6,000 kDa. The dialysis was repeated three times to ensure that all small molecules were removed. After dialysis, all samples were filtered using a 0.2-micron filter to remove any debris. Protein concentrations were measured using a kit provided by Bio-Rad (Hercules, CA, USA). Different peptides were purchased from Bio-Synthesis (Lewisville, TX, USA). Lectin and agglutinins were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Preparation of Dietary Oleosin Antigens

To purify the oleosin from peanuts, corn, safflower, sunflower, and soybean, the foods were prepared according to the method described by Vojdani (Vojdani A. Immune reactivities to peanut proteins, agglutinins and oleosins. *Alternative Therapies in Health*

and Medicine, 21(Suppl 1): 73–79, 2015). A total of 100 mL of chloroform/methanol (2/1, v/v) was then added and blended for two minutes using a food processor. The mixture was put in a 50-mL tube and centrifuged at 14,000 RPM for five minutes. The liquid on the upper phase was filtered through two 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 re-suspended in diethyl ether. At this point, 10 mL of water was added to each bottle, which was centrifuged at 20,000 g for five 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 five 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 a 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, USA) using chloroform/methanol as the solvent. The collected fractions of oleosins were checked by sodium dodecyl sulfate (SDS)-gel electrophoresis.

2.4 Preparation of Dietary Gum Antigens

Mastic gum, carrageenan, xanthan gum, guar gum, gum tragacanth, locust bean gum, and β-glucan were purchased from Sigma Aldrich (Saint Louis, MO, USA). Extracts from these items were prepared according to the procedures described by Vojdani (Vojdani A, Vojdani C. Immune reactivities against gums. Alternative Therapies in Health and Medicine, 21(Suppl 1): 64–72, 2015). Ten grams of each gum were extracted in a 500 mL of buffer pH 4.6 by mixing them for eight hours at 25°C on a magnetic stirrer. The solution was centrifuged at 20000 g, and supernatant was removed and concentrated on 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 the time of usage. Different gum extracts were dissolved in 0.1M PBS. These antigens were diluted on a 1:50 ratio in a 0.1M carbonate buffer at a pH of 9.2 and 100 μL of each gum antigen.

2.5 Enzyme-Linked Immunorsorbent Assay (ELISA) for Demonstration of Immune Reactivity

Food antigens and peptides were dissolved in PBS or methanol at a concentration of 1.0 mg/mL, then diluted on a 1:100 ratio in a 0.1 M carbonate-bicarbonate buffer at a pH of 9.5, and 100 uL was added to each well of the polystyrene flat-bottom ELISA plate. Plates were incubated overnight at 4°C and then washed three times with 200 uL Tris-buffered Saline (TBS) containing 0.05% Tween 20 at a pH of 7.4. The non-specific binding of immunoglobulins was prevented by adding a mixture of 2% bovine serum

albumin (BSA) into the TBS, and then incubating overnight at 4°C. Plates were washed as described above, and the serum samples diluted on a 1:100 ratio in a 0.1 M; PBS Tween containing 2% BSA was added to duplicate wells and incubated for one hour at room temperature.

Plates were washed again, and then, polyclonal and monoclonal antibodies diluted at an optimal dilution of 1:500 were added to duplicate antigen-coated wells; plates were incubated for an additional one hour at room temperature. The plates were then washed five times with TBS-Tween buffer. The enzyme reaction was started by adding 100 uL of paranitrophenylphosphate in 0.1 mL diethanolamine buffer 1 mg/mL containing 1 mM $MgCl_2$ and sodium aside at a pH of 9.8. The reaction was stopped 45 minutes later with 50 uL of 1 N NaOH, the samples were read by an ELISA reader, and the optical densities (OD) were recorded.

For the determination of the specificity of monoclonal and affinity-purified polyclonal antibodies in reaction with various food antigens, four wells of each 96-well plate were coated with 2% BSA alone but not the food antigens. After the addition of all the other antigens, the mean OD of these wells were subtracted from all other reactions.

2.6 Determination of Immune Reactivity Scale

Two hundred and four proteins (**see Table 1**) were tested for seven target tissue antibodies in duplicate, leading to 2,856 antigen-antibody OD measurements. The results of each duplicate OD were averaged together for one OD value, and the CD of control wells, which was less than 0.15, was subtracted from all other measurements. Of the 2,856 OD measurements, the mean OD was 0.33 with a standard deviation (SD) of 0.53.

The OD of 0.87 represented two standard deviations from the mean, the OD of 1.41 represented three standard deviations from the mean, and an OD of 1.95 represented four standard deviations from the mean. OD values below two standard deviations or less than 0.53 were labeled non-significant. OD values above two standard deviations but below three standard deviations at OD values of 0.66-0.86 were categorized as 1+ reaction. OD values above three standard deviations but less than four standard deviations at OD values of 0.87–1.07 were categorized as 2+ reactions. OD values above four standard deviations with OD values greater than 1.08 were categorized as 3+ reactions (**see Table 2**).

2.7 Binding of serially diluted thyroid antibody to fixed concentrations of various food antigens

For the demonstration of anti-thyroid antibodies binding to food antigens, different wells of microtiter plates were first coated with optimal concentrations (10 mg/well) of cashew, roasted cashew, latex hevein, cooked egg yolk, and seaweed. After the completion of all the steps necessary for coating the plates, each monoclonal antibody in dilutions of 1:500–1:512000 were added to two different rows of microtiter plates, and after the completion of all ELISA steps, the optical densities (ODs) were measured.

2.8 Binding of fixed concentration of antibody to serially diluted food antigens

Different food antigens in a concentration of 400 mg/mL were serially diluted. Microtiter plates were coated with 0.03–40 mg of each antigen in duplicate rows. After

the incubation and blocking steps, the addition of a 1:500 dilution of monoclonal anti-thyroid antibody, and the completion of all ELISA steps, the ODs were recorded.

2.9 Inhibition of anti-thyroid antibody binding to food antigen-coated plates by different concentrations of food antigens

Four different rows of three different microtiter plates were coated in this pattern: wells A1, B1, C1, and D1 were coated with T_3; the other eleven wells of each row (A2-A12, B2-B12, C2-C12, D2-D12) were coated either with roasted cashew, egg, seaweed, or latex hevein. Controls and inhibitors were then added as follows: 100 mL of serum diluent to wells A1-A2, B1-B2, C1-C2, and D1-D2; 100 mL of diluent containing 100 mg of T_3 to wells A3, B3, C3, and D3; and 0.5-120 mg of cashew, egg, seaweed, and latex hevein to, respectively, A4-A12, B4-B12, C4-C12, and D4-D12.

Plates were incubated at 37°C for one hour, and a 100 mL of mouse monoclonal anti-T_3 was added to all 48 wells. After repeated incubation, washing, the addition of the secondary antibody, and completion of all the ELISA steps, the optical densities were recorded. Plate #2 was used for the addition of T_4 and anti-T_4 antibody. Plate #3 was used for the addition of Tg and anti-Tg with proper controls.

Table 1: Dietary Proteins Screened for Immune Reaction

DIETARY PROTEINS SCREENED FOR IMMUNE REACTION			
DAIRY and EGGS, Modified	Cashew Vicilin	Spinach + Aquaporin	Oyster, boiled
	Chia Seed	Tomato + Aquaporin	Scallops, seared
Alpha-Casein & Beta-Casein	Flax Seed	Tomato Paste	Squid (Calamari), seared
Cow's Milk	Hazelnut, raw + roasted	Yam + Sweet Potato, baked	Shrimp, seared
Chocolate Milk	Macadamia Nut, raw + roasted	Zucchini, boiled	Shrimp Tropomyosin
Egg White, boiled	Mustard Seed		Parvalbumin
Egg Yolk, boiled	Pecan, raw + roasted	**FRUIT, Raw and Modified**	
Goat's Milk	Peanut, roasted		**MEAT, Modified**
Milk Butyrophilin	Peanut Butter	Apple	
Soft Cheese + Hard Cheese	Peanut Agglutinin	Apple Cider	Beef, boiled medium
Whey Protein	Peanut Oleosin	Apricot	Chicken, boiled
Yogurt	Pistachio, raw + roasted	Avocado	Lamb, baked
	Pumpkin Seeds, roasted	Banana	Pork, baked
GRAINS, Raw and Modified	Sesame Albumin	Banana, boiled	Turkey, baked
	Sesame Oleosin	Latex Hevein	Gelatin
Amaranth	Sunflower Seeds, roasted	Blueberry	Meat Glue
Buckwheat	Walnut	Cantaloupe + Honeydew Melon	
Casomorphin		Cherry	**HERBS, Raw**
Oats	**VEGETABLES, Raw and Modified**	Coconut, meat + water	
Quinoa		Cranberry	Basil
Rice	Artichoke, boiled	Date	Cilantro
Rice, white + brown, boiled	Asparagus	Fig	Cumin
Rice Cake	Asparagus, boiled	Grape, red + green	Dill
Rice Protein	Beet, boiled	Red Wine	Ginger
Rice Endochitinase	Bell Pepper	White Wine	Oregano
Rye, Barley, Spelt, Polish Wheat	Broccoli	Grapefruit	Parsley
Sesame	Broccoli, boiled	Kiwi	Rosemary
Sorghum	Brussels Sprouts, boiled	Lemon + Lime	Thyme
Tapioca	Cabbage, red + green	Mango	
Teff	Cabbage, boiled	Orange	**SPICES, Raw**
Wild Rice, boiled	Canola Oleosin	Orange Juice	
Wheat + Alpha-Gliadins	Carrot	Papaya	Cinnamon
Yeast Miller	Carrot, boiled	Peach + Nectarine	Clove
Hemp	Cauliflower, boiled	Pear	Mint
	Celery	Pineapple	Nutmeg
BEANS, Modified	Chili Pepper	Pineapple Bromelain	Paprika
	Corn + Aquaporin, boiled	Plum	Turmeric (Curcumin)
Black Bean, boiled	Popped Corn	Pomegranate	Vanilla
Bean Agglutinins	Corn Oleosin	Strawberry	
Dark Chocolate + Cocoa	Cucumber, pickled	Watermelon	**GUMS**
Fava Bean, boiled	Eggplant, boiled		
Garbanzo Bean, boiled	Garlic	**FISH and SEAFOOD, Raw and Modified**	Carrageenan
Kidney Bean, boiled	Garlic, boiled		Gum Guar
Lentil, boiled	Green Bean, boiled	Cod, baked	Gum Tragacanth
Lentil Lectin	Lettuce	Halibut, baked	Locust Bean Gum
Lima Bean, boiled	Mushroom, raw + boiled	Mackerel, baked	Mastic Gum + Gum Arabic
Pinto Bean, boiled	Okra, boiled	Red Snapper, baked	Xanthan Gum
Soybean Agglutinin	Olive, green + black, pickled	Salmon	
Soybean Oleosin + Aquaporin	Onion + Scallion	Salmon, baked	**BREWED BEVERAGES and ADDITIVES**
Soy Sauce, gluten-free	Onion + Scallion, boiled	Sardine + Anchovy, cooked	
Tofu	Pea, boiled	Sea Bass, seared	Coffee Bean Protein, brewed
	Pea Protein	Tilapia, baked	Instant Coffee
NUTS and SEEDS, Raw and Modified	Pea Lectin	Trout, baked	Black Tea, brewed
	Potato, white, baked	Tuna	Green Tea, brewed
Almond	Potato, white, fried	Tuna, seared	Honey, raw + processed
Almond, roasted	Pumpkin + Squash, boiled	Whitefish, baked	Beta-Glucan
Brazil Nut, raw + roasted	Radish	Crab + Lobster, boiled	Food Coloring
Cashew	Safflower + Sunflower Oleosin	Imitation Crab	
Cashew, roasted	Seaweed	Clam, boiled	

3. Results

3.1 Immune Reactivity between Affinity-Purified Polyclonal Antibodies and Food Antigens

The affinity-purified polyclonal antibody made against TSH-R and the monoclonal antibody made against TPO did not react with any of 204 food proteins and TBG. But the polyclonal antibody made against DIO2 had a 1+ reaction only with buckwheat.

3.2 Reaction of Monoclonal Antibodies Made Against Thyroglobulin, T_3 and T_4 with 204 Food Proteins

Using this monoclonal antibody against Tg resulted in a 3+ immune reaction with latex hevein (**see Table 2**). By using monoclonal antibody against T_4, our study found a significant list of food proteins that directly demonstrated immune reactivity with thyroxine. These foods include avocado, lemon and lime, cooked Brussels sprouts, seaweed, cooked zucchini, roasted and raw almond, cooked black bean, raw and roasted Brazil nut, cashew, roasted cashew, cashew vicilin, raw and roasted hazelnut, raw and roasted macadamia nut, mustard seed, roasted peanut, peanut butter, raw and roasted pistachio, gluten-free soy sauce, tofu, gelatin, cooked egg yolk, raw salmon, cooked tilapia, raw tuna, cooked tuna, cooked clam, cooked scallops, cooked squid (calamari), cooked shrimp, amaranth, and oats (**see Table 2**).

Similarly using the monoclonal antibody made against T_3, we found a significant list of food proteins that directly demonstrated an immune reactivity with triiodothyronine. These foods include avocado, latex hevein, lemon and lime, orange

juice (pasteurized and concentrate), cooked Brussels sprouts, baked white potato, seaweed, radish, roasted almond, raw and roasted Brazil nut, cashew, roasted cashew, cashew vicilin, raw and roasted macadamia nut, mustard seeds, roasted peanut, peanut butter, raw and roasted pistachio, soy bean agglutinin, gluten-free soy sauce, tofu, roasted sunflower seeds, gelatin, cooked egg yolk, raw salmon, cooked tilapia, raw tuna, cooked tuna, cooked clam, cooked scallops, cooked squid (calamari), cooked shrimp, cow's milk, casein (alpha and beta), sesame, hemp, rye, barley, kamut, buckwheat, sorghum, millet, spelt, amaranth, quinoa, yeast, oats, corn, and rice (**see Table 2**).

3.3 Simultaneous Reactivity of Monoclonal Antibodies Against Thyroid Target Sites

We selected ten foods from Table 1 to see if the same immune reactivity with a specific food, for instance latex hevein, elicited by a monoclonal antibody made against T_3, for example, would occur with a different monoclonal antibody. The data clearly shows that each monoclonal antibody shows different patterns of immune reactivity to the food antigens (see Table 3). In fact, TPO and TBG had no reactions to any food at all.

3.4 Demonstration of the specificity of anti-thyroid antibodies binding to different food antigens

The specificity of these monoclonal anti-T_3, T_4 and Tg antibodies in binding to various food antigens was confirmed by dilution and inhibition studies. As shown in Figure 1–A, –B and –C, the ODs or immune reactions to the food antigens decline significantly in proportion to the dilutions of the monoclonal antibodies. For example, the reaction of anti-T_3 antibody at a dilution of 1:500 with seaweed gives an OD of 2.8, a

dilution of 1:16,000 gives an OD of 1.23, and a dilution of 1:512,000 gives an OD of 0.2, which is equivalent to the background of the ELISA (see Figure 1–A). Similar results were obtained from the reaction of diluted T_4 with various food antigens (see Figure 1–B). Interestingly, using an anti-T_4 antibody, we found significant differences between the immune reactivity of raw cashew and roasted cashew (Figure 1-B). The binding of anti-Tg antibody to latex hevein, but not to seaweed, egg, or cashew, and the proportion of the binding to antibody dilution are shown in Figure 1–C. Furthermore, when fixed amounts of antibodies made against T_3, T_4 and Tg were added to plates coated with food concentrations of 0.037–400 mg/mL, the ELISA ODs increased in proportion to the concentration of the food antigens (see Figure 2–A, –B and –C). The reaction of anti-T_4 with serially diluted roasted cashew was stronger than its reaction with raw cashew (see Figure 2-B). To further demonstrate the specificity of these antigen-antibody reactions, different amounts of food antigens (inhibitors) in concentrations of 0.5–120 mg or controls were added in the liquid phase of plates that contained the optimal concentrations of food antigens in solid phase. The addition of anti-T3, -T4 or -Tg antibodies to the mixture resulted in a significant inhibition of thyroid antibody binding to some food antigens on the plates. This inhibition of antigen-antibody reaction was more obvious when higher concentrations of food antigens were used in the liquid phase (Figure 3–A, –B, –C).

Table 2. Immunoreactivity of monoclonal antibodies to T3 and T4 with food proteins

Food Protein	T_3	T_4	Food Protein	T_3	T_4
Almond	-	++	Orange Juice pasteurized or concentrate	+++	-
Almond roasted	++	+++	Peanut Butter	++	++
Amaranth	+++	+	Peanut roasted	++	+
Avocado	+	+	Pistachio raw & roasted	+	++
Barley	++	-	Potato	+++	-
Black Bean cooked	-	+	Potato White baked	+	-
Brazil Nut raw & roasted	+	+++	Quinoa	+++	-
Brussels Sprouts cooked	+	++	Radish	+	-
Buckwheat	+++	-	Rice	+++	-
Casein (a & b)	+	-	Rye	++	-
Cashew	++	+	Salmon raw	+	+++
Cashew roasted	++	+++	Scallops cooked	+++	+++
Cashew Vicilin	+	+++	Seaweed	+++	+++
Chocolate	++		Sesame	+	-
Clam cooked	+	+++	Shrimp cooked	++	+++
Coffee	+++	-	Sorghum	+++	-
Corn	+++	-	Soy Bean Agglutinin	+	-
Cow's Milk	++		Soy Sauce Gluten-Free	+	+++
Egg Yolk cooked	++	+++	Spelt	++	
Gelatin	++	+++	Squid (Calamari) cooked	+++	+++
Hazelnut raw & roasted	-	+	Sunflower Seeds roasted	+	-
Hemp	+++	-	Tapioca	+++	-
Kamut	+++	-	Tofu	+++	+++
Latex Hevein	+++	-	Tilapia cooked	+	+++
Lemon & Lime	+	+	Tuna cooked	+	+++
Macadamia Nut raw & roasted	++	++	Tuna raw	++	+++
Millet	++	-	Yeast	+	-
Mustard Seed	+++	+++	Zucchini cooked	-	++
Oats	+++	++			

+ = 0.66-0.86 ++ = 0.87-0.107 +++ = >1.08

Table 3. Example of 10 selected foods and their degrees of reactivity with monoclonal antibodies against Tg, T3 and T4

	Tg	T_3	T_4
Latex hevein	+++	+++	-
Kamut	-	+++	-
Soy sauce	-	+	+++
Gelatin	-	++	+++
Scallops	-	+++	+++
Cashew, roasted	-	++	+++
Cashew, vicilin	-	+	+++
Coffee protein	-	+++	-
Brazil nut	-	+	+++
Almond	-	-	++

Table 4. Reaction of monoclonal and polyclonal antibodies against 204 different foods

Antibodies	**Degree of reaction**			
	-	+	++	+++
Polyclonal antibody against TSH-R	204	0	0	0
Polyclonal antibody against DIO_2	203	1	0	0
Monoclonal antibody against TPO	204	0	0	0
Polyclonal antibody against TBG	204	0	0	0
Polyclonal antibody against Tg	203	0	0	1
Polyclonal antibody against T3	151	18	16	19
Polyclonal antibody against T4	172	7	7	18

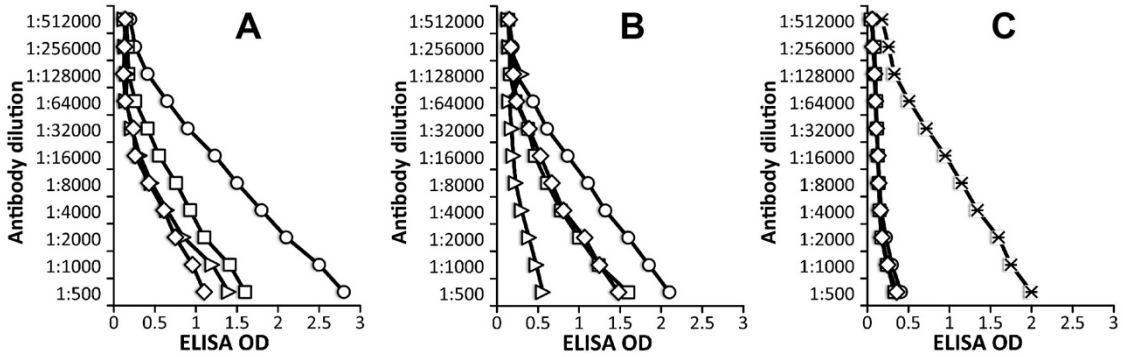

Figure 1. The binding of serially diluted T3 (A), T4 (B), and Tg (C) monoclonal antibodies to the same concentration of different food antigens. Seaweed = l. Roasted cashew = □. Cashew = r. Egg = u. Latex hevein = 5.

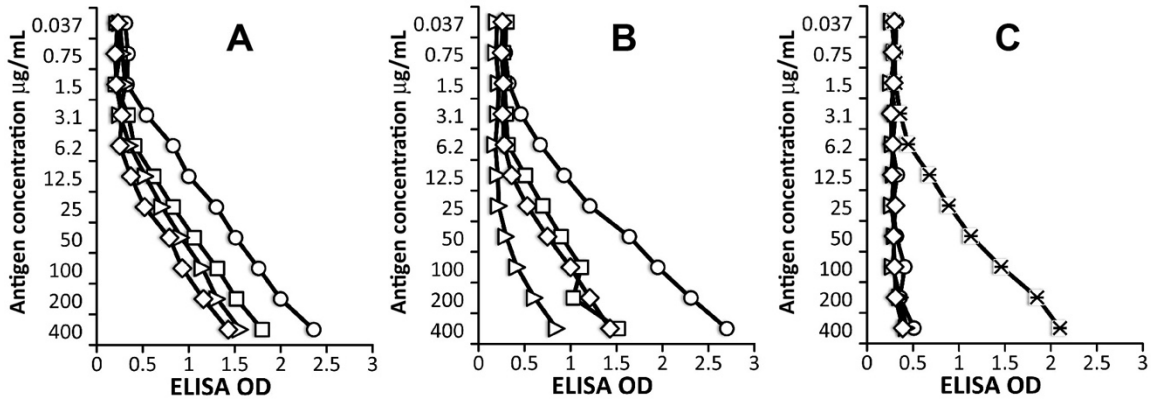

Figure 2. The binding of monoclonal antibody against T3 (A), T4 (B), and Tg (C) to serially diluted food antigens. Seaweed = l. Roasted cashew = □. Cashew = r. Egg = u. Latex hevein = 5.

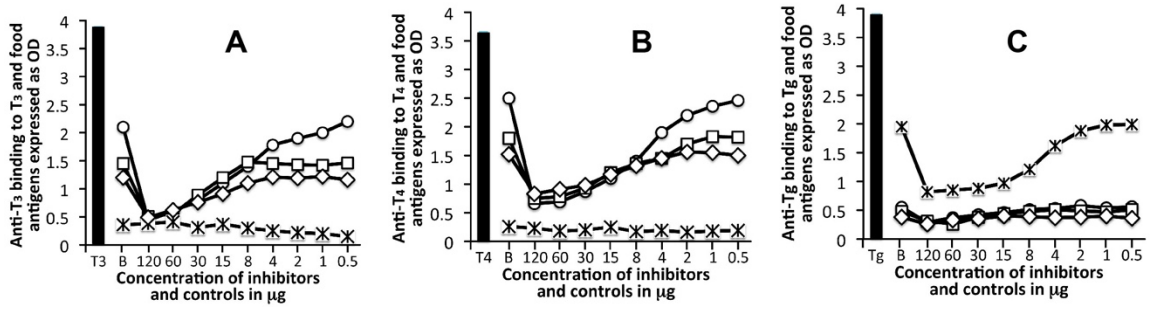

Figure 3. Inhibition of T3 (A), T4 (B), and Tg (C) antibodies with different concentrations of food antigens. Seaweed = l. Roasted cashew = □. Egg = u. Latex hevein = 5.

4. Discussion

Our laboratory study found dietary proteins that share amino acid sequence homology and have the potential to play a role in cross-reactivity with the thyroid target sites. This may serve to fill a knowledge gap and may have identified a starting place for further research into the relationship between immunological responses to dietary proteins and autoimmune thyroid diseases. We identified immune reactivity between antibodies made against various thyroid target antigens and food proteins and target sites on various locations of the thyroid axis. This potential antibody against specific thyroid axis sites with food antigens can lead to the possibilities that some dietary proteins may play a role in autoimmune thyroid diseases. Our study identified T_4, T_3, Tg, and 5-deiodinase antibodies that reacted with purified food antigens. These reactions suggest immunological cross-reactivity between food immune reactions and thyroid axis sites.

Interestingly, although many pathogenic organisms have shown direct cross-reactivity with both TSH-R and TPO, our study found that none of the 204 foods tested demonstrated any immune reactive response to these key autoimmune target sites associated with Graves' and Hashimoto's diseases, with the exception of latex hevein for thyroglobulin. Hevein is a lectin-like protein derived from *Havea brasillensis* (rubber tree). Although this lectin-like protein is not found in food, it is associated with the latex-fruit syndrome. Approximately 30–35% of individuals, who are allergic to natural rubber latex, show cross-reactive IgE hypersensitivity to plant sources such as avocado, chestnut, bell pepper, kiwi, peach, and tomato. Immune cross-reactivity with latex or latex-fruit syndrome may potentially lead to Tg cross-reactivity in susceptible individuals who are reactive to natural rubber latex and associated foods.[25]

Although direct food reactions did not occur with the TSH-R and TPO most commonly associated with Graves' and Hashimoto's diseases, there was significant cross-reactivity between both T_4 and T_3 hormones found within the thyroid gland with 25–35 food antigens (Table 2).[26 27]

We also did not identify immune reactions to TBG in our study, suggesting that food immune reactivity does not appear to have a role in thyroid hormone transport dysregulation or on the ratios of thyroid hormones bound to protein, and, therefore, foods should not impact the ratios of bound compared to unbound thyroid hormones or biomarkers such as T_3 uptake.

One reaction was identified between Type II 5-deiodinase (DIO_2), thereby suggesting that food immune reactivity with buckwheat may potentially impact T_4 to T_3 thyroid conversion associated with non-thyroidal illness syndrome (NTI) or euthyroid sick syndrome. These findings suggest a potential new mechanism for NTI that has not been reported in the literature, to the best of our knowledge.

Most of the cross-reactive reactions occurred with T_4 and T_3, and many of the cross-reactive foods overlapped with both hormones, as T_4 and T_3 have very similar amino acid homology, especially involving iodine sequencing. (REF) Some common patterns were identified with these reactions, including cross-reactions to many gluten-containing foods and grains and foods that contain iodine structural sequences similar to thyroid hormones, such as seaweed, white potato, nuts (almond, cashew, hazelnut, and peanut), seafood, and more (Table 2).

There was also an overlap between T_4 and T_3 foods not associated with iodine, such as cooked Brussels sprouts, lemon and lime, radish, tofu, gluten-free soy sauce, and

cooked zucchini. Some of these food proteins have been classified as goitrogens; however, their impact on the thyroid function may be due to an immune cross-reactivity instead of an interference with iodine uptake, as proposed in the goitrogenic model. This is especially probable given that some of these goitrogenic foods were tested in a modified cooked state to remove goitrogenic properties such as glucosinolates. Immunological cross-reactivity with foods listed as goitrogenic may explain some of the observed adverse reactions that occur in small subsets of thyroid patients, although the mechanisms now appear to be immunological rather than goitrogenic.

Additionally, there were many cross-reactive foods with T_4 and T_3 that did not overlap with each other. Although T_3 and T_4 have some structural similarity with each other, T_4 has two independent conformations in the crystal lattice with significant differences in the outer phenyl ring geometry, when compared to T_3. The major differences between T_4 and T_3 structures are shortened to C4'-O4' bond contraction of the C3'-C4'-C5' angle and an increase in the C3' and carbon C5' angles of T_4.[28] These differences may explain why certain food proteins only have cross-reactive reactions with either T_3 or T_4. Overall, this simultaneous reaction of three different monoclonal antibodies made against Tg, T_3, and T_4 with some food antigens but not others is the best indication for the specificity of the immune reactions between antibodies made against thyroid target antigens and different food proteins (**see Table 2, Table 4**).

The specificity of these anti-thyroid antibodies binding to antigenic determinants of food antigens was shown by a serial dilution of food antigens or different concentrations of monoclonal antibody with the same concentration of food antigens in the solid phase. The dose response curves shown in Figures 1 & 2, –A, and –B, first with

seaweed followed by roasted cashew, and cooked egg yolk, and the curves in Figure 1–C that show a strong response from latex hevein are supportive of the specificity of these antigen-antibody reactions. To further confirm this reaction of antibodies with food antigens, inhibition studies were conducted with different concentrations of food antigens in the liquid phase and the addition of monoclonal thyroid antibodies. As shown in Figures 3–A and –B, the addition of 120 mg of seaweed to the liquid phase resulted in 76% inhibition in the binding of anti-T_3 and 80% in the binding of anti-T4 to the food antigens (Figure 3-A, -B). The inhibition of Tg antibody to seaweed was not significant since, to begin with, the anti-Tg antibody did not react to seaweed (see Figure 3-C). In contrast, the inhibition of anti-Tg binding to latex hevein was very significant (>50%) when 15–120 mg of antigen was added to the liquid phase. Overall, this inhibition of anti-thyroid antibody binding to different food antigens was reversed in proportion to the lower concentration of immune reactive foods in the liquid phase. The results of these experiments support the proposition that the binding of monoclonal antibodies to T3, T4 and Tg to different foods as shown in Tables 1–3 is specific, and hence cross-reactivity between thyroid tissue antigens and various food antigens should be taken seriously.

5. Conclusions

The results of our study identified immune reactivity between T_3, T_4, DIO_2, thyroglobulin, and many food proteins. These immune reactions may explain the etiology of some cases of food interactions with both thyroid hormone replacement and thyroid hormone metabolism. Theoretically, these food protein reactions may contribute to the

autoimmune reactivity in a subset of autoimmune thyroid disease patients, which is a subject that warrants further research.

It is doubtful that the consumption of potentially reactive food proteins alone would induce an inflammatory response on thyroid axis target sites. The consumption of dietary proteins in combination with other factors such as digestive enzymes is necessary to induce immunological reactions. First, dietary proteins cannot have an impact on antigenic-antibody models if properly digested, unless the individual is immunologically reactive to those foods, thereby producing antibodies to those specific dietary proteins due to a failure in oral tolerance to those specific undigested food antigens. [29] If the individual has the oral tolerance to the ingested proteins and does not produce significant antibodies to those proteins, then no reaction would be expected. Second, antigen-antibody reactions alone are not solely responsible for pathogenic reactions. Cellular immunity, immunological tolerance, human leukocyte antigen (HLA) allele, and other factors are involved in pathogenic immune reactions to dietary proteins. Therefore, antibodies binding to antigens are not always pathogenic; however, the ability of specific monoclonal and polyclonal antibodies to bind with purified proteins suggests the potential for immunological cross-reactivity in a subset of susceptible individuals.

Further research should be conducted to evaluate the specific epitope for each of these foods and thyroid axis target sites. Additionally, immunological factors such as tolerance, T-cell polarization, and other factors that may induce susceptibility to dietary protein immune reactivity need to be investigated. Individual case studies and clinical trials will be required to assess whether these food proteins have any actual clinical role in thyroid-associated reactions. The results of our research provide a list of susceptible

dietary proteins that may immunologically impact thyroid interactions and warrant further study. These results provide a first step in narrowing down a list of specific dietary proteins that, due to protein cross-reactivity, may potentially have an impact on autoimmune thyroid diseases.

Chapter 3: Manuscript 2

Reaction between antibodies to thyroid axis target sites and chemicals bound to human serum albumin

Abstract

When chemicals bind to proteins, they induce a conformational change in the macromolecule that has the potential to create new peptide structures that may interact with various target sites of the body and lead to antibody production. Molecular mimicry can occur if epitopes of the protein share surface topology to similar binding sites. The alteration of peptides that share topological equivalence with alternating side chains can lead to the formation of binding surfaces that can mimic the antigenic structure of a variant peptide. In addition to peptide similarity with models of cross-reactivity, binding sites are polyfunctional and can accommodate more than one antigenic epitope. We investigated how antibodies made against thyroid target sites may bind to various chemicals bound to human serum albumin (HSA). We found that monoclonal or polyclonal antibodies developed against the thyroid-stimulating hormone (TSH) receptor, 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, and triiodothyronine bound to various chemical-HSA compounds. Our study identified a new mechanism in which chemicals bound-to-albumin lead to structural protein misfolding. This, in turn, creates neoantigens, which cause the development of antibodies that bind to key target proteins of the thyroid axis through protein misfolding. This laboratory analysis of immune reactivity between thyroid target sites and chemicals bound to HSA is the initial step for determining whether chemicals may play a potential immunoreactive role in the thyroid disease.

1. Introduction

Immunological cross-reactivity is expressed when antibodies with similar amino acid homology or similar antibody surface topology bind to the same binding site.[29] [30] The interactions of multiple antigenic antibodies with the same binding site is known as molecular mimicry.[31] Molecular mimicry of various antigens with self-tissue proteins can induce tissue-specific autoimmune diseases in susceptible subjects. [32] [33] These molecular interactions with the antigen/antibody binding sites can occur from a diverse list of antigen-promoted antibodies.[34] Cross-reactive antibodies from various infections have been found to play a role in autoimmune thyroid diseases and thyroid metabolism dysfunction by binding to multiple target sites of the thyroid axis via molecular mimicry.[35] [36] [37] [38] [39] [40] [41] [42] [43] [44] Furthermore, many antibody binding sites are polyfunctional and can accommodate more than one antigenic epitope and play a role in autoimmune diseases.[45] Cross-reactive interactions with various target sites of the thyroid axis may also lead to thyroid metabolism disruption.

Cross-reactivity with antibodies from chemicals bound to proteins potentially play a cross-reactive role in autoimmune thyroid diseases and thyroid metabolism disruption. In addition, the binding of chemicals to self-proteins such as albumin, globulin, or hemoglobin leads to protein misfolding and induce a conformational change in the macromolecule. The alteration of protein topography leads to the binding of antibody to the protein in the target sites.[46] Molecular mimicry can occur if epitopes of the protein share surface topology to similar bindings sites.[47]

Research on how chemicals bound to protein play a role in the cross-reactive model of thyroid metabolism and autoimmune thyroid diseases is currently limited. In this laboratory study, we evaluated the potential for the binding of antibodies that are made against the thyroid axis to various chemicals bound to albumin through chemical induction of misfolding of HSA, which immunologically acts similar to thyroid target site antigens such as TSH receptor, 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, triiodothyronine, and various chemicals bound to albumin.

Chemical molecules can bind directly to circulating proteins or bind indirectly to circulating proteins after hepatic or extrahepatic conversion from prohapten to haptens, generating hapten-protein adducts. This leads to neoantigen formation resulting in systemic T-cell or antibody immune responses against the haptens and self-proteins.[48 49] The potential for neoantigens to develop from environmental chemicals after binding to human tissue antigens and the role this may play in cross-reactivity against thyroid axis sites was investigated in our study. We evaluated the immune reactivity of chemicals bound to human albumin that included chemical compounds found in plastic products, foams, cosmetics, upholstery, dry cleaning agents, fire retardants, metal products, and chemicals commonly found in water and soil contamination.

Cross-reactivity between chemically-induced misfolded HSA with the various target sites of the thyroid axis may play a role in the pathophysiology of thyroid autoimmunity. This could impact various aspects of thyroid metabolism, which may interfere with the proper dosage of thyroid hormones, impair thyroid peripheral metabolism, disrupt thyroid feedback loops, and alter thyroid hormone transport. In this

study, we attempt to take the initial first laboratory step in determining if any patterns of cross-reactivity occur from hapten-protein adducts with specific thyroid axis target sites.

2. Materials and Methods

2.1 Polyclonal and Monoclonal Antibodies

We purchased mouse monoclonal antibody to thyroxine-binding globulin, thyroxine antibody, triiodothyronine antibody, and thyroid peroxidase antibody from MyBioSource Inc. (San Diego, CA USA). Affinity-purified goat polyclonal thyroxine 5-deiodinase (DIO2) antibody was produced from MyBioSource and affinity purified antibody to rabbit polyclonal thyroid-stimulating hormone receptor was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Proteins and Chemicals

Human serum albumin (HSA), bovine serum albumin (BSA), hemoglobin, formaldehyde, tolylene-2.4-diisocyanate, trimellitic anhydride, p-amino benzoic acid, bisphenol-A (BPA), tetrabromobisphenol-A, isopropyl benzoic acid, cyanoethyl benzoic acid, propyl, 4-hydroxy benzoic acid, permethrin, mercury chloride, nickel sulfate, cobalt acetate, cadmium chloride, lead acetate, and arsenic oxide were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.3 Preparation of Formaldehyde-Human Serum Albumin

Because there are copious amounts of HSA in the serum, which is found in the majority of tissues in the human body, we selected HSA as our model protein. HSA has a well-defined monomeric and three-dimensional structure. Additionally, in their vivo

studies, Chipinda, Hettick, and Siegel reported that HSA is a target of hapten binding.[50] We prepared a formaldehyde-human serum albumin (F-HSA) using the method published by Patterson.[51] We separately exposed 1 mg of HSA in a phosphate-buffered saline to 1 mg of formaldehyde at a pH of 7.4. We then incubated the mixture at 37 °C for 30 minutes and copiously dialyzed it against phosphate-buffered saline. We then sterilized F-HSA with a 0.2-μm filter. We then performed the electrophoretic and immunoelectrophoretic analysis to compare F-HSA and HSA to determine conjugation. The conjugation was confirmed by comparative differences in altered mobility for HSA and F-HSA.

2.4 Preparation of Tolylene-2.4-Diisocyanate-Human Serum Albumin

We used a similar method as published by Pezzini et al. in the formation of tolylene-2,4-diisocyanate-human serum albumin (TDI-HSA).[52] We dissolved 1g of HSA in 10 ml of a buffer solution containing potassium chloride (0.05mol 1–1) and sodium borate (0.05mol 1–1) at a pH of 9.4 that was cooled to 4°C. We then added Dioxane (10ml) containing 0.15ml of tolylene-2,4-diisocyanate in a dropwise method while stirring the solution over a 3-hour period, followed by the addition of 2 ml of ethanolamine, centrifugation, and then dialysis filtration and lyophilization. We confirmed the conjugation through electrophoresis.

2.5 Preparation of Trimellitic Anhydride-Human Serum Albumin

For the preparation of trimellitic anhydride-human serum albumin A (TMA-HSA), we dissolved 25 mg of trimellitic anhydride in a 0.5 ml of dioxane. We then added that dropwise to 25 mg of HSA dissolved in 5 ml of cold 7% $NaHCO_3$ in water and

stirred it for one hour at 4°C. The conjugates were then dialyzed against four changes of 0.1M $NaHCO_3$ and one change of buffer. We then used the method published by Pien to filter the mixture and keep it at -20°C.[53]

2.6 Preparation of 2,4-dinitrophenol-HSA Conjugates

For the preparation of 2,4-dinitrophenol ring-HSA (DNP-HSA) conjugates, we dissolved 40 mg each of p-aminobenzoic acid, isopropyl benzoic acid, cyanoethyl benzoic acid, or permethrin in 2 ml of 1 N HCL using an ice bath emersion. In parallel, we dissolved 1 g of HSA in boric acid 0.16M sodium chloride (0.15M buffer pH9.0 [pH was raised with NaOH]). While keeping the beaker on ice, we added the diazonium salt in a dropwise method while rapidly stirring the solution. The pH of the mixture was readjusted from 9.0 to 9.5 after the addition of each drop. The mixture was stirred slowly for at least one hour after all the solution was added, while making additions of NaOH solution to maintain the pH at a range of 9.0 to 9.5. We removed the non-reacted small molecules using a molecular cutoff of 8000 daltons with the dialysis. We conjugated the DNP to BSA and hemoglobulin similar to the preparation of DNP-HSA in specificity studies.

2.7 Preparation of Bisphenol-A, Tetrabromobisphenol-A, Tetrachloroethylene, and Parabens-HSA Conjugates

For the preparation of bisphenol-A-HSA, tetraobrombisphenol-A-HSA, and parabens-HSA conjugates, we dissolved 1 g of HSA in 100 ml of 0.01M PBS at a pH of 7.4. We then added 100 mg each of bisphenol-A, tetrabromobisphenol-A, tetrachloroethylene, or propyl 4-hydroxybenzoate (parabens) dissolved in 10 mL of

0.01M PBS. Each chemical was added in a dropwise method to the protein mixture. We kept the mixture at room temperature for one hour and then at 4°C for four hours. We removed the non-reacted small molecules using a molecular cut of 8000 daltons with dialysis.

2.8 Binding of Mercury, Mixed Heavy Metals to HSA

For the preparation of mercury-HSA and mixed heavy-metals HSA, we dissolved 250 mg of HSA with potassium chloride and sodium borate 0.05 ml l–1 in a 9-ml buffer solution containing 0.1 Na OH with an adjusted pH of 9.4. We dissolved 25 mg of Thimerosal and mercury chloride in 1 ml of a buffer and added it in a dropwise method to the HSA mixture. The mixture was stirred overnight and dialyzed against 0.1M PBS utilizing tubing with a cutoff of 8000 daltons. We used the method published by Vojdani by confirming the conjugation of the haptenic chemical by sodium dodecyl sulfate (SDS) gel electrophoresis and a shift in the HSA band.[54] We also confirmed that the chemicals became covalently linked by an increase in absorption from 230 to 260nM.

2.9 Demonstration of Anti-Thyroid Antibody Binding to Various Chemicals Bound to HSA Using ELISA

HSA or chemical bound to HSA at a concentration of 1.0 mg/mL–1 was diluted 1:100 in 0.1M carbonate-bicarbonate buffer with a pH of 9.5. We added 100 µl to each well of a polystyrene flat-bottom ELISA plate. The plates were incubated overnight at 4°C and then washed with 200 µl Tris-buffered saline (TBS) containing 0.05% Tween 20 at a pH of 7.4. Non-specific binding of immunoglobulin was prevented by adding 2% BSA in PBS and then incubating it overnight at 4°C. The plates were washed again, and

then, polyclonal and monolclonal antibodies at an optimal dilution of 1:500 in 0.1M PBS Tween containing 2% BSA were added to duplicate wells and incubated for one hour at room temperature. The plates were washed, and then, alkaline phosphatase goat anti-rabbit or anti-mouse IgG F(ab')2 fragments (KPI, Gaithersburg, MS, USA) at an optimal dilution of 1:400–1:2000 in 1% BSA-TBS were added to each well. The plates were then incubated for an additional one hour at room temperature and washed with TBS-Tween buffer five times. The enzyme reaction was started by adding 100 µl of paranitrophenylphosphate in diethanolamine buffer 1 mg/mL–1 containing 1mM $MgCl_2$ and sodium azide at a pH 9.8. The reaction was stopped 30 minutes later with 50 µl of 1N NaOH. The optical density (OD) was read at 405nm using a microtiter reader. To ensure non-specific binding, we used several control wells that contained all reagents or had wells that were coated with HSA alone, followed by all other reagents, to determine the specificity of antigen-antibody reactions.

3. Determination of Immune Reactivity Scale

Eleven chemicals bound to HSA and the enzyme PDI were tested with seven target tissue antibodies, which lead to 84 antigen-antibody OD measurements. The OD range was 0.1–2.02 and the mean OD was 0.46 with a standard deviation of 0.45. The OD of 0.91 represented one standard deviation from the mean. The OD of 1.35 represented two standard deviations from the mean. The OD of 1.81 represented three standard deviations from the mean. OD values below 0.90 represented non-significant reactions. OD values between 0.91–1.35 represented 1+ reactions. OD values between

1.36–1.80 represented 2+ reactions. OD values between 1.81–2.02 represented 3+ reactions.

4. Results

Immunological reactivity using monoclonal and polyclonal antibodies made against thyroid target antigens showed various degrees of reaction (Table 1). We identified immune reactivity of aflatoxin bound-to-albumin (1+) isocyanate bound-to-albumin (2+) with TSH-R (Figure 1). Immune reactivity of aflatoxin bound-to-albumin (1+) and formaldehyde bound-to-albumin (1+) with thyroxine 5-deiodinase (Figure 2) was identified. Immune reactivity was identified in aflatoxin bound-to-albumin (2+), formaldehyde bound-to-albumin (1+), isocyanate bound-to-albumin (1+), 2,4-dinitrophenol bound-to-albumin (1+), protein disulfide isomerase (3+), bisphenol-A (1+), tetrabromobisphenol-A bound-to-albumin (2+), tetrachloroethylene bound-to-albumin (1+), mercury bound-to-albumin (1+), parabens bound-to-albumin (3+), and heavy-metal composite bound-to-albumin (2+) with T3 (Figure 3). Immunological reactivity was identified in aflatoxin bound-to-albumin with thyroglobulin (2+) (Figure 4). Our laboratory investigation found no immune reactivity between any chemicals and thyroid peroxidase (TPO) (Figure 5), thyroxine-binding globulin (TBG) (Figure 6), and thyroid hormones thyroxine **(**T4) (Figure 7).

Table 1. Summary of Chemicals with Immune Reactivity to Thyroid Axis Target Sites

Chemicals	Thyroid Axis Target Site
Aflatoxins-HSA	Thyroxine 5-Deiodinase + Thyroid Stimulating Hormone Receptors + Thyroglobulin ++ Triiodothyronine ++
Formaldehyde-HSA	Thyroxine 5-Deiodinase + Triiodothyronine +
Isocyanate-HSA	Triiodothyronine + Thyroid Stimulating Hormone Receptors ++
2,4-Dinitrophenol-HSA	Triiodothyronine +
Bisphenol-A-HSA	Triiodothyronine +
Protein Disulfide Isomerase	Triiodothyronine +++
Tetrabromobisphenol-A-HSA	Triiodothyronine ++
Tetrachloroethylene-HSA	Triiodothyronine +
Mercury-HSA	Triiodothyronine +
Parabens-HSA	Triiodothyronine +++
Heavy Metal Composite-HSA	Triiodothyronine ++

0.91 – 1.35 = + 1.36 – 1.80 ++ 1.81-2.02 = +++

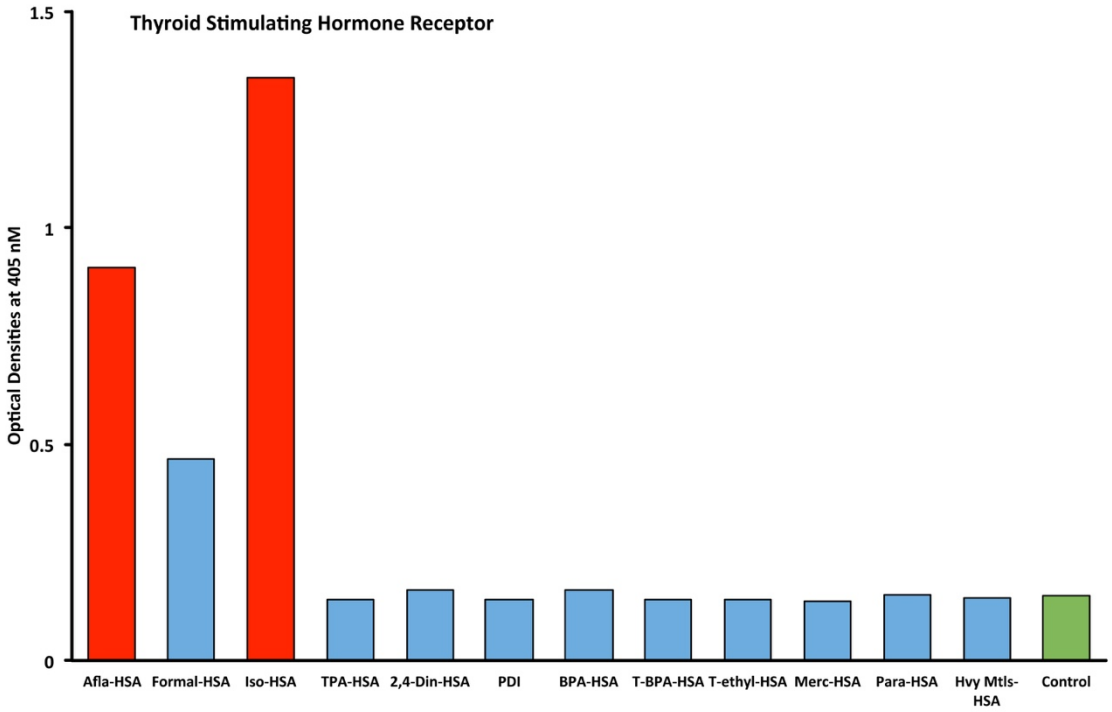

Figure 1: Reaction of thyroid stimulation receptor polyclonal antibodies with different chemicals bound to human serum albumin. The antigens in red are above 1 standard deviation of the mean. The control is in green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

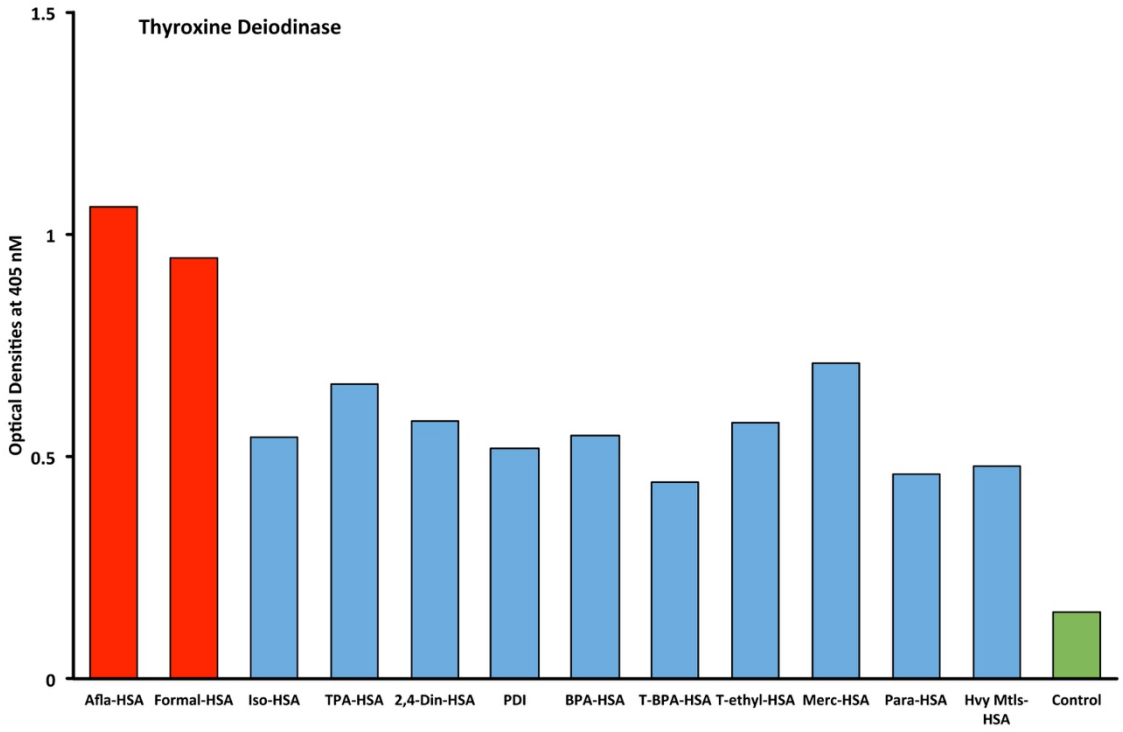

Figure 2: Reaction of thyroxine deiodinase polyclonal antibodies with different chemicals bound to human serum albumin. The antigens in red are above 1 standard deviation of the mean. The control is in green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

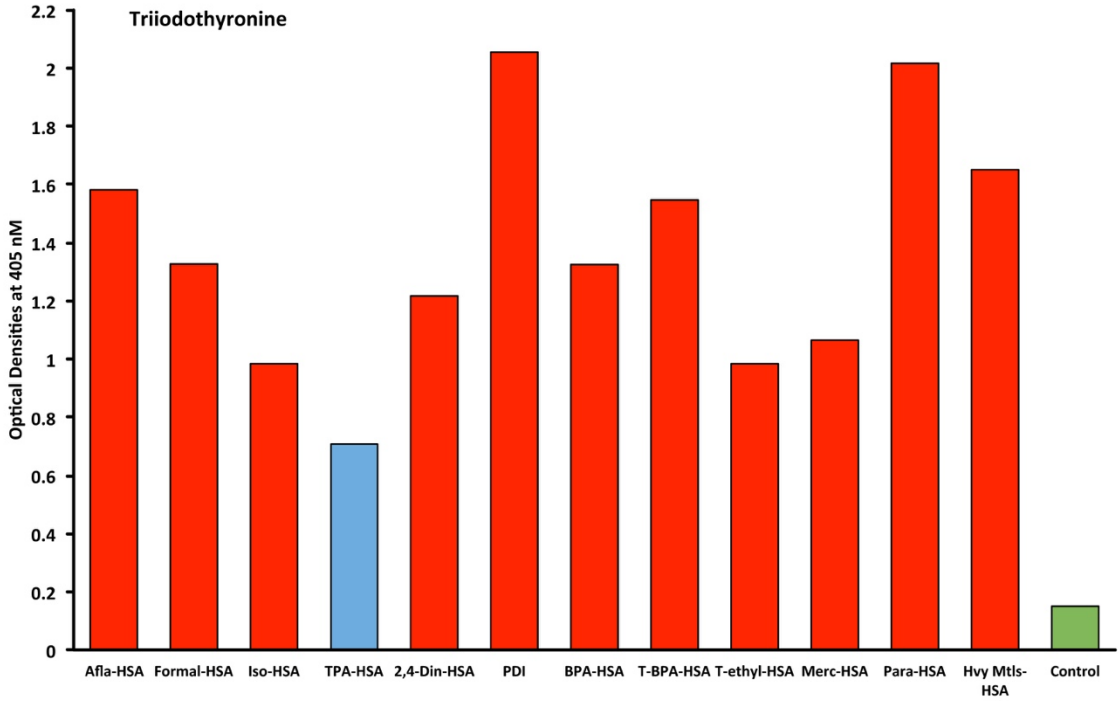

Figure 3: Reaction of triiodothyronine monoclonal antibodies with different chemicals bound to human serum albumin. The antigens in red are above 1 standard deviation of the mean. The control is in green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

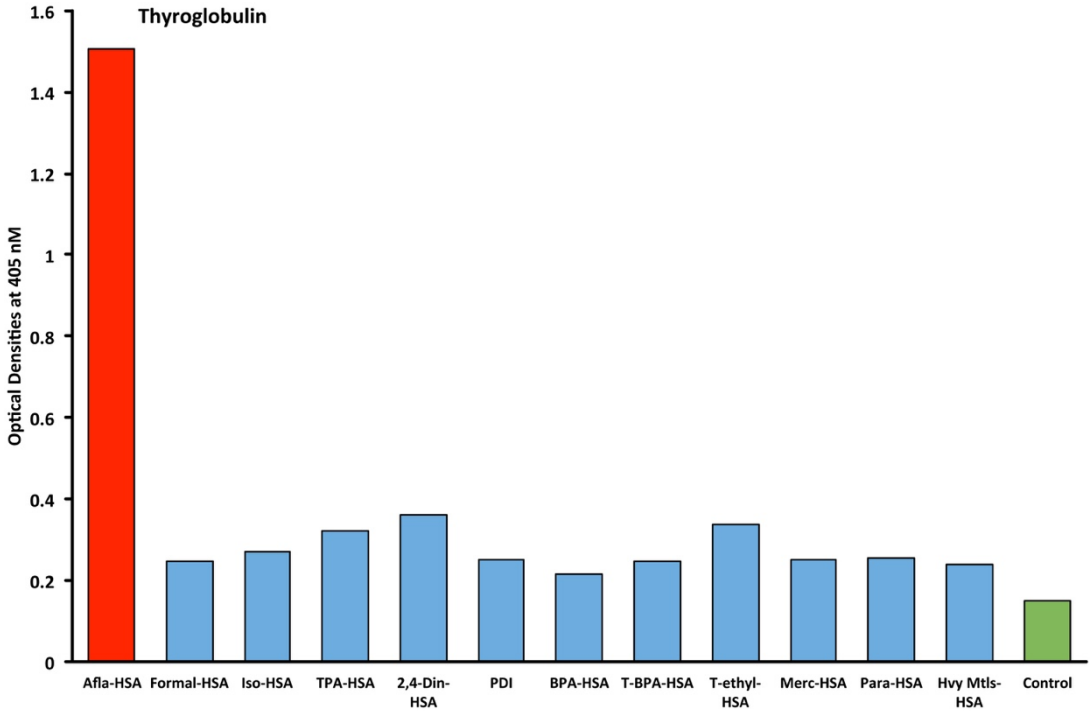

Figure 4: Reaction of thyroxine deiodinase to monoclonal antibodies with different chemicals bound to human serum albumin. The antigen in red are above 1 standard deviation of the mean. The control is in green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

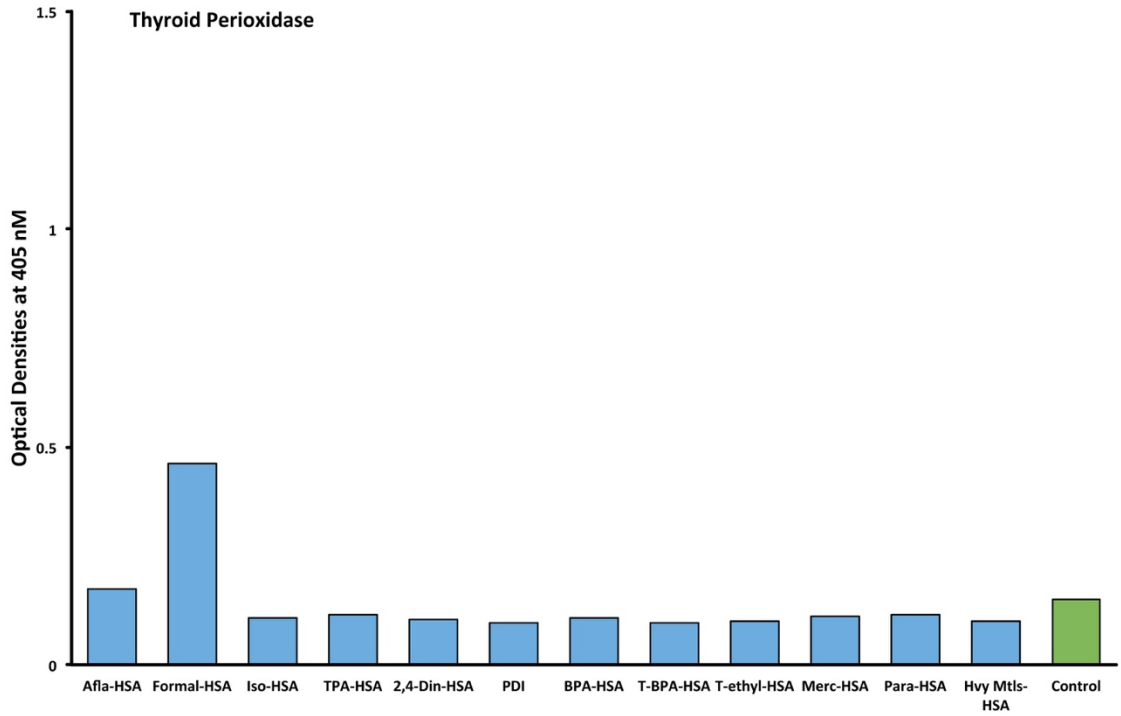

Figure 5: Reaction of thyroid peroxidase monoclonal antibodies with different chemicals bound to human serum albumin. The control is in green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

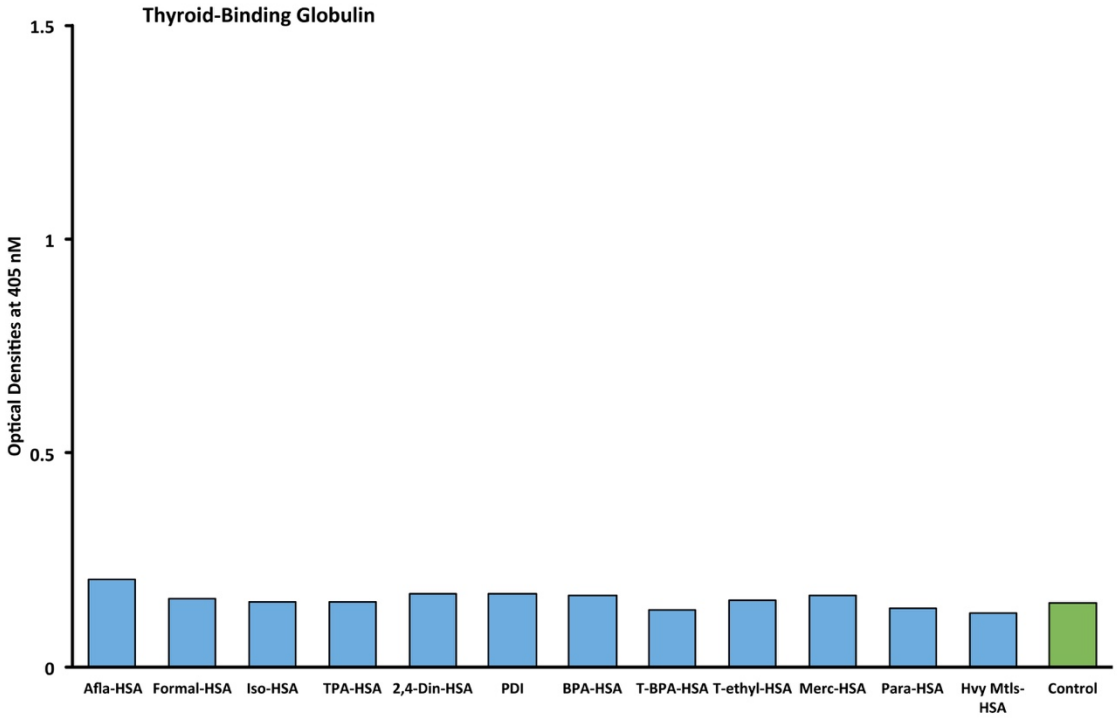

Figure 6: Reaction of thyroid-binding globulin monoclonal antibodies with different chemicals bound to human serum albumin. The control is green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

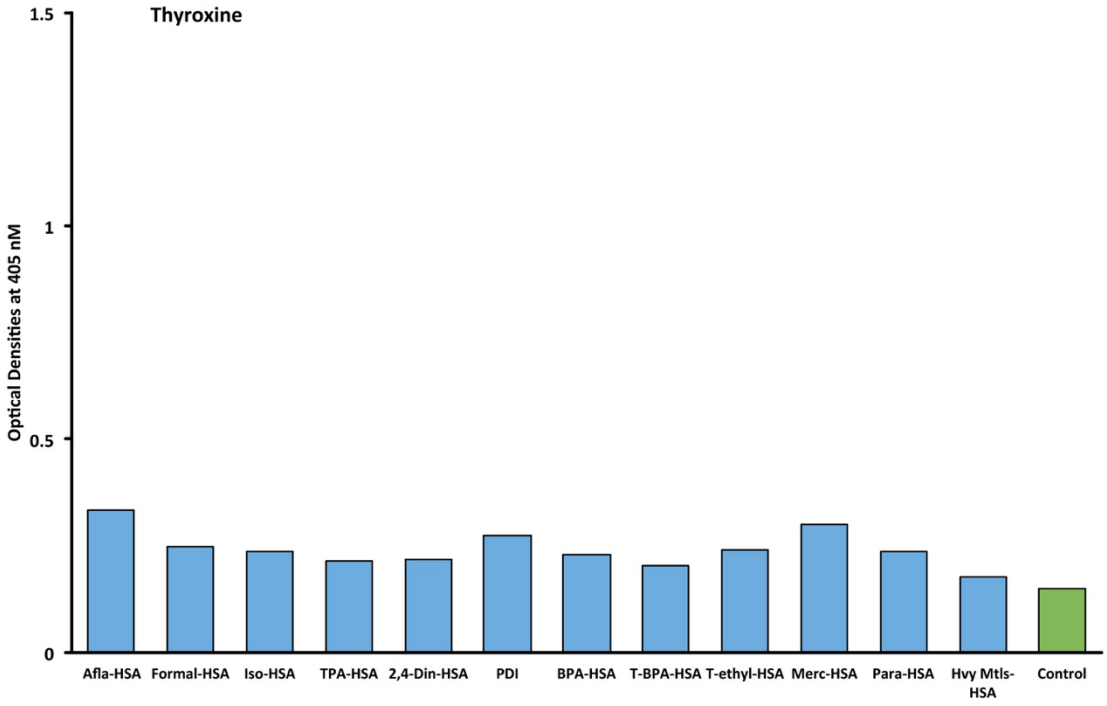

Figure 7: Reaction of thyroxine monoclonal antibodies with different chemicals bound to human serum albumin. The control is in green. Afla-HSA = Aflatoxin-HSA; Formal-HSA = Formaldehyde-HSA; Iso-HSA = Isocyanates-HSA; TPA-HSA = Trimellitic + Phthalic Anhydride-HSA; 2,4-Din-HSA = 2,4-Dinitrophenol-HSA; PDI = Protein Disulfide Isomerase; BPA-HSA = Bisphenol-A-HSA; T-BPA-HSA = Tetrabromobisphenol-A-HSA; T-ethyl-HSA = Tetrachloroethylene-HSA; Merc-HSA = Mercury-HSA; Para-HSA = Parabens-HSA; Hvy Mtls-HSA = Heavy Metal Composite-HSA.

5. Discussion

Previous models of how chemicals play a role in disrupting thyroid metabolism include binding of the chemicals with nuclear hormone receptors, orphan and neurotransmitter receptors, as well as direct chemical alteration of enzymatic pathways.[55] Our study identified a new mechanism in which chemicals bound-to-albumin lead to structural protein misfolding. This, in turn, creates a neoantigens that lead to the development of antibodies, which bind to key target proteins of the thyroid axis through molecular mimicry. The outcomes of this study may serve to fill a knowledge gap of how

chemicals may influence thyroid function through a novel pathway that has not yet been identified in the thyroid literature to the best of your knowledge.

As chemicals bind to proteins and induce a conformational change in the macromolecule, there are potentials to create new structures that may react with various target sites of antibodies.[56] Molecular mimicry can occur if epitopes of the protein share surface topology to similar bindings sites.[57] Alterations of peptides that share topological equivalence of alternating side chains can lead to the formation of binding surfaces that can mimic the antigenic structure of a variant peptide.[58] In addition to peptide similarity with models of cross-reactivity, it is now clear that binding sites are polyfunctional and can accommodate more than one antigenic epitope.[59] The cross-reactive binding of specific epitopes with thyroid axis target sites may explain a previously uninvestigated mechanism of how chemicals may interfere with thyroid hormone metabolism, interfere with thyroid medication dosage, or potentially promote autoimmune reactivity. Our study found that antibodies made against TSH-R, DIO2, thyroglobulin, and T3 interact with chemicals bound to HSA (Table 1).

Several chemicals bound to albumin exhibited specific cross-reactivity with T3. These chemicals included aflatoxins, formaldehyde, isocyanate, 2,4-dinitrophenol, protein disulfide isomerase, bisphenol-A, and tetrabromobisphenol-A. The structural similarity between them may explain their roles as both thyromimetics and immunological cross-reactive target proteins. The structure of thyromimetics is based on those of endogenous thyroid hormones, which consist of a biaryl ether skeleton substituted with iodine, alpha-alanine moiety, and a hydroxyl group at two 2,4-dinitrophenol rings.[60]

Interestingly, our study found that antibodies made against T3 reacted with several chemicals, but antibodies made against T4 did not. Although T3 and T4 have some structural similarity, there are significant biochemical structural differences between them. When differentiated with T3, there are two conformations that are independent with T4 in the outer phenyl ring structure and independent confirmations in the crystal lattice. The significant distinctions between T3 and T4 structures are condensed C4'-O4' bond contraction of the C3'-C4'-C5' angle and in an enlargement in the C3' and carbon C5' angles of T4.[61] These differences may explain why antibodies made against T3 but not against T4 reacted with chemicals bound to HSA. This chemical-protein-adduct cross-reactivity with T3 may impact thyroid medication dosage and impact thyroid metabolism by interfering with T3 directly in the periphery.

Our investigation found immune reactivity between BPA and antibody to T3. This may suggest that immune reactivity to compounds in plastic products may impact circulating T3 levels and thyroid autoimmunity. BPA and triiodothyronine (T3) possess such a high degree of molecular structure similarity that BPA may act as an antagonist compound on T3 receptor sites.[62] In particular, hydrocarbon rings found both on BPA and T3 with anchor-ring-like similarities may induce immune reactivity.[63] When compounds have structural similarity, it may potentially lead to immune reactivity, with the formation of antigen-antibody complexes, which may lead to inflammation and autoimmunity.[64] The potential for BPA to induce thyroid autoimmunity due to immune reactivity has been reported previously.[65] In our study, we found that antibodies formed against BPA bound to human serum albumin may play a role in molecular mimicry with T3 leading to potential thyroid metabolism disruption.

Additionally, we identified cross-reactivity between anti-T3 antibody and the enzyme, protein disulfide isomerase (PDI). PDI directly acts to catalyze protein folding and the multimerization of thyroglobulin in the follicular lumen of the thyroid gland.[66 67] PDI also has a role in the biosynthesis of T3 by inactivating type-2 iodothyronine 5'-deiodinase involved with converting T4 into bioactive T3.[68 69] BPA binds to the PDI, which is located throughout the body and is potentially accountable for the diverse list of physiological influences of BPA due to enzyme function disruption.[70] Therefore, demonstration of cross-reactivity between T3 and PDI may explain how the binding of BPA to PDI may be an additional mechanism of interference with the thyroid function due to molecular mimicry as we have identified in our study.

Tetrabromobisphenol A (TBBPA) is a fire-retardant compound. Our study found that when TBBPA binds to human serum albumin (HSA), it forms a conformational change in the peptide that exhibits molecular mimicry reactions with T3. It should be noted that TBBPA has already been found to share structural similarity with thyroid hormones and can interfere with thyroid hormone physiology.[61 71] This structural similarity between TBBPA and T3 not only allows it to compete with thyroid receptor sites, but also allows for potential immunological cross-reactivity, as identified in our laboratory study.

We identified cross-reactivity between T3 and 2,4-dinitrophenol bound to HSA. 2,4-dinitrophenol has been found to have structural similarity to thyroid hormones and hence has an affinity to bind to thyroid transport proteins.[72] Exposure to 2,4-dinitrophenol has been reported to induce thyroid insufficiency. The immunological mechanisms of cross reactivity that we have identified in our study between 2,4-dinitrophenol bound to

HSA and T3 may explain the mechanism behind thyroid metabolism disruption that is reported in the literature. [73] [74]

Our study also identified cross-reactivity between TSH-R and T3 with isocyanates-HSA. Several studies have demonstrated that isocyanates have the potential to disrupt thyroid metabolism. [75] [76] [77] It is possible that the structural similarity of isocyanates to T3 acts as an endocrine disruptor and may play a potential cross-reactive role that we identified in our study using TSH-R and T3. A similar mechanism of structural similarity between formaldehyde and T3 may occur since our study identified cross-reactivity between formaldehyde-HSA and both T3 as well as 5-deiodinase. These immunological reactions may explain the findings of an animal study in which formaldehyde exposure altered the thyroid function and reduced the circulating T3 levels.[78]

Immunological reactivity was identified between formaldehyde HSA and thyroxine 5-deiodinase and T3. In a small animal study, rats exposed to formaldehyde showed alterations in thyroid function and reduced T3 levels.[79] The reaction between antibodies to T3, 5-deiodinase, and formaldehyde bound to HSA suggests that this immunological reactivity has the potential to interfere with circulating T3 levels and the thyroid conversion of T4 and T3.

We propose that chemicals can bind to proteins in the serum, such as human serum albumin, leading to protein misfolding and the development of new epitopes. These epitopes can lead to cross-reactive interactions with various target sites of the thyroid axis and the formation of antibodies that potentially generate immunological reactivity with various target sites of the thyroid axis, which may promote autoimmune

thyroid reactivity. The results of our study provide new insights into how hapten-protein-adducts can develop into neoantigens that may lead to immunological interactions with various target proteins of the thyroid axis due to cross-reactivity, and how it can potentially lead to thyroid metabolism disruption. Further research evolving into animal and human models would be necessary to investigate the exact clinical role of these interactions.

Chapter 4: Summary of Manuscripts and Thesis Conclusions

The findings of this thesis identified that environmental triggers have the potential to impact humoral immunity involved in mechanisms of autoimmunity. The rates of autoimmunity are growing rapidly, worldwide, and, currently, there is no treatment for the disease. It is unlikely that a curative treatment will be found in the near future. Current therapies for autoimmunity are focused on reducing the inflammatory expression of the disease and for the most part is not effective and expensive. There is a growing need to identify environmental triggers and potential lifestyle and dietary habits that may play a role in reducing the expression or the onset of the disease in susceptible subpopulations.

In the first manuscript, we identified immune reactivity between dietary proteins and target sites on the thyroid axis that includes thyroid hormones, thyroid receptors, enzymes, and transport proteins. We also measured immune reactivity of either target specific monoclonal or polyclonal antibodies to the thyroid-stimulating hormone (TSH) receptor, 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, and triiodothyronine in 204 purified dietary proteins commonly consumed in cooked and raw forms. Dietary protein determinants included unmodified (raw) and modified (cooked and roasted) foods, herbs, spices, food gums, brewed beverages, and additives. The results of the study identified immune reactivity between T3, T4, DIO2, and thyroglobulin and many food proteins. These immune reactions may explain the etiology of some cases of food interactions with both thyroid hormone replacement and thyroid hormone metabolism. Theoretically, these food protein reactions may contribute to autoimmune reactivity in a subset of autoimmune thyroid disease patients.

In the second manuscript, we explored a different mechanism of cross-reactivity also known as molecular mimicry that occurs if epitopes of the protein share surface topology with similar binding sites. The alteration of peptides that share topological equivalence with alternating side chains can lead to the formation of binding surfaces that can mimic the antigenic structure of a variant peptide. Once chemicals bind to proteins, they induce a conformational change in the macromolecule, which has the potential to create new peptide structures that may interact with various target sites of the body and lead to antibody production. In addition to peptide similarity with models of cross-reactivity, binding sites are polyfunctional and can accommodate more than one antigenic epitope.

In the study, we investigated how antibodies made against thyroid target sites may bind to various chemicals bound to human serum albumin (HSA). These are specific monoclonal or polyclonal antibodies developed against thyroid-stimulating hormone (TSH) receptor, 5'deiodinase, thyroid peroxidase, thyroglobulin, thyroxine-binding globulin, thyroxine, and triiodothyronine. Our study identified a new mechanism in which chemicals bound-to-albumin lead to structural protein misfolding. This, in turn, creates a neoantigens that lead to the development of antibodies, which bind to key target proteins of the thyroid axis through protein misfolding.

Cross-reactivity is an established and a known mechanism in autoimmune reactions and inflammatory responses. The exploration of these mechanisms was conducted on thyroid autoimmunity in this thesis, because it is the most prevalent autoimmune disease in the world. However, the same methodology can be applied to target sites for virtually all other autoimmune diseases. In this thesis, we presented a body

of work that identified how cross-reactivity could occur due to amino acid sequence homology and how chemicals can bind to albumin and induce conformational changes in the protein, thereby creating neoantigens that may then cross-react with specific autoimmune target sites. Identifying a clear list of dietary protein triggers and environmental chemicals to specific autoimmune target sites of a determined autoimmune disease can lead to an entirely new model of how diet and lifestyle medicine may approach autoimmune disease. These efforts may potentially lead to clinical models that can improve the quality of life, reduce medical expenses, and lower the impact of chronic illness on society.

Chapter 5: Discussion and Perspectives

This thesis has identified several cross-reactive environmental and food protein interactions between the thyroid axis based on the models of cross-reactivity also known as molecular mimicry in the laboratory. It should be noted that it is doubtful that the exposure to the identified sources alone would induce immunological responses. Cross-reactivity is only a concern if individuals produce antibodies to these sources. Individuals that have immunological tolerance to dietary proteins and do not react to these proteins are not susceptible to cross-reactivity interactions. Furthermore, antigen-antibody reactions alone are not solely responsible for pathogenic reactions. Cellular immunity, immunological tolerance, human leukocyte antigen (HLA) allele, and other factors are involved with pathogenic immune reactions to dietary proteins. Therefore, antibodies binding to antigens are not always pathogenic; however, the ability of specific monoclonal and polyclonal antibodies to bind with purified proteins suggests the potential for immunological cross-reactivity and serves as an initial step to identify food and environmental risk factors in susceptible populations.

Further research should be conducted to evaluate the specific epitope for each of these foods and thyroid axis target sites. Additionally, immunological factors such as tolerance, T-cell polarization, and other factors that may induce susceptibility to dietary protein immune reactivity need to be investigated. Individual case studies, prospective studies, and clinical trials will be required to assess whether these food proteins in thyroid-associated reactions have any actual clinical role.

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