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DEVELOPMENT, CHARACTERIZATION AND VALIDATION OF A MOUSE MODEL OF TREE-NUT ALLERGY USING HAZELNUT AS A MODEL ALLERGENIC FOOD

By

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ABSTRACT

DEVELOPMENT, CHARACTERIZATION AND VALIDATION OF A MOUSE MODEL OF TREE-NUT ALLERGY USING HAZELNUT AS A MODEL ALLERGENIC FOOD

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Food allergy prevalence is increasing and the public has become increasingly aware of the problem. The mechanisms of tree-nut allergy are not completely known and a mouse model to study tree-nut allergy is unavailable. The overall hypotheses driving my research were i) mice can develop tree-nut allergy that mimic certain phenotypes of human tree-nut allergy and ii) a validated mouse model of tree-nut allergy is useful to study impact of dietary modification on various markers of this disease. The aims of my studies to test these hypotheses were 1) to develop an ELISA based method to measure allergen specific IgE as an alternative to the passive cutaneous anaphylaxis assay (PCA); 2) to determine if hazelnut can directly elicit a specific IgE antibody response via activating IL-4 in mice; 3) to characterize the systemic immune response following transdermal hazelnut protein exposure in BALB/c mice; 4) to develop an adjuvant free model of hazelnut protein induced systemic anaphylaxis; and 5) to study the effect of a diet rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on systemic immune responses to hazelnut. During our studies we demonstrated that 1) Food specific IgE levels can be measured by an ELISA based method that is comparable to PCA; 2) Hazelnut itself can be an allergenic food, capable of directly eliciting hazelnut binding specific IgE antibodies via activation of Type-2 cytokines in mice; 3) Mice can respond in an allergic manner to transdermal hazelnut protein exposure without the use of

adjuvant; 4) Transdermal exposure of mice with hazelnut protein sensitizes them for systemic anaphylaxis when challenged either i.p. or orally with hazelnut; and 5) EPA and DHA supplementation together can enhance hazelnut specific IgE antibodies via altering the INF-γ / IL-4 ratio in favor of IL-4. Taken together, the mouse model described in this thesis might be a useful tool for determination of mechanisms of tree-nut allergy. Future studies that could be proposed using this model include using tools such as gene knock out strains of mice and RNA interference to study mechanisms associated with tree-nut allergy; to further therapeutic and prophylactic studies using pharmaceutical or dietary interventions.

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ABBREVATIATIONS USED

ASIgE, Allergen specific IgE; Ig, immunoglobulin; OD, optical density; ELISA, enzyme linked immunosorbent assay; PCA, passive cutaneous anaphylaxis assay; BSA, bovine serum albumin; PBS, phosphate buffered saline; SD, standard deviation, SE, standard error; RAST, radio allegro sorbent test

CHAPTER ONE

1.0 Introduction

Allergy or immediate hypersensitivity can be described as an abnormal response of the immune system to substances that are normally harmless (pollens, foods, drugs). Food allergy is an immune mediated adverse reaction to food that occurs when the immune system misidentifies food as a foreign chemical in the body and responds robustly to one or more specific proteins in that food.

An adverse reaction to food encompasses any abnormal reaction after the food is ingested. It may be due to a food intolerance (adverse physiologic response), or to a food allergy (immunological reaction) [1]. Food intolerances might be due to toxic contaminants (e.g. histamine in scombroid fish poisoning) or pharmacologic properties of the food (e.g. tyramine in aged cheese), or it may be due to metabolic disorders in the host (e.g. lactase deficiency) or even possible idiosyncratic responses [2]. Food allergies, however, are immune-mediated reactions to food, commonly called food hypersensitivities. These can be due to IgE mediated or non-IgE mediated immune mechanisms. Therefore, the involvement of the immune system is a key-deciding factor to determine if an adverse reaction to food is truly a food allergy.

Hypersensitivity has been defined as an inappropriate response of the immune system to antigens (a substance, usually a protein, that stimulates an antibody immune response). According to the scheme of Gell and Coombs [3, 4] , there are four types of

hypersensitivity reactions classified as Type-I to Type-IV depending on both types of antigens involved and mechanisms of disease. Type I hypersensitivity, commonly called "immediate type hypersensitivity", is a rapid adverse reaction mediated by IgE antibody against a soluble allergen (subclass of antigen, where antibody production is of the IgE isotype). Here IgE antibodies are bound to high affinity receptors on mast cells and basophils. Upon antigen cross-linking, cells degranulate, releasing various chemical mediators (e.g. histamine), which results in onset of disease symptoms. Food allergy belongs to this type of hypersensitivity with peanut allergy being an example widely seen. Clinically, Type I hypersensitivities are expressed extremely fast with symptoms such as hives, rashes, asthma, vomiting, diarrhea etc. Skin prick testing and serum screening for specific IgE antibodies diagnose this type of hypersensitivity.

In Type II hypersensitivity, IgG, IgM and complement play key roles, here with an insoluble antigen. Antibodies bind to the antigen and activate the complement system, which in turn initiates the release of mediators of disease. Examples of type II hypersensitivity include autoimmune hemolytic anemia, rheumatic fever, drug allergies etc. [5]. Currently, it is unclear if this mechanism plays a role in food allergy.

Type III hypersensitivity is associated with IgG and IgM binding to circulating antigens, then forming immune complexes. These immune complexes become large mesh works with antigen bridges. Complement is activated and the immune complex is cleared. If failure to clear these immune complexes persists, disease events occur. An example of

type III hypersensitivity is systemic lupus erythematosus. It is not known whether this mechanism may play role in food allergies.

Type IV hypersensitivity, commonly called "delayed type hypersensitivity", is mediated by CD4+ T-cells and intact antigen presenting cells. Reactions such as poison ivy, contact dermatitis and nickel allergy all are type IV hypersensitivities. Gluten enteropathy (or Celiac disease) is also a Type IV hypersensitivity and is mediated by a non-IgE mechanism involving T cells and monocytes [6, 7].

Food allergy has been estimated to affect 3.7% of American adults [8]. Its prevalence is at its highest during the first few years of life, affecting about 6% of infants less than 3 years of age and then decreases during childhood [9]. Whereas some food allergies such as cow's milk are likely to be outgrown [1], several food allergies, such as tree-nut and peanut allergy can be chronic, often lasting a lifetime [8]. Recently, Sampson assessed the prevalence of food allergy in the United States and is summarized in Table 1.1 [8].

Table 1.1 Prevalence of common food allergies in the United States*

Food	Young Children	Adults
Milk	2.5%	0.3%
Egg	1.3%	0.2%
Peanut	0.8%	0.6%
Tree nuts	0.2%	0.5%
Fish	0.1%	0.4%
Shellfish	0.1%	2.0%
Overall	6.0%	3.7%

* From Sampson et al. 2004 [8].

Food allergy remains the leading cause of systemic anaphylaxis treated in emergency departments in a number of countries, including the United States and the public has become increasingly aware of the problem [8]. Food-related allergic reactions account for around 30,000 emergency room visits [2] and 150-200 deaths in America alone each year [8]. Peanuts and tree-nuts (e.g., hazelnuts, almonds etc) are the major food types that cause systemic anaphylaxis with fatal or near fatal consequences [2, 10].

The pathogenesis of food allergy may be broken down to two phases, the sensitization phase and the effector phase. In the sensitization phase, allergen is encountered for the first time, allergen is presented to the B-lymphocytes by antigen presenting cells (macrophages, dendritic cells) that then produce allergen specific IgE with the aid of T-lymphocytes (Th2) [11]. These IgE antibodies then bind to high affinity receptors (FCεRI) on both mast cells and basophils. In the effector phase, recurrent exposure to the allergen, causes cross-linking of two bound IgE antibodies causing degranulation of the mast cells and basophils, releasing mediators of disease (histamine, prostaglandins, leukotrienes etc. [12].

Most symptoms of food allergy are immediate, occurring within minutes to hours after food ingestion [4]. They range from skin reactions (hives, itching, eczema, swelling), to gastrointestinal distress (nausea, vomiting, diarrhea, cramps), to respiratory troubles (wheezing, sneezing, asthma, rhinitis, laryngeal edema, labored breathing, tightness of

throat) and systemic symptoms including anaphylactic shock with decreased body temperature and blood pressure, which could potentially lead to death [13, 14].

While more than 100 different food types have been documented to trigger allergic reactions in sensitized humans, 90% of food allergies are caused by only eight food types, often called the red-flag foods (Egg, milk, wheat, soy, peanuts, tree-nuts, fish and shellfish) [15]. The scientific reasons as to why only eight food types account for a vast majority of food allergies are unclear. Despite the potential for a fatal outcome, food avoidance is the only sure way to prevent food allergy episodes. Epinephrine is usually prescribed to patients with food and can be used to stop an anaphylactic reaction.

There is growing concern and indication that the prevalence of food allergy might be increasing consistent with asthma and other allergic diseases for reasons that are not clear [8, 16, 17]. Even though extensive research is ongoing about food allergies, mechanisms driving this increasing trend are unclear at present. Therefore more research needs to be done so that this increasing trend can be stopped. To do this, well-characterized animal models need to be developed. Then potential therapies can be developed and studied. Currently there are several animal models available to study food allergy, including rodent models for several foods including peanut, cow's milk and egg. However, **a mouse model to study tree-nut allergy was not available when we began our work in 2001.**

With this gap in the knowledge, I sought out to develop and characterize a mouse model of tree-nut allergy, using hazelnut as a model tree-nut, which might be useful to study tree-nut allergy. In this study, my guiding hypotheses were i) mice can develop tree-nut allergy that mimic certain phenotypes of human tree-nut allergy and ii) a validated mouse model of tree-nut allergy is useful to study impact of dietary modification on various markers of this disease.

The aims of my studies to test these hypotheses were 1) to develop an ELISA based method to measure allergen specific IgE as an alternative to the passive cutaneous anaphylaxis assay; 2) to determine if hazelnut can directly elicit a specific IgE antibody response via activating IL-4 in mice; 3) to characterize the systemic immune response following transdermal hazelnut protein exposure in BALB/c mice; 4) to develop an adjuvant free model of hazelnut protein induced systemic anaphylaxis; 5) to study the effect of a diet rich in EPA and DHA on systemic immune responses to hazelnut.

In the following chapters I review the published animal models of food allergy and then show the detailed studies done to achieve my study aims.

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CHAPTER TWO

2.0 Review of Literature

2.1 Animal models of food allergy

With this increasing trend in food allergy prevalence and the fact the human prospective sensitization studies are not ethically possible, animal models that mimic human allergic responses need to be studied and developed. Animal models hold the potential to be extremely valuable tools for determining mechanisms, predicting triggers, and testing possible treatments/therapies.

Development of an animal model for food allergy should take into account the following parameters: 1) the concentration of the allergen (high doses are known to induce tolerance); 2) the allergen should be taken in context with the food source; 3) the route and duration of allergen exposure; 4) genetic predisposition (high and low IgE responders); 5) the use of adjuvants (agents which not having any specific antigenic effects itself, stimulates the immune system, increasing the immune response to what it is in contex with) (natural or artificial-alum, cholera toxin, *Bordetella pertussis*, and carrageenan are known IgE-selective adjuvants); 6) isotype specificity response (mice respond with two anaphylactic antibodies, IgG1 and IgE; rats with IgG2a and IgE; guinea pigs with IgG1 and IgE; dogs with IgE; and pigs, likely with IgE; and 7) the Th1/Th2 polarization (mice have very delineated Th1/Th2 polarization, whereas in humans polarization is not as discrete) [1].

Animal models currently available include mice, rats, guinea pig, atopic dog, and neonatal swine. **Reviewed here is a list of animal models that have been developed for food allergy.**

2.2. Rat model of Ovalbumin allergy

A number of studies have used the Brown Norway rat for development of a model of OVA allergy [2-5]. A group headed by Leon Knippels at the TNO nutrition and Food Research Institute have developed a rat model of OVA allergy [3, 4]. They used Brown Norway rats and exposed to ovalbumin either ad libitum via the drinking water (0.002 to 20 mg/mL) continuously for 6 weeks or by gavage (1 mg/mL per rat) without the use of adjuvant. Gavage was performed daily, twice a week, once a week or once every 2 weeks during a period of 6 weeks. Following sensitization, animals were assessed for OVA specific IgE and IgG1.

After intra-gastric administration of ovalbumin once or twice a week or once every two weeks, a very low frequency of ovalbumin-specific antibody responses were detected. Daily intra-gastric dosing with ovalbumin resulted in antigen-specific IgG as well as IgE responses in almost all animals tested (7/8 at day 42 of sensitization). With ad libitum exposure, ovalbumin-specific IgG was noted, but ovalbumin-specific IgE was less than detectable.

These studies show that the BN rat may provide a suitable animal model for inducing specific IgG and IgE responses to OVA upon exposure via the enteral route without the use of adjuvants.

Furthering the earlier work, efforts were made to characterize the models immune-mediated effects after oral challenge with OVA [3]. Here Brown Norway rats were exposed to ovalbumin (OVA) by daily gavage dosing (1 mg OVA/rat/day) for 6 weeks, without the use of an adjuvant, or by i.p. injections (positive control) with OVA (0.2mg/ml) together with alum (5mg). Subsequently, effects on breathing frequency, blood pressure, and gastrointestinal permeability were investigated upon an oral challenge with 10 to 100 mg OVA in vivo.

In both i.p. and orally sensitized rats, an increase in gut permeability (increased passage of beta-lactoglobulin as bystander protein) was determined between 0.5 and 1 h after an oral OVA challenge was given. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of the animals. Whereas, i.p. sensitized animals had a significant drop in blood pressure following i.v. challenge with OVA. Upon oral challenge with OVA of orally and parenterally sensitized animals, local effects were observed in all animals whereas systemic effects were observed at a low frequency, which reflects the situation in food allergic patients after an oral challenge.

The authors conclude from these studies that the Brown Norway rat provides a suitable animal model to study oral sensitization to food proteins as well as immune-mediated effects after oral challenge with food proteins.

Limitations of these studies include the lack of cytokine data to support the Th2 dominated response. Also, systemic effects after oral challenge were minimal even after rather large challenge doses of 100 mg of a purified allergen (OVA). Further analysis into the local effects could have been done using histological analysis on the gastrointestinal tract.

2.3 The Guinea pig model of cow's milk proteins

A group located at the University of Texas Medical Branch has developed a model to study the allergenicity of cow's milk proteins in a guinea pig model [6]. In this study, the allergenicity of milk-based infant formula and cow's milk proteins were evaluated by examining altered intestinal permeability, intestinal anaphylaxis, and PCA after oral sensitization with cow's milk proteins and challenge with β-lactoglobulin. Colonic segments from cow's milk–sensitized or milk formula–sensitized animals were challenged with β-lactoglobulin in Ussing chambers. Cow's milk–sensitized animals responded with an antigen-induced, anaphylactically mediated elevation in the transmural short-circuit current as measured by net chloride secretion, whereas only 60% of animals fed infant formula responded to challenge. Bronchospasm developed in all animals fed cow's milk; however, only those animals fed infant formula that responded to intestinal challenge developed bronchospasm.

The authors concluded that cow's milk–based infant formula had less sensitizing power than whole cow's milk and that the model was effective in testing allergenicity at the intestinal level.

The strengths of this study are the fact that they fed the allergen without the use of adjuvant. Limitations of this study are that they did not assess antibodies driving these responses or cytokine profile leading to the response, therefore a true allergy is not evident unless further work is done. Furthermore difficulties associated with passive cutaneous anaphylaxis (PCA) testing and the fact that the antibody response is of the IgG1a subtype and not IgE limit the use of guinea pigs as a suitable model with which to study food allergy associated mechanisms [7, 8]. Finally, reagents and gene knockout strains are not readily available as they are for other species of animal.

2.4 Atopic dog model for multiply foods (Cow's milk, beef, ragweed, and wheat)

An atopic dog colony at the University of California has been under development as a model of food allergy [9-11]. This colony is a spaniel/basenji-type dog that was selected with a genetic predisposition to allergy and that had histories of sensitivity to pollens and foods. Ermel et al. [11] used newborn pups that they subcutaneously injected into the axilla a mixture of allergens containing 1 μg each (cow's milk, beef, ragweed, or wheat) commercial extract, with alum (200 μl) as an adjuvant. At ages 3, 7, and 11 weeks, pups were vaccinated with attenuated distemper-hepatitis vaccine. At 2 and 9 days after each vaccination, pups received a boost of the same allergen: alum injection they received as newborns. Booster injections were 10μg of allergen extract in 200μl alum.

Immunized pups responded with allergen-specific IgE by week 3, which peaked at week 26, and could be maintained with injections of antigen with alum every other month and daily feeding of diet containing small amounts of the allergen. Skin tests were positive when challenged with the immunized allergen as evidenced by a wheal-and-flare reaction and negative with a control, un-immunized allergen. Analysis of gastric food sensitivity was done through an endoscope by injecting allergenic food extracts into the gastric mucosa after intravenous injection of Evans blue dye. Tissue examination showed marked mucosal swelling and persistent erythema at the site of this allergen injection. Furthermore, late biopsy specimens revealed eosinophil infiltration into the lamina propria and migration through the endothelium. From these results the authors conclude that the dog may serve as a useful model to study food allergy [11].

Strengths of this model include that the dog is one of the few species other than humans in which allergies develop naturally on normal environmental exposure to a broad spectrum of allergens, including pollens, house dust mites, human dander, fleas, and foods [12-14]. Also, the dog can respond both vomiting and diarrhea in response to oral challenge due to food allergy [15, 16]. Furthermore, food allergy has identified by single-ingredient elimination testing in 25 dogs with histories and cutaneous signs were consistent with food-induced allergic dermatitis [17].

Some of the limitations associated with this model include the high cost of care and housing of a large animal like a dog would limit the studies. Having a large number of

animals per study would be difficult. The ease of getting reagents and gene knock out animals would be difficult. They did not perform cytokine analysis to see if a Th1/Th2 imbalance is driving this allergy. Therefore, using the atopic dog as a model of mechanistic and therapeutic analysis of food allergy is challenging.

2.5 Atopic dog model for multiply foods (Peanut, walnut, Brazil nut)

Further work was done on the atopic dog colony at the University of California with the goal being the development of an animal model for peanut and tree-nut allergy [16]. They used their colony of a spaniel/basenji-type dog that had been selected with a genetic predisposition to allergy and that had histories of sensitivity to pollens and foods. Here they used a group of eleven dogs and put them through the previous protocol, injecting them subcutaneously with 1μg of peanut, English walnut, soy and Brazil nut (commercial extract) with alum (200 μl) as an adjuvant. The dogs were also sensitized to either wheat or barley (1μg in 200μl alum). At ages 3, 7, and 11 weeks, pups were vaccinated with attenuated distemper-hepatitis vaccine. At 2 and 9 days after each vaccination, pups received a boost of the same allergen: alum injection they received as newborns. Booster injections were 10μg of allergen extract in 200μl alum. Skin testing, IgE immunoblotting, and oral challenges to allergen were preformed.

Dogs responded at 6 months of age with positive intradermal skin reactions to the nut allergens. IgE immunoblotting showed a strong recognition to peanut, walnut and Brazil nut in the aqueous preparations. Proteins binding to the IgE are similar to the profile of major allergens seen in human food allergy (peanut-Ara h 1, walnut-Jug r 2, Brazil nut-

Ber e 1). At 2 years of age, each of the 4 peanut and the 3 Brazil nut sensitized dogs and 3 out of the 4 walnut sensitized dogs reacted to oral challenge with the allergen they were sensitized to with symptoms such as vomiting and lethargy.

Strengths of this study include that the dog is one of the few species other than humans in which allergies develop naturally on normal environmental exposure to a broad spectrum of allergens, including pollens, house dust mites, human dander, fleas, and foods [12-14]. Also, the dog can respond both vomiting and diarrhea in response to oral challenge due to food allergy [15, 16].

Limitations of this study include the length between sensitization and oral challenge. Dogs are challenged at 2 years of age, thus this model would be a long and costly one that would delay scientific progress. Also, the high cost of care and housing of a large animal like a dog would limit the studies making having a large number of animals per study difficult. The ease of acquiring reagents and gene knock out animals would be challenging. Mechanistic studies to assess cytokine profile were not done. Furthermore, multiple allergens are injected at the same time, thus making it difficult to assess the true allergenic potential of each individual food due to competition and possible cross-reactivity. Therefore, using the atopic dog as a model of mechanistic and therapeutic analysis of food allergy is challenging.

2.6 The Neonatal swine model of peanut allergy

A group lead by Ricki Helm at the University of Arkansas has been using the neonatal swine to develop a model for peanut allergy [18]. Initially, they used both intragastric (i.g.) and intraperitoneal (i.p.) sensitizations followed by oral challenge with peanut to optimize a sensitization/challenge protocol. From the early studies they found that approximately 25% of i.g. sensitized animals and 75–90% of i.p. sensitized animals responded to an oral challenge of peanut meal. Thus, they concluded that the optimal experimental protocol was to use i.p. sensitization of peanut extract and oral challenge with peanut meal. From that they came up with the following protocol. Out bred Large White/Landrace pregnant sows at day 108 of gestation are allowed to nurse under normal conditions on a soybean/peanut-free diet. Following birth, piglets at days 9–11, 17, and 25 of age were i.p. sensitized with 500 μg of peanut extract with 100 μg cholera toxin. Random selection of animals in each litter to receive control treatments, either phosphate buffered saline (PBS) or PBS with 100 μg of cholera toxin was done. I.g. challenge with peanut meal and intradermal skin testing was performed every other week starting 2 weeks after the final sensitization. Blood was taken weekly to assess the immune responses to the sensitization protocol.

Oral challenges were preformed on days 39 and 53 with 10 or 20 g of peanut meal and resulted in symptoms in 75–100% of animals by the second oral challenge within 30–60 minutes of the challenge. Symptoms following oral challenge included emesis, malaise, tremors, and convulsions with major and minor rashes. There was evidence of respiratory distress and anaphylactic shock in approximately 10–20% of sensitized animals whereas

no shock was noted in control animals. Animals entering shock were treated with epinephrine to alleviate all symptoms. When oral challenge was repeated up to day 90 sensitized animals responded with increasing degrees of physical symptoms, whereas the control animals challenged with peanut meal did not respond with any physical, gastrointestinal or systemic sign of allergy. Peanut-sensitized animals challenged with a irrelevant allergenic food (soybean/peanut-free diet) did not show any symptoms, thus showing the specificity to the previous peanut challenge.

Skin testing was done on alternating weeks with peanut confirmed the persistence of the allergic state throughout a 14-week period. Using either the native or recombinant forms of the major peanut allergens, Ara h 1 and Ara h 2, induced a positive skin test when compared to rice extracts, thus showing reactivity to allergens similar to what is seen in human peanut allergy. Skin prick tests with peanut extracts intradermally were also positive showing a wheal and flare > 5–15 mm. The PBS and PBS/cholera toxin control groups skin prick tests were negative (2 mm) with peanut extract, whereas the histamine positive control showed positive wheal and flare.

The animals were assessed for the production of antigen-specific IgG and IgE by passive cutaneous anaphylaxis. Peanut-specific IgG values measured in peanut-sensitized animals reached levels > 1,000 μg/mL (range, 26–7,700 μg/mL) by day 37 and maintained values of > 500 μg/mL (range, 51–1,500 μg/mL) at day 60. Non-peanut-sensitized animals had < 50 μg/mL antigen-specific IgG. To prove that peanut specific IgE is the responsible isotype causing the allergic symptoms following oral challenge,

passive cutaneous anaphylaxis tests were performed in naive animals. One hundred micro liters of unheated and serial heat-inactivated serum from peanut-sensitized pigs was administered intradermally into the back of naïve animals. 24 hours later, 5 mg of peanut extract was administered by i.v. injection. After 30 minutes the responses were noted. Intradermal skin sites with the unheat-inactivated serum responded with a wheal and flare > 10 mm at the site of injection, whereas heat-inactivated serum showed no reaction, confirming that native IgE was responsible for the reaction because the peanut specific IgE is denatured in heat, while the IgG was left intact.

Following the last oral challenge, the gastrointestinal tract was taken and assessed pathological alterations. The histologic findings were vascular congestion, hemorrhage, and epithelial denudation that in the proximal small intestine. Other acute markers included mucus extrusion and submucosal edema in the stomach. The colon seemed normal in most piglets, with occasional vascular congestion and crypt abscesses. From these results the authors conclude that the neonatal pig model of peanut allergy mimics the physical and immunological characteristics of peanut allergy in humans. Therefore, this model should be useful for determining IgE-mediated mechanisms and immunotherapeutic intervention strategies with repeated allergen challenges.

Some of the strengths of this model include i) how the swine closely resemble humans in gastrointestinal physiology and the development of mucosal immunity is also similar to that seen in humans [1]; ii) The developing piglet has similar anatomy and nutritional requirements, a distribution and maturation of intestinal enzymes, and an enteric

absorption of antibody that is similar to that of the developing infant [1]; iii) The newborn piglets are born immunocompetent, thus allowing for assessment of immune responses [19]. iv) Hypersensitivity responses similar to those of human allergic disease have been demonstrated in swine [20]. Furthermore, studies in veterinary medicine have shown swine to have an IgE-mediated-like response to parasites, legumes, and pollens reminiscent of that in humans

Limitations of using the neonatal pig model include how the pig is not a routinely used animal model; therefore reagents and gene knock out strains are sparse, making some research impossible. Also the size of the pig makes studies with large numbers extremely costly. Here, mechanisms (cytokine profile) driving this allergic response were not studied. Finally, the use of adjuvant and route of sensitization are also drawbacks of this model because human exposure is not likely by an injection, a better route of sensitization would be desired. Therefore, the use of the neonatal pig as a model of food allergy could pose to be difficult.

2.7 Mouse models of food allergy

The mouse is one of the widest used animals in laboratory sciences today. With the ever increasing number of gene knock out strains available and reagents, more and more molecular and mechanistic studies can be done that can not be done in other, larger species. For reasons like these, mouse models of allergy are valuable tools in allergy research and need to be profiled further.

There has been several strains of inbred mice that have been characterized as being either high or low IgE-responder animals for food allergens [18]. As in humans, two separate events are required: the first event is a sensitization phase and, in the case of mice, the production of two anaphylactic antibodies, IgE and IgG1; the second phase (effector) is characterized as the allergic response following allergen challenge [18].

There are numerous models of food allergy using different allergens, routes of exposure, with or without adjuvant and strain of mice. Here I review the models most widely used that encompass both phases of allergy, the sensitization phase and the effector phase.

2.8 Mouse model of Cow's milk allergy

A group at The Mount Sinai School of Medicine headed by Hugh Sampson has developed a mouse model of Cow's milk allergy. To do this Li et al., [21] used several different strategies to overcome oral tolerance in a mouse model and induce IgE-mediated cow's milk hypersensitivity. They used three-week-old C3H/HeJ female mice and sensitized them intragastrically with Homogenized Cow's milk (0.01 mg, 0.1mg, or 1.0 mg/g body weight) plus cholera toxin (0.3 μg/g) as an adjuvant and were boosted five times at weekly intervals with the same dose of allergen and cholera toxin. Six weeks after the initial sensitization dose, mice were fasted and then intragastrically challenged with two doses of cow's milk (30 mg/ml) given 30 minutes apart. Hypersensitivity responses were assessed based on systemic anaphylaxis symptom scores, vascular leakage, plasma histamine release, PCA, serum antibody titers, skin testing, and histological examination.

Their findings were that these mice exhibit several characteristics of human IgE-mediated cow's milk–induced food allergy. Mice had elevated cow's milk specific IgE antibody levels at 3 weeks and peaked at 6 weeks after initial sensitization. Elevated allergen-specific IgE levels were shown to be associated with systemic anaphylaxis, whereas levels of IgG1 were not; heating of serum from sensitized mice eliminated PCA reactions in naive mice; and mast cell degranulation was evident because of elevated plasma histamine levels (CM-sensitized (1mg/g plus cholera toxin) mice (4144 +/-1244) vs. (661 +/- 72 nmol/L) in sham sensitized mice), all of which are important features of IgE-mediated food allergy. Levels of serum casein after oral challenge were consistent with intestinal permeability studies and histological examination revealed changes in both the GI (vascular congestion, edema, enterocyte sloughing) and respiratory (increased perivascular and peribronchial lymphocytes, monocytes, and eosinophils systems. Furthermore, the development of this IgE-mediated hypersensitivity is likely to be Th2 cell mediated because in vitro stimulation of spleen cells from sensitized mice to cow's milk induced significant increased in the levels of IL-4 (44 pg/ml), IL-5 (68 pg/ml) but not INF-γ (4 pg/ml).

The authors conclude from this study that this model should provide a useful tool for evaluating the immunopathogenic mechanisms involved in cow's milk allergy and for exploring new therapeutic approaches [21].

Strengths of this model include i) The use of a dose study to determine amount of allergen to sensitize with; ii) Determination of cytokine profile, showing mechanism driving IgE; iii) Did oral challenge after sensitization and studied systemic anaphylaxis.

Some limitations of this study include i) The use of cholera toxin as an adjuvant does not mimic how humans are likely sensitized to peanut allergens; ii) A profile of the IL-4 to INF-γ ratio could have been done to show the balance in Th1 and Th2 cytokines.

2.9 Mouse model of peanut allergy

As in the cow's milk allergy model, in able to mimic the clinical and immunological characteristics of peanut allergy, Sampson's group used female C3H/HeJ mice and sensitized them orally with freshly ground whole peanut and cholera toxin as adjuvant [22]. Five-week-old mice were sensitized by intragastric gavage with either a low dose of 5 mg/ml of ground whole peanut (1 mg of peanut protein) or high dose of 25 mg of ground whole peanut (5 mg of peanut protein) together with 10 μg cholera toxin on day 0 and day 7. Three and five weeks after initial sensitization, mice were fasted overnight and challenged intragastrically with 10 mg crude peanut extract divided into two doses at 30- to 40-minute intervals.

Following challenge mice had fatal or near fatal anaphylaxis that occurred in 12.5% of sensitized mice at 3 weeks. At the second challenge (five weeks challenge) symptoms following challenge were more severe, increasing the fatality rate to 21%., whereas the colera toxin alone control group had no responses. Peanut-specific IgE and titers were

significantly increased at weeks 1–5 with the low dose eliciting more peanut specific IgE. IgG1 levels did not differ between low-dose and high-dose sensitizations, suggesting this antibody did not play a significant role in inducing anaphylaxis in this model. PCA reactions were done to confirm what isotypes were contributing to the systemic anaphylaxis. Heat-inactivated serum did not cause a positive response, whereas serum from the peanut sensitized mice did, confirming that the anaphylactic response to be IgE-induced and not IgG1. IgG2a levels were significantly higher in the high-dose versus low dose sensitization, suggesting that IgG2a was inversely related to the severity of peanut hypersensitivity. Mast cell degranulation and histamine levels in the plasma were also assessed and both associated with peanut sensitization and peanut challenge.

From this study the authors concluded that this model of peanut allergy mimics the clinical and immunological characteristics of peanut allergy in humans and should serve as a useful tool for developing therapeutic approaches for the treatment of peanut allergy.

Strengths of this model include a number of hallmarks of human allergy are seen such as, increased plasma histamine, allergen specific IgE levels, and anaphylaxis following challenge. Also, a dose study was conducted to determine optimum dose of peanut sensitization.

Again a major drawback of this model is the fact that they use cholera toxin as an adjuvant to induce allergy. An adjuvant free-model could be better at getting to the true sensitization seen in human allergy.

2.10 Mouse model of Ovalbumin induced food allergy

A group from the National Taiwan University Hospital headed by Rong-Hwa Lin has developed a mouse model of Ovalbumin food allergy. Wang et al. [23] for the first time found that epicutaneous allergen can induce a Th2 response, without the use of adjuvant. They exposed both C57BL/6J and BALB/c mice to various concentrations of OVA (100mg/ml, 100μg/ml, and 10μg/ml) by a patch method of applying a patch with the allergen and securing it with an elastic bandage.

They found that epicutaneous exposure to both strains of mice results in high OVA specific IgE levels. They saw antibody titers of OVA specific IgE in BALB/c mice between 0 and 1800 for 1μg after 5 courses of immunization. Overall specific IgE levels in BALB/c mice around 5000 in their 100 μg exposed group. A rather weak IgG2a response was noted (peak IgG2a titer of around 2000 at 4^{th} response in 100μg exposed group) from the BALB/c strain with very few animals making even detectable levels. They did not report on the C57BL/6 strain. They report a Th-2 predominant response, but there was only a 4-5picogram increase in IL-4 levels following OVA ex vivo stimulation. Two courses of OVA epicutaneous stimulation lead to a barely detectable level of INF-γ from ex vivo lymph node cell stimulation with OVA.

They found that epicutaneous exposure to both strains of mice results in high OVA specific IgE levels as well as ex vivo IL-4 production by lymph node cells. Further very little IgG2a and INF-γ production was seen.

Hsieh et al. 2003 in continuation of the earlier mentioned work confirmed that food allergy may be through skin sensitization [24]. In this study they sensitized BALB/c mice epicutaneously through the shaved skin of the back. A patch impregnated with 100μg of ovalbumin was applied for a 1-week period and then removed. After three courses of sensitization, OVA-specific antibodies were measured and then mice were challenged with 50mg of OVA orally. Anaphylactic responses, plasma histamine levels, and histology of the intestines and lungs were then preformed. They found that BALB/c mice elicit OVA-specific IgE when a patch impregnated with OVA is applied to the shaved dorsal skin. Following oral challenge with allergen, symptoms of systemic anaphylaxis occurred, plasma histamine increased, and marked changes were seen in both the intestine (vascular congestion, edema, enterocyte sloughing at villis tips) and lungs (perivascular and peribronchial inflammatory infiltrates, which consisted of lymphycytes, monocytes, and eosinophils).

The major strength of this model is the fact that the sensitization phase is adjuvant free. Because no adjuvant is used therapeutic and prophylactic methods to fight food allergy can be better studied without the adjuvant effect altering the response (is the therapeutic effect actually weakening the adjuvant).

A possible weakness of this model is the fact that a purified protein was used and not a food allergen and Th1/Th2 balance was not assessed. Further analysis of a time course of

ex vivo stimulation would give a better representation of the cytokine response in this model.

2.11 Anti-ulcer drugs promote oral sensitization to hazelnut allergens

The only mouse based model using tree-nut and assessing hypersensitivity, use anti-ulcer drugs to promote hypersensitivity [25]. In the recent years, parallel with our studies a group from the Medical University of Vienna developed a protocol to induce immune responses to oral hazelnut. In there studies, they feed BALB/c mice hazelnut (2mg) with or with different antiulcer drugs (sucralfate 2mg). They report hazelnut specific IgG1, but no detectable levels of IgE when mice were orally fed hazelnut extract with a pretreatment of anti-ulcer drugs. Although oral sensitization is highly sought after, without adjuvant there is no response and mice develop oral tolerance. They claim that this treatment did sensitize mice for type I skin reactivity to hazelnut extract, as evidenced by passive cutaneous anaphylaxis (PCA) reactions using naïve mice and hazelnut specific IgG1 purified from plasma from the anti-ulcer group of mice. They do show that the IgG1 specific to hazelnut is anaphylactogenic in naïve animals when concentrated, but do not show if there is an in vivo consequence after challenge.

Conclusions of this study are that the use of anti-ulcer drugs may promote the induction of immediate type food hypersensitivity towards hazelnut by protecting against gastric digestion.

The strength of this study is that they use the oral route of sensitization to induce hypersensitivity. Although a novel and important finding, this model does not follow classical allergy mechanisms, showing allergen specific IgE, therefore it is not a validated tree-nut allergy model. Furthermore, they do not show an in vivo effector phase after allergen challenge. Finally, they do not assess mechanisms associated with hypersensitivity development; cytokine profile assessment would have been interesting if profiled.

2.12 Gap in the knowledge

Even though extensive research is ongoing about food allergies, mechanisms driving this increasing trend are unclear at present. Therefore more research needs to be done so that this increasing trend can be stopped. To do this, well-characterized animal models need to be developed. Then potential therapies can be developed and studied. Reviewed here was a list of current animal models available to study food allergy. They include mouse models for several foods including peanut, cow's milk and egg. However, **a mouse model to study tree-nut allergy was not available when we began our work in 2001.**

With this gap in the knowledge, I sought out to develop and characterize a mouse model of tree-nut allergy, using hazelnut as a model tree-nut, which might be useful to study tree-nut allergy. In the next five chapters, efforts were made to characterize a mouse model of hazelnut allergy with objectives being 1) to develop an ELISA based method to measure allergen specific IgE as an alternative to the passive cutaneous anaphylaxis assay; 2) to determine if hazelnut can directly elicit a specific IgE antibody response via activating IL-4 in mice; 3) to characterize the systemic immune response following

transdermal hazelnut protein exposure in BALB/c mice; 4) to develop an adjuvant free model of hazelnut protein induced systemic anaphylaxis; 5) to study the effect of a diet rich in EPA and DHA on systemic immune responses to hazelnut.

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CHAPTER THREE

3.0 An ELISA based method for food specific IgE antibody measurement in mouse serum: An alternative to the passive cutaneous anaphylaxis assay[1]

3.1 Abstract

Background: Passive cutaneous anaphylaxis (PCA) assay has been a gold standard method to measure allergen specific IgE antibody levels in allergy mouse models. Many factors including stringent guidelines for laboratory animal use make PCA a difficult choice. Therefore, alternative methods are needed that can be readily applied for measurement of specific IgE antibody levels in mouse serum.

Aims: The aim of this study was to develop and optimize an ELISA based system that is comparable to PCA and can be used to measure specific IgE in mouse plasma or serum.

Results: Herein we describe a novel ELISA based method that is more-sensitive in comparison to PCA, IgE isotype specific (because it has little cross-reactivity with IgG1 or IgG2a isotype) and highly reproducible (<10% inter or intra assay variation). Furthermore, we demonstrate the utility of this assay to measure specific IgE Ab against a variety of food extracts including chicken egg, peanut, almond, filbert/hazelnut and sweet potato.

Conclusions: These findings are of particular interest to those who are seeking (i) to measure food extract specific IgE antibody in animal models and (ii) an alternative to the animal based PCA method to measure mouse IgE antibodies.

[1] This work was published in *J Immunol Methods*. 2003 Apr 1;275(1-2):89-98.

3.2 Introduction

Allergen specific IgE antibody (ASIgE Ab) production is a central event in the pathogenesis of atopic disorders that include allergic asthma, -rhinitis, -dermatitis, -conjunctivitis, food and drug allergies and anaphylaxis [1-3]. Consequently, presence of elevated levels of specific IgE Ab in the serum is a diagnostic factor for immediate hypersensitivity response to environmental antigens in humans and animal allergy models [4]. Therefore, accurate, reliable and user-friendly methods are needed to measure serum levels of allergen specific IgE antibodies.

Passive cutaneous assay (PCA) performed in mice or rats has been widely used to measure allergen specific IgE antibody levels in animal models for half a century [5-8] The PCA method has the advantage of measuring not only the biologically active IgE Ab but also the consequence of allergen/IgE interaction leading to the inflammatory mediator release from mast cells and the clinical expression of cutaneous anaphylaxis. However, many factors including stringent guidelines for laboratory animal use, its labor-intensive nature and the capacity of murine IgG1 to trigger PCA responses, limit the utility of this assay. Therefore, alternative *in vitro* methods that are inexpensive, easy to perform and comparable in sensitivity to PCA are needed.

A number of ELISA based methods have been described for measuring IgE Ab specific to individual purified proteins such as ovalbumin, milk proteins (casein, beta-lacto globulin), peanut major allergens (Ara h1, Ara h2), or haptens [7, 9-17]. However, we are not aware of methods available for measuring whole food extract specific IgE antibody in

mouse serum. In an effort to fill this need, here we describe an ELISA based method for food extract specific IgE Ab detection that is comparable in sensitivity to PCA assay. Furthermore, we demonstrate the utility of this method by performing specific IgE Ab detection using a variety of food extracts including chicken egg, peanut, almond, filbert/hazelnut and sweet potato.

3.3 Materials and methods

3.3.1 Materials

The following materials were purchased from sources as indicated in parenthesis. Food extracts (Greer Labs, Lenoir, NC, USA); Biotin labeled anti-mouse IgG1 (Southern Biotech, Birmingham, AL), anti-mouse IgG2a (Southern Biotech, Birmingham, AL) and anti-mouse IgE antibody (Serotec, Raleigh, NC; BD PharMingen, San Deigo, CA, USA); Ig isotype standards (Southern Biotech, Birmingham, AL); *p*-nitro-phenyl phosphate (Sigma, St Louis, MO, USA); Streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); Protein-G Sepharose (Pharmacia Biotech); Ovalbumin (ICN Bio-medicals, Montreal).

3.3.2 Mice

All mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). Only adult female mice (6-7 weeks age) were used in the study. All animal procedures used were in accordance with the Michigan State University policies.

3.3.3 Animal immunization and serum collection

Various standardized food protein extracts purchased from Greer labs were sterilized by filtration and total protein content was determined by Lowry's method. A group of adult mice (n=5) received intraperitoneal injection of 100 ug of protein extract from peanut, almond, filbert/hazelnut, walnut and chicken egg plus alum (2.5 mg/mouse) as an adjuvant. Another group (n=5) received 100 ug of protein extract from chicken egg, wheat, soy, coffee and sweet potato plus alum (2.5 mg/mouse). Control mice (n=3)

received sterile saline injections. All mice were bled on day 11 after first injection. Animals received booster injection and bled on days 7, 15, 32 and 62 after the booster injection. The serum was used in antibody estimations.

3.3.4 Food specific IgE, IgG1, IgG2a antibody measurement

Enzyme linked immunosorbent assay (ELISA) was optimized for each of the food extracts taking into account the background activity (i.e., all reagents added except for the sample). All reagents were used at a final volume of 50 uL/well except for blocking buffer that was used at 75 uL/well. Washing was done with 200 uL/well using an automatic ELISA washer (Dynex Technologies Inc, Ultra-wash Plus). Each food extract was analyzed for protein content by Lowry's method and then used in ELISA for coating at concentrations ranging from 10 to 5000 ug/mL. Briefly, ELISA plates (96 well EIA/RIA plate, 96 well easy wash ^{TM}, high binding, Corning Inc., NY) were coated with food extracts diluted in carbonate buffer (0.05 M, pH 9.6) and incubated at 4 ^{o} C, over night. Unbound extract was discarded and the plates were blocked (0.17% BSA/PBS) at 37 ^{o} C. For peanut IgE assay, blocking was performed with 5% gelatin. After washing (0.05% Tween 20 in PBS), serum samples were added at various two-fold dilutions from 1/20 to 1/640 or in some experiments at 1/30 to 1/61,440 in dilution buffer (0.085% BSA, 0.05% Tween 20 in PBS). Following incubation, plates were washed and a biotin labeled anti-mouse IgG1 or IgG2a or IgE antibody added. After incubation, plates were washed and streptavidin alkaline phosphatase conjugate was added at 1/4000 (in dilution buffer). Subsequently, plates were washed and *p*-nitro phenyl phosphate (PNPP) substrate added (1 tablet per 5 mL substrate buffer, according to manufacturers instruction; Sigma).

Reactions were allowed to develop at the room temperature in the dark and absorbance measured in a microplate reader using dual mode of wavelength at 405 nm (peak) minus 690 nm (background) (Microplate ELISA Reader, SoftMax program, Molecular Devices;). According to manufacturer's instructions, dual mode provides relatively better measurements since it adjusts the reading for background interference (Personal communication, Technical Services, Molecular Devices). All plates included negative controls (no mouse serum sample background and no antigen coating control) and a positive internal control (A reference mouse serum sample containing known levels of ovalbumin specific IgE antibody; a kind gift from Prof. Kent HayGlass, The University of Manitoba). All samples were analyzed a minimum of 2 to 3 times.

3.3.5 Determination of assay sensitivity and serum antibody titer

The assay sensitivity was defined as the background optical density from wells to which all reagents but no mouse serum sample had been added, + 3 SD. Antibody titer was defined as the reciprocal of the serum dilution that closely matched this value.

3.3.6 Passive cutaneous anaphylaxis assay

Anti-ovalbumin IgE levels were determined by 48-hr PCA assay in female S-D rats as previously described [18]. Means of duplicate or triplicate analyses are presented.

3.4 Results

3.4.1 Impact of coating antigen concentration on food specific IgE antibody detection

In order to determine the optimal coating antigen concentration for IgE antibody detection, initially an ELISA was set-up using various amounts of egg extract. As evident from the results, a coating antigen concentration of 500 ug/ml yielded maximal absorbance (OD). (Figure 1A). However, the assay took 24 hr incubation for complete color development. Furthermore, the OD was off the scale implying off-accuracy and off precision. We rationalized that increasing the concentration of coating antigen might reduce the developing time it takes to get a reasonably good assay with a good detection window. Therefore, we used 2 to 10 fold higher antigen amounts for coating. As evident (Figure 1B), a combination of higher coating antigen concentration (at 5000 ug/mL instead of 500 ug/mL) and a lower developing time of 2 hr (instead of 24 hr) provided an assay with a reasonably good window of detection. Furthermore, the background activity was not significantly enhanced with higher coating antigen concentrations. Thus, coating food extract at a protein concentration of 5000 ug/mL and a developing time of 1-3 hours was used in all subsequent assays. This data suggests that the antigen did not bind to the microplate well in sufficient quantities except at high concentration.

3.4.2 Immunoglobulin epsilon isotype specificity of the assay

We tested the isotype specificity of the assay by two different ways: (i) first, we compared the isotype specificity of the detection antibody. In this experiment, we coated wells with mouse IgE (at 250 ng/mL), IgG1 (at 500 ng/mL) and IgG2a (at 500 ng/mL) Ig

isotype standards or egg and then added serum samples from egg sensitive mice to egg coated wells and buffer to isotype standard coated wells. All wells were developed with a biotin conjugated anti-IgE antibody. As evident from the results (Figure 2A), biotin antibody detected egg specific IgE antibody. Furthermore, biotin antibody reacted with IgE but not with IgG1 or IgG2a isotype standards verifying the epsilon isotype specificity of the assay; (ii) second, we depleted IgG1 and IgG2a from mouse serum by treating it with protein-G Sepharose following manufacturer's instructions (Pharmacia Biotech) and tested its impact on IgE detection by the assay (Figure 2B). Removal of IgG1 and IgG2a from mouse serum indeed enhanced the detection window of the assay (i.e., peak signal minus the background OD) although it had no significant impact on the assay sensitivity. A control experiment was performed to make sure that protein-G treatment indeed removed most of the egg specific IgG1 and IgG2a from mouse serum (Figure 3).

3.4.3 Comparison between ELISA and PCA assays

In order to compare the relative sensitivity of ELISA vs. PCA to measure mouse IgE antibodies, we measured ovalbumin specific IgE Ab titer of serum sample by both methods. As evident (Figure 4), ELISA titer of the sample (15,360) was ~two titers higher compared to that of PCA titer (3,900). These data suggest that the ELISA method described here might be a suitable alternative to PCA method for measuring mouse IgE antibodies.

3.4.4 Reproducibility of the ELISA method

We examined the inter-assay and intra-assay variation of this assay when performed by two different individuals. As evident from the data, co-efficient of variation in all cases was <10 % (Figure 5).

3.4.5 Application of the assay to measure food specific IgE antibody levels in mouse serum

We tested the potential utility of the ELISA method to measure food extract specific IgE antibody against a variety of food types using food sensitized mouse serum. As evident in Figure 6 this method was useful to measure specific IgE Ab levels using extracts from chicken egg, peanut, almond, hazelnut and sweet potato. The average background activity (OD) for various food types at 2 hour reading was as follows: Egg ~</= 0.3, peanut extract, </=0.26), filbert/hazelnut </= 0.38), almond </=0.3, and sweet potato </= 0.06).

3.5 Discussion

We have described an ELISA based method for measurement of food specific IgE antibody in mouse serum using a variety of food types. The data presented demonstrates that the ELISA based method is more sensitive than PCA, isotype specific (because it has little cross reactivity with IgG1, IgG2a) and highly reproducible (<10% inter-assay and intra-assay variation). We are not aware of any previously described ELISA methods for food extract specific IgE antibody detection in mouse serum.

Three methods have been widely used to measure allergen specific IgE antibody in the serum samples --PCA, RAST and ELISA, although several other methods (e.g., in vitro histamine release assay, fluorometric reverse (IgE-capture) ELISA, resetting etc) have been described in the literature [4, 7, 9-15, 19-25]. PCA has several advantages including: (i) high sensitivity; (ii) high specificity (if challenged 48 hour post sensitization); and (iii) measurement of biological consequence of allergen-IgE interaction. However, it suffers from many practical disadvantages such as (i) high cost; (ii) animal use that is labor intensive; and (ii) concerns about animal welfare. The RAST is routinely used clinically for measurement of specific IgE Ab in the human serum [3, 4, 24, 26]. Although it is applicable to a mouse system, its utility is limited by the use of radioisotopes and low level of precision (i.e., 1+, 2+ as readouts). In contrast, this ELISA method addresses the many shortcomings of PCA and RAST methods.

Currently there are few ELISA based methods available for determination of specific IgE Ab against purified proteins derived from foods including ovalbumin (chicken egg

derived), milk proteins (casein, beta-lacto globulin) and Ara h1, Ara h2 (peanut derived) [9, 10, 16, 17]. While these methods are very useful for individual purified proteins, their suitability for whole food extracts from different sources has not been demonstrated. Furthermore, we are not aware of previous reports on measuring specific IgE antibody in mouse serum for either whole extract or purified proteins from almond, hazelnut and sweet potato.

Some of the specific IgE antibody measuring ELISA methods employ use of protein-G to remove competing IgG1 from the serum [10]. We found that depleting IgG1 and IgG2a from the mouse serum enhanced detection window of the assay (Figure 3 suggesting competition between specific IgG1/IgG2a and IgE antibody for common epitopes. However, the sensitivity of the assay apparently was not affected by this competition.

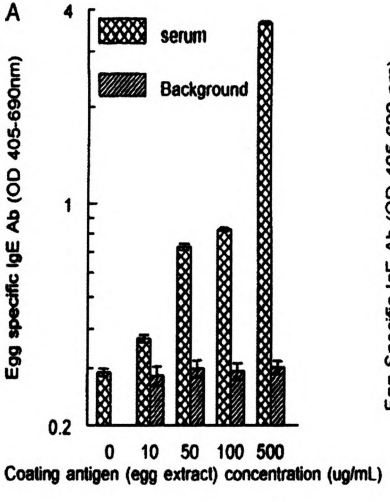
In the present method we used alkaline phosphatase-dependent substrate (p-nitro- phenyl phosphate) for color development. It may be possible to further enhance the sensitivity of this method using alkaline phosphatase dependent fluorescence substrates (e.g., 4-methyl umbelliferylphosphate).

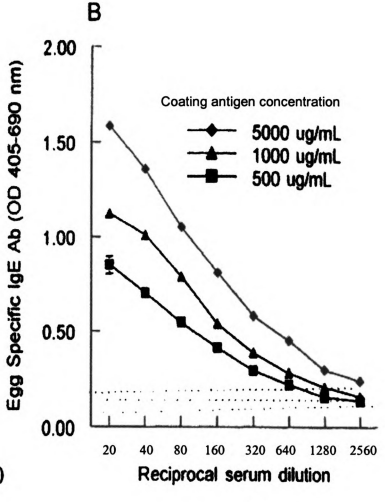
In the present study, the ELISA method described here was tested for general applicability using five different food types. We noticed that peanuts consistently exhibited higher background activity when BSA was used as a blocking agent. In contrast, use of 5% gelatin as a blocking agent dramatically reduced background and increased assay sensitivity. We have also found that the method described here is suitable for a number of other food types (data not shown). We conclude that the method

described here may be applied to detect IgE antibodies in mouse serum against a variety of food types—not necessarily all food types.

In summary, we have described an ELISA based assay that would be very useful to those who are seeking: (i) to measure food extract specific IgE antibody in mouse models of food allergy; and (ii) an alternative to animal based PCA method to measure mouse IgE antibodies.

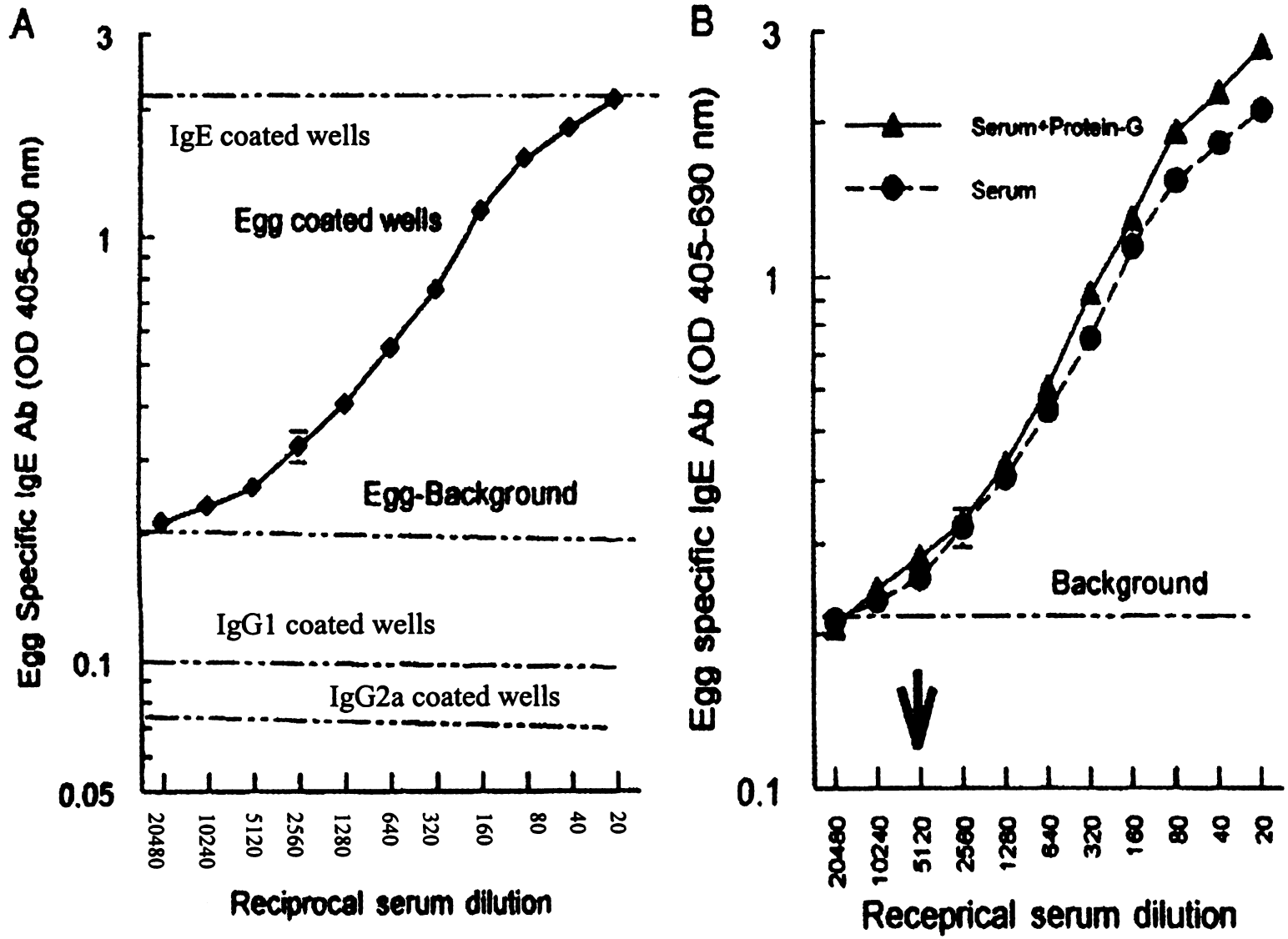
3.6 Figures


A
4
1
0.2
serum
Background
Egg specific IgE Ab (OD 405-690nm)
0 10 50 100 500
Coating antigen (egg extract) concentration (ug/mL)


B
2.00
1.50
1.00
0.50
0.00
Coating antigen concentration
5000 ug/mL
1000 ug/mL
500 ug/mL
Egg Specific IgE Ab (OD 405-690 nm)
20 40 80 160 320 640 1280 2560
Reciprocal serum dilution

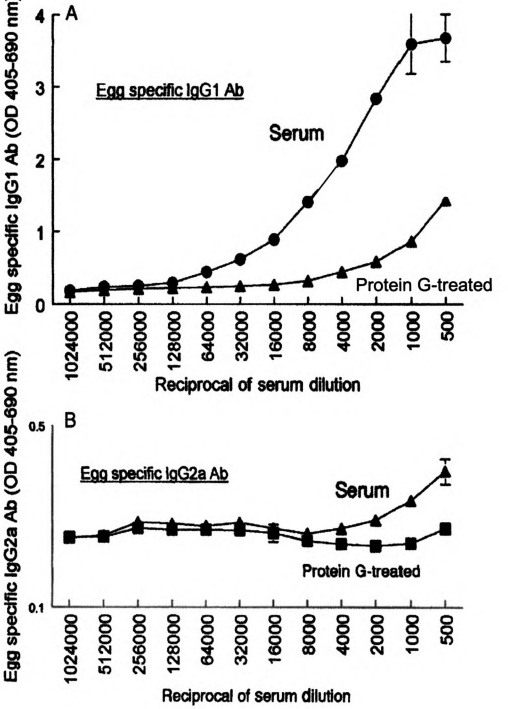
Figures 3.6.1(A, B): Impact of coating antigen concentration on assay sensitivity

(A) Wells were coated with various amounts of egg protein extract (10-500 ug/mL) and egg specific IgE Ab in the serum obtained from egg sensitized mice was measured after overnight (A) or ~2 hour developing time (B). For figure A, serum was used at 1/10 dilution. The same serum sample was used at indicated dilutions in Figure B. Data represent mean+/- SE of duplicates.


A
3
1
0.1
0.05
Egg Specific IgE Ab (OD 405-690 nm)
IgE coated wells
Egg coated wells
Egg-Background
IgG1 coated wells
IgG2a coated wells
20480
10240
5120
2560
1280
640
320
160
80
40
20
Reciprocal serum dilution
B
3
1
0.1
Egg specific IgE Ab (OD 405-690 nm)
Serum+Protein-G
Serum
Background
20480
10240
5120
2560
1280
640
320
160
80
40
20
Receprical serum dilution

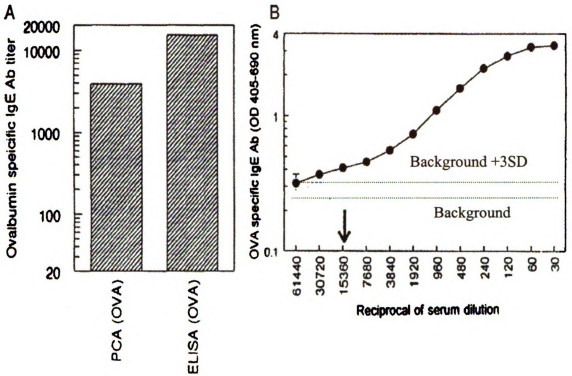
Figures 3.6.2 (A-B): Immunoglobulin (Ig) epsilon isotype specificity of the assay

(A) Wells were coated with either the egg extract or Ig isotype controls (IgG1 at 500 ng/mL, IgG2a at 500 ng/mL, IgE at 250 ng/mL), followed by serum (from egg sensitized mice) addition to egg coated wells only and developed with anti-mouse IgE antibody. (B) Serum from egg-sensitized mice was pre-treated with 50% slurry of Protein-G Sepharose following manufacturer's instructions (Pharmacia Biotech) and then subjected to egg specific IgE ELISA. Data represent mean+/- SE of duplicates or triplicates. Arrow indicates the egg specific IgE Ab titer.


A
Egg specific IgG1 Ab
Serum
Protein G-treated
Egg specific IgG1 Ab (OD 405-690 nm)
4
3
2
1
0
1024000
512000
256000
128000
64000
32000
16000
8000
4000
2000
1000
500
Reciprocal of serum dilution
B
Egg specific IgG2a Ab
Serum
Protein G-treated
Egg specific IgG2a Ab (OD 405-690 nm)
0.5
0.1
1024000
512000
256000
128000
64000
32000
16000
8000
4000
2000
1000
500
Reciprocal of serum dilution

Figures 3.6.3 (A-B): Impact of Protein-G treatment on egg specific IgG1 and IgG2a antibody levels in serum from mice sensitized with egg

Wells were coated with the egg extract followed by addition of serum with or without Protein-G treatment (50% slurry), following manufacturer's instructions (Pharmacia Biotech) and developed with biotin labeled ant-mouse IgG1 (Figure A) or IgG2a (Figure B) antibody. Serum sample was identical to the one used in Figure 2. Data represent mean+/- SE of duplicates or triplicates.



Figures 3.6.4 (A-B): Comparison of assay sensitivity: ELISA vs. PCA

(A) An identical mouse serum sample was subjected to ELISA and PCA and ovalbumin (OVA) specific IgE antibody titer determined. (B). This figure demonstrates the method used to deduce ovalbumin specific IgE Ab titer by the ELISA method that is shown in Figure A. Data from triplicate experiments is presented (mean +/- SE). Arrow indicates the OVA specific IgE Ab titer.

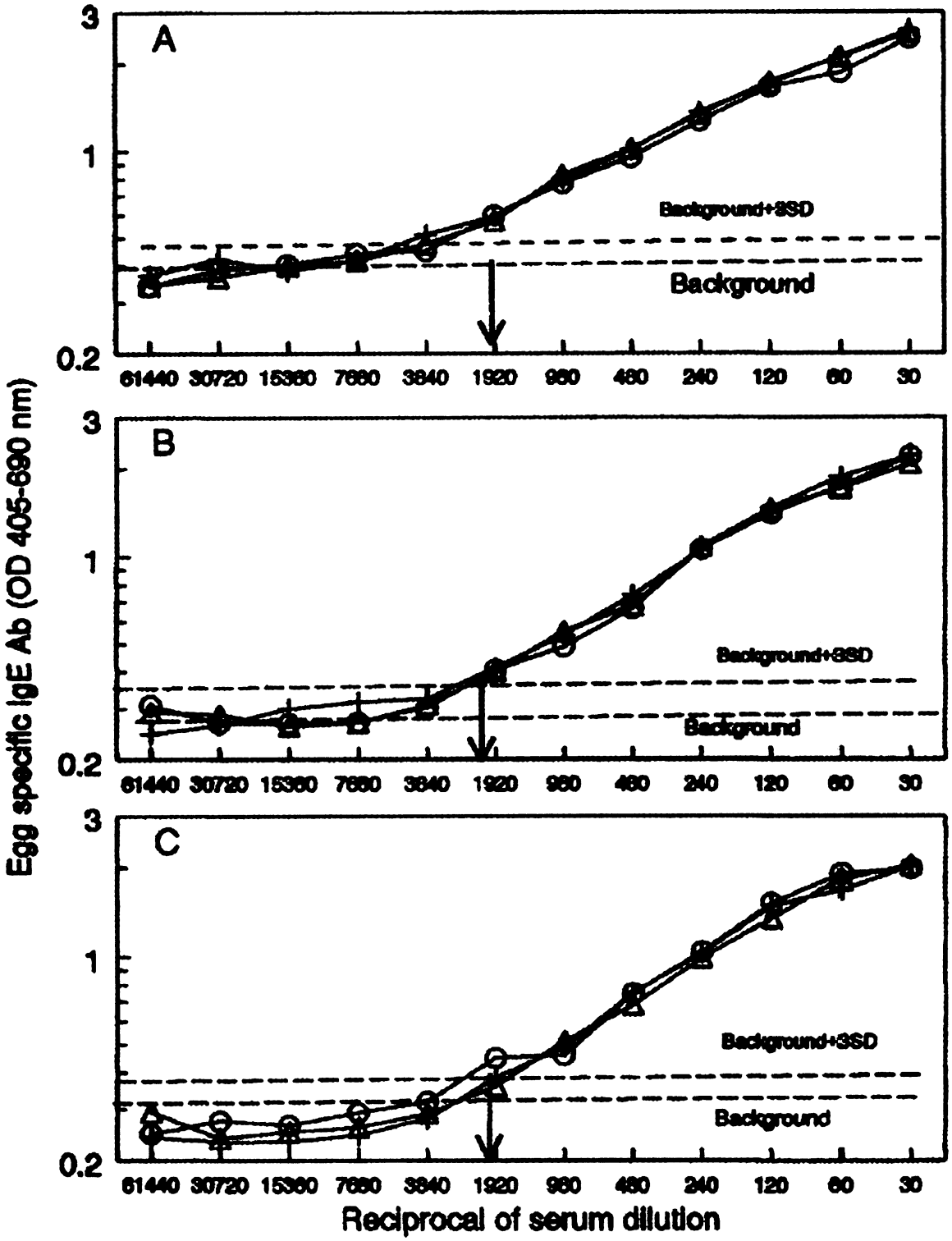

A
B
C
3
1
0.2
Background+3SD
Background
61440 30720 15360 7680 3840 1920 960 480 240 120 60 30
Egg specific IgE Ab (OD 405-690 nm)
Reciprocal of serum dilution

Figure 3.6.5 (A-C): Inter and Intra assay variation of the ELISA method

(A) This Figure illustrates three sets of data obtained by screening an identical sample on the same ELISA plate (Intra-assay variation). (B, C) Data shown illustrates three sets of data obtained by screening an identical sample on different ELISA plates on different days by two different persons (Inter-Assay variation). Data represent mean+/- SE of triplicates. Arrow indicates the egg specific IgE Ab titer.

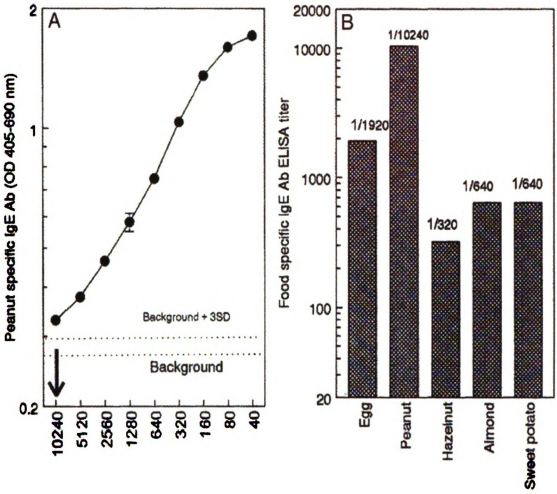


Figure 3.6.6: Applications of the ELISA method to measure food extract specific IgE Ab in mouse serum

(A) Data shown in this figure illustrates the utility of this method to determine peanut specific IgE antibody titers in the serum of mice sensitized with peanut. Reciprocal of the serum dilution with OD that closely matched the background OD + 3SD, was defined as the IgE antibody titer of the serum sample (shown as an arrow); (B) Similar method as shown in Figure A, was used to deduce IgE titers against other food types using serum from mice sensitized with various food types. Data represent mean+/- SE.

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CHAPTER FOUR

4.0 Hazelnut allergy: Evidence that hazelnut can directly elicit specific IgE antibody response via activating Type-2 cytokines in mice[2]

4.1 Abstract

Background: Hazelnut is one of the major tree-nuts that potentially causes fatal food allergy, with underlying mechanisms unclear at present. One suggestion is that hazelnut allergy results from immune cross-reactivity of IgE antibodies produced against certain aeroallergens.

Hypothesis, approach: Here we tested the hypothesis that hazelnut is intrinsically capable of eliciting an allergic response using a mouse model. Groups of mice were injected i.p. with hazelnut/filbert protein extract with or without alum as an adjuvant and hazelnut specific antibody (IgE, IgG1) responses were examined using optimized ELISA. Hazelnut specific Type-2 and Type-1 cytokine responses were evaluated by *ex vivo* antigen mediated activation of spleen cells.

Results: Hazelnut elicited robust IgE and IgG1 antibody responses. Time-course and dose response analyses further provided evidence for memory Type-2 dependent antibody responses to hazelnuts. Hazelnut specific IgE response in two strains of mice with different MHC haplotypes and IgE response to hazelnut without the use of alum adjuvant asserted that hazelnut is intrinsically an allergenic food. The Type-2 cytokine analyses revealed that hazelnut sensitization results from activation of IL-4 and IL-5, thus providing mechanistic basis for hazelnut specific IgE response.

Conclusion: Our data argue that hazelnut—a widely consumed food, is intrinsically an allergenic food capable of directly eliciting hazelnut binding specific IgE antibodies *via* activation of Type-2 cytokines in mice.

[2] This work has been published in Int Arch Allergy Immunol.2005 Aug;137(4):295-302.

4.2 Introduction

Immediate hypersensitivity to food, commonly called food allergy, afflicts 6-8 % children and 2% adults in Westernized countries such as the United States[1, 2]. Incidence of these diseases is believed to be increasing consistent with asthma and other allergic diseases for reasons that are not clear[2-4]. Food allergies typically start in infancy and some food allergies such as tree-nut allergies tend to be chronic often lasting for lifetime[2]. Peanuts and tree-nuts (e.g., hazelnuts, almonds etc) are the major food types that often cause systemic anaphylaxis with fatal or near fatal consequences[1, 2].

Scientific evidence based on double blind placebo controlled oral hazelnut challenge test clearly confirms that hazelnut can cause immediate hypersensitivity in humans[5-7]. Hazelnut allergy is more common in Europe than in the US. One reason often cited for this geographic difference is cross-reactivity between birch pollen (and hazel pollen) allergens and nut proteins[8, 9]. Given this cross-reactivity, it is assumed that many cases of hazelnut allergy are actually secondary allergies, and that one or more of the aeroallergens is/are the primary sensitizer. Nevertheless, it is not clear whether this is always the case, particularly in the US where birch pollen allergy is relatively rare. Therefore, it is important to know whether hazelnut proteins can act as primary sensitizers. Thus, it is unclear at present whether hazelnut by itself is capable of directly eliciting specific IgE antibodies.

In this study we tested the hypothesis that hazelnut by itself is capable of eliciting IgE response via activating prototypic Type-2 cytokines (IL-4 and IL-5) in a mouse model. Our results provide the evidence that hazelnut is capable of eliciting both hall-marks of

an allergic response—i.e., hazelnut binding allergenic (IgE) antibodies as well as Type-2 cytokine response (IL-4 and IL-5) in mice.

4.3 Materials and Methods

The following materials were purchased from sources as indicated in the parenthesis: hazelnut/filbert protein extract (Greer Labs, Lenoir, NC, USA); hazelnut extract was tested for protein content by Lowry's method; biotin conjugated Rat anti-mouse IgG1, IgG2a and IgE antibody; paired antibodies and recombinant standards for mouse IL-4, IL-5 and IFN-γ (BD PharMingen, San Deigo, CA, USA); *p* nitro phenyl phosphate (Sigma, St Louis, MO, USA); Streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); and Con-A (Sigma, St Louis, MO, USA).

4.3.1 Mice immunization and bleeding

All mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). Only adult female mice (6-7 weeks age) were used in the study. All animal procedures used were in accordance with the Michigan State University policies. A group of adult mice (n=4) received intraperitoneal (i.p.) injection of 100 μg of hazelnut protein extract plus alum (2.5 mg) as an adjuvant. Another group (n=4) received 1000 μg of hazelnut protein extract plus alum. A third control group of mice (n=6) received saline plus alum alone. All mice were bled three days before injections (pre-immune bleeding), on day 11 after first injection (primary response). Animals received booster injections and were bled on indicated days during secondary and tertiary immune responses. Experiments where hazelnut alone was used involved 4 mice per group per dose per strain.

Epicutaneous exposure experiments were performed using a modified method described for ovalbumin[10]. Groups of mice (n=8) were exposed to saline or hazelnut extract

(n=12; 500 ug/100 μL in saline per mouse per application); each mouse was applied with the reagent on the back skin that had been clipped-off hair and covered with non-latex bandage for 3 days. Mice were rested for 4 days. Then the cycle of exposure to saline or hazelnut was continued for six weeks. Weekly blood samples were collected and plasma was used in the antibody analysis.

4.3.2 Measurement of hazelnut specific IgG1, IgE, IgG2a antibodies in the plasma

We have previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgG1, IgG2a and IgE antibody analyses (chapter 3) [11, 12].

4.3.3 Spleen cell culture and cytokine analyses

Spleen cells were harvested and standard cell cultures were setup previously as described [13]. Briefly, spleen cells were cultured (7.5 million/mL) in the absence and presence of hazelnut protein extract (100 and 500 μg/mL). Cell culture in presence of medium alone served as a negative control and Con-A (2 μg/mL) served as a positive control. Cell culture supernatants were harvested at various time points for use in cytokine analyses using pre-optimized ultrasensitive assays (Assay sensitivity: IL-4, 0.13 pg/mL; IL-5: 2.8 pg/mL; IFN-γ: 0.72 pg/mL).

4.3.4 Statistical Analysis

Student's t test was used to evaluate significance using Analyse-It™ software program (Analyse-It software Ltd, Leeds, UK). The statistical significance level was set at 0.05.

4.4 Results

4.4.1 Characterization of hazelnut specific antibody responses in C57BL/6 mice

Mice were evaluated for the appearance of hazelnut binding Type-2-dependent specific IgG1 and IgE antibodies in the plasma during primary (day 11), secondary (days 7 and 15 after 2nd injection), and tertiary (day 40 after 3rd injection) immune responses. Pre-immune serum had neither detectable hazelnut specific IgE antibodies (mean titer <40) nor significant amounts of hazelnut binding IgG1 antibodies (mean titer <800). Following injection with hazelnut extract (100 μg), elevated levels of both IgE and IgG1 antibodies appeared (primary response: day11, mean IgE titer: 160; mean IgG1 titer: 1,024,000; secondary response: day 15, mean IgE titer: 452 +/- 160, mean IgG1 titer: 32,768,000). We next examined the dose-response and time-course of Type-2-dependent antibody responses over a three-month period. As evident from the results (Figure 2A) low doses of hazelnut (100 μg) consistently elicited higher primary as well as higher memory IgE antibody responses relative to the high dose of 1000 μg per mouse. In contrast, although there was a memory IgG1 response to hazelnut, the intensity of response was comparable at both doses tested (Figure 2B). These data provide direct evidence that hazelnut is capable of eliciting Type-2 dependent antibody responses in mice.

4.4.2 IgE antibody response to hazelnut: Role of host strain, alum adjuvant and route of exposure

In order to test whether hazelnut is intrinsically an allergenic food, immune response to hazelnut was examined in two inbred strains of mice with different MHC haplotypes

(C57BL/6 H-2^b; and BALB/c H-2^d). As evident, hazelnut elicited a robust primary as well as memory IgE antibody response in both strains although BALB/c exhibited higher IgE titers (Figure 3A). Hazelnut specific IgG1 and IgG2a antibody titers are shown in Figure 3B, C. Furthermore, we tested whether hazelnut can elicit IgE antibody response in the absence of alum adjuvant. Results (Table 1) demonstrated that hazelnut in the absence of adjuvant is capable of eliciting IgE antibody response in BALB/c but not C57Bl/6 mouse when injected by i.p. route. We then found that exposure to hazelnut in BALB/c mice by physiologically relevant epicutaneous route also elicits robust IgE antibody response (Figure 3D) confirming that hazelnut is intrinsically an allergenic food.

4.4.3 Characterization of hazelnut specific Type-2 cytokine responses in mice

In order to study the mechanism of IgE and IgG1 response to hazelnuts, we examined Type-2 cytokine responses in hazelnut-sensitized mice. As evident, hazelnut significantly activated the prototypic Type-2 cytokine (IL-4 and IL-5) responses in spleen cells from hazelnut plus alum sensitized C57Bl/6 mice (Figure 4A-B). Cytokine response from alum alone control mice shown in Figure 4C-D. Similar responses were also evident in BALB/c mice sensitized with hazelnut alone in the absence of alum (data not shown). Hazelnut also elicited a modest IFN-γ response in C57Bl/6 model of hazelnut plus alum sensitization: Culture medium: 51.8 ±4; hazelnut 100 μg/mL: 159 ±2; hazelnut 500 μg/mL: 251 ±35 pg/mL (12hr); Culture medium: 87 ±6; hazelnut 100 μg/mL: 252 ±2; hazelnut 500 μg/mL: 404 ±23 (48hr).

4.5 Discussion

There are three important findings from this study: (i) Hazelnut can directly elicit hazelnut binding IgE antibodies in mice; (ii) This ability of hazelnut is an intrinsic property since IgE response was demonstrable in two different strains of mice with different MHC haplotype; and in the absence of alum adjuvant in BALB/c mice by i.p. and epicutaneous route; and (iii) hazelnut is capable of activating prototypic Type-2 cytokine (IL-4 and IL-5) responses thus explaining the mechanism underlying the observed IgE and IgG1 responses.

In this study we chose C57BL/6 and BALB/c mice to examine allergenic immune response to hazelnuts. This is because these strains have been widely used in the allergy studies earlier although it was not clear whether they respond to hazelnut with specific IgE antibodies and Type-2 cytokine responses [12, 14-16].

We chose to examine IgG1, IgE responses to hazelnut as readouts of Type-2 responses in this study. This is because both IgG1 and IgE isotype of antibody production is dependent on the prototypic Type-2 cytokine IL-4, although dependence of IgG1 is not complete[17-19]. Interestingly, hazelnut also elicited Type-1 cytokine (IFN-γ) dependent IgG2a antibody isotype as well.

In the present study we found that hazelnut elicited very high levels of specific IgG1 antibodies with titers in several millions. In mice IgG1 antibodies participate in systemic

anaphylaxis [20]. However, at present it remains unclear whether hazelnut specific IgG antibodies have any disease relevance in humans.

We chose to inject hazelnut to mice because, earlier studies in mice using other allergens (such as ovalbumin) have shown that i.p. injection is a more reliable and sensitive method for inducing IgE antibodies compared to oral exposure [21]. Since our objective was to test whether hazelnut elicits IgE antibodies in mice, we decided to choose the i.p., route over the oral exposure. Oral sensitization to hazelnut has not succeeded in our hands so far using very young (day 8 of birth) C57Bl/6 mice (Birmingham et al. unpublished data). A very recent study (published subsequent to the submission of our manuscript) reported that oral administration of hazelnut in BALB/c mice did not lead to sensitization unless antiulcer drugs were administered [22]. Notably, this study demonstrated that antiulcer drugs facilitate induction of hazelnut specific IgG1 antibodies with immediate hypersensitivity skin reactions although IgE antibodies were not detectable. In contrast to this study, besides i.p., route, using physiologically relevant epicutaneous route of exposure to hazelnut, we found robust specific IgE antibody response confirming the intrinsic allergenic nature of hazelnuts.

There is also a previous study examining antibody response to hazelnut in rabbits[23, 24]. They also reported that hazelnut elicited IgG antibodies. However they did not study IgE or Type-2 cytokine responses to hazelnuts.

It is noteworthy that hazelnut is an allergenic food (not an allergen *per se*) as it represents a source of allergens some of which have been characterized : *Cor a 1* (a *Bet v 1* homologue)[8]; *Cor a 2* profilin[8]; 11S albumin[25]; 2S albumin, lipid transfer protein and proteins with 47 kDa, 35 kDa and 32 kDa identified as allergens from hazelnut[7]. For some of them it is known that they are present in the pollen as well as in the seed explaining immune cross-reactivity. It should be noted that results from our study does *not* provide evidence against the immune cross-reactivity hypothesis of hazelnut sensitization. Rather, we have only demonstrated that hazelnut by itself is also capable of eliciting specific IgE antibody responses in mice.

In summary, data from this study argue that hazelnut is capable of directly eliciting hazelnut binding allergenic (IgE) antibodies *via* activating Type-2 cytokine responses in mice.

4.6 Figures

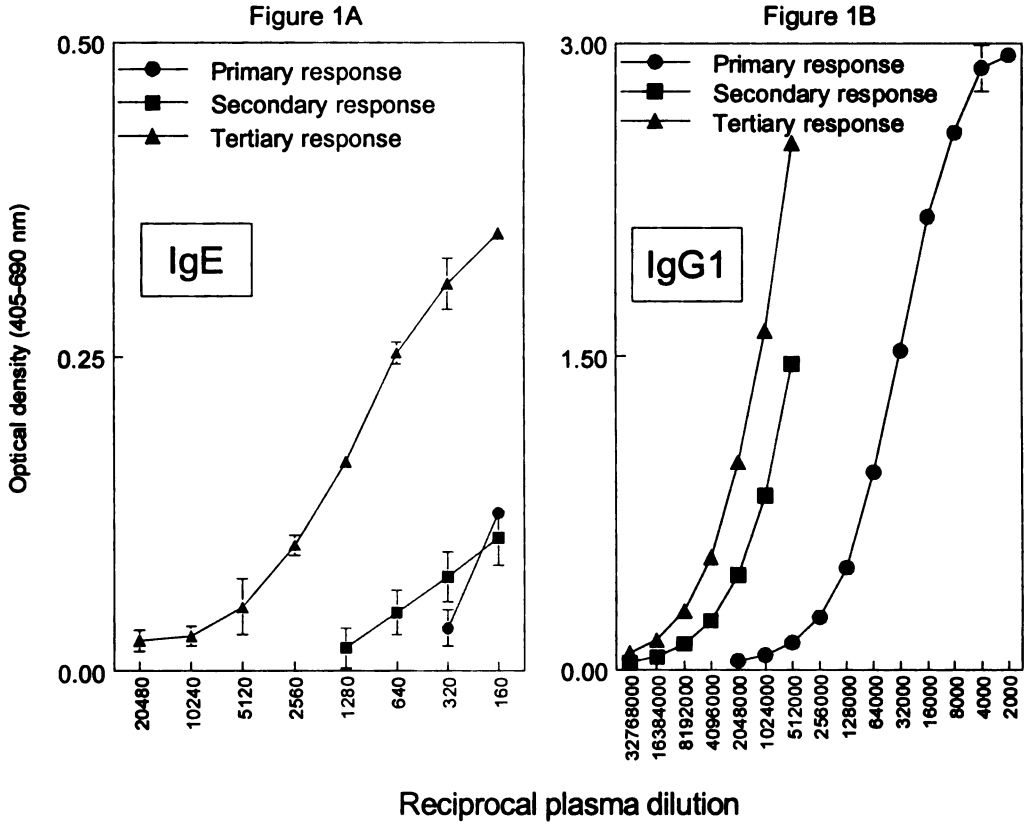

Figure 4.6.1 (A & B): Immune response to hazelnut in C57BL/6 mice.

Groups of mice (n=4) were injected with hazelnut protein extracts plus alum and hazelnut binding specific IgE (Figure A) and IgG1 (Figure B) antibodies were measured. Primary response: Antibody levels on day 11 after first injection; Secondary response: antibody levels on day 7 after the second injection; Tertiary response: antibody levels on day 40 after third injection. Data shown is OD (mean +/- SE). At some points error bars are not visible.

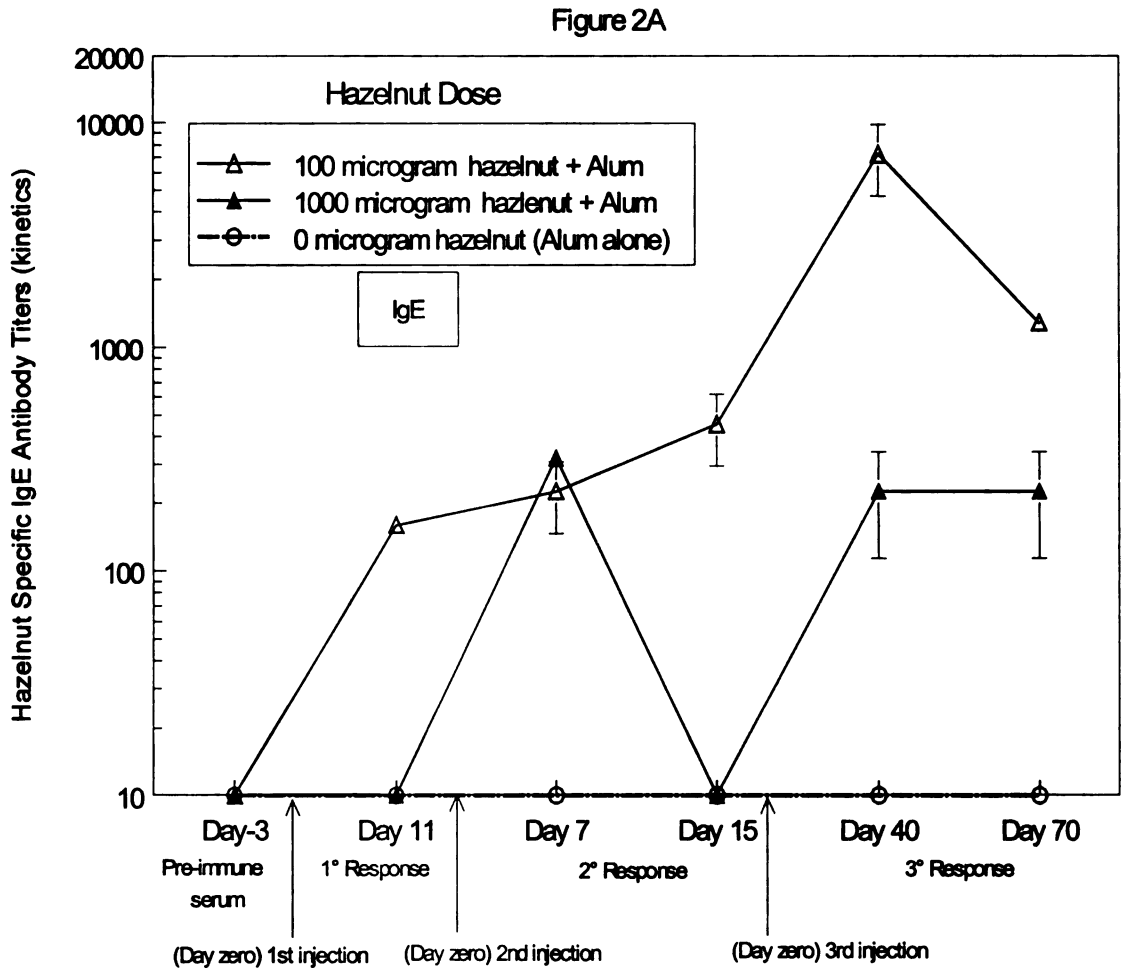

Figure 4.6.2A: Dose-response and time-course analyses of Type-2-dependent allergenic response to hazelnuts in C57BL/6 mice.

Groups of mice (n=4) were injected with hazelnut protein extracts plus alum and hazelnut binding specific allergenic antibody, IgE, responses were measured over a three month period with three injections of hazelnut extracts. Data shown is geometric mean +/- SE specific antibody titers. Where titers were identical error bars are not shown.

Figure 2B

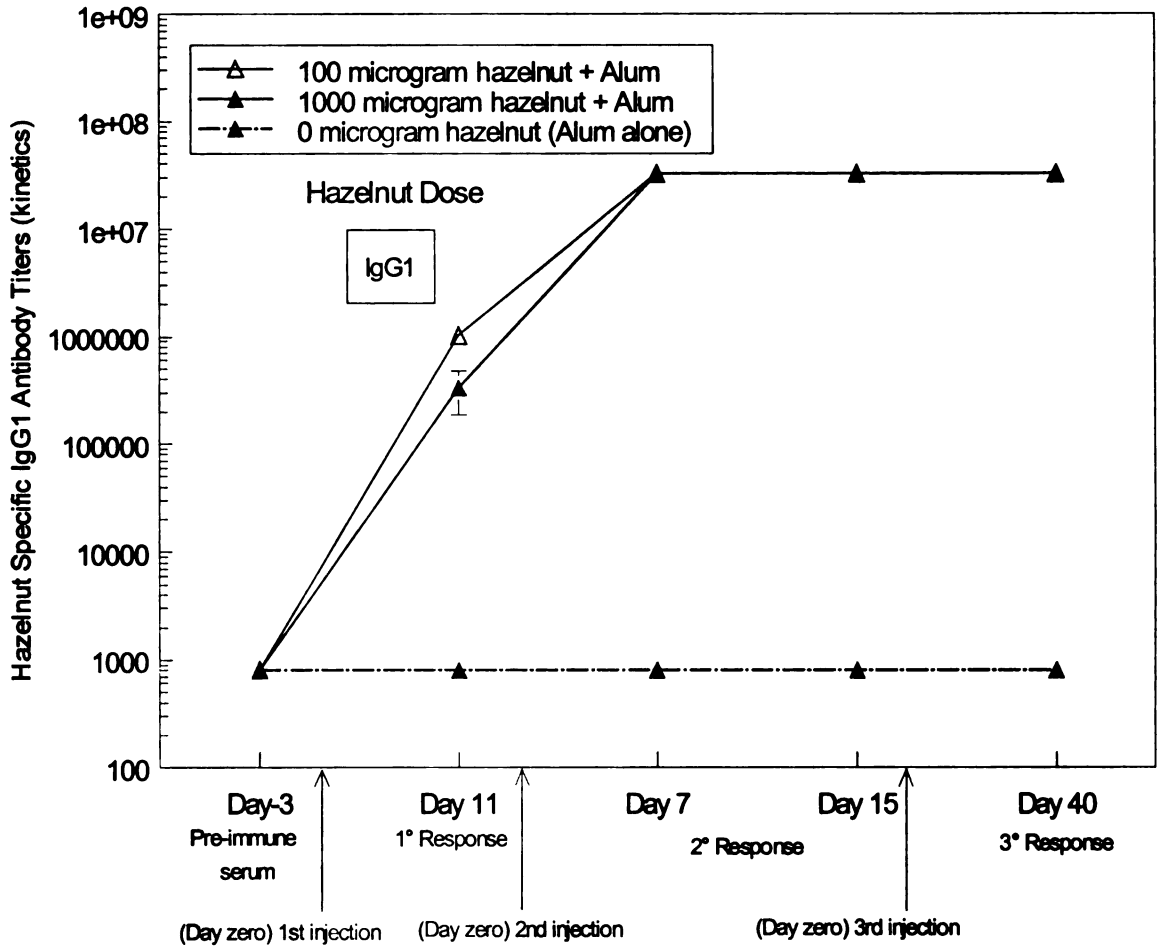

Figure 4.6.2B: Dose-response and time-course analyses of Type-2-dependent antigenic response to hazelnuts in C57BL/6 mice.

Groups of mice (n=4) were injected with hazelnut protein extracts plus alum and hazelnut binding specific antigenic, IgG1, responses were measured over a three-month period with three injections of hazelnut extracts. Data shown is geometric mean +/- SE specific antibody titers. Where titers were identical error bars are not shown.

Figure 3

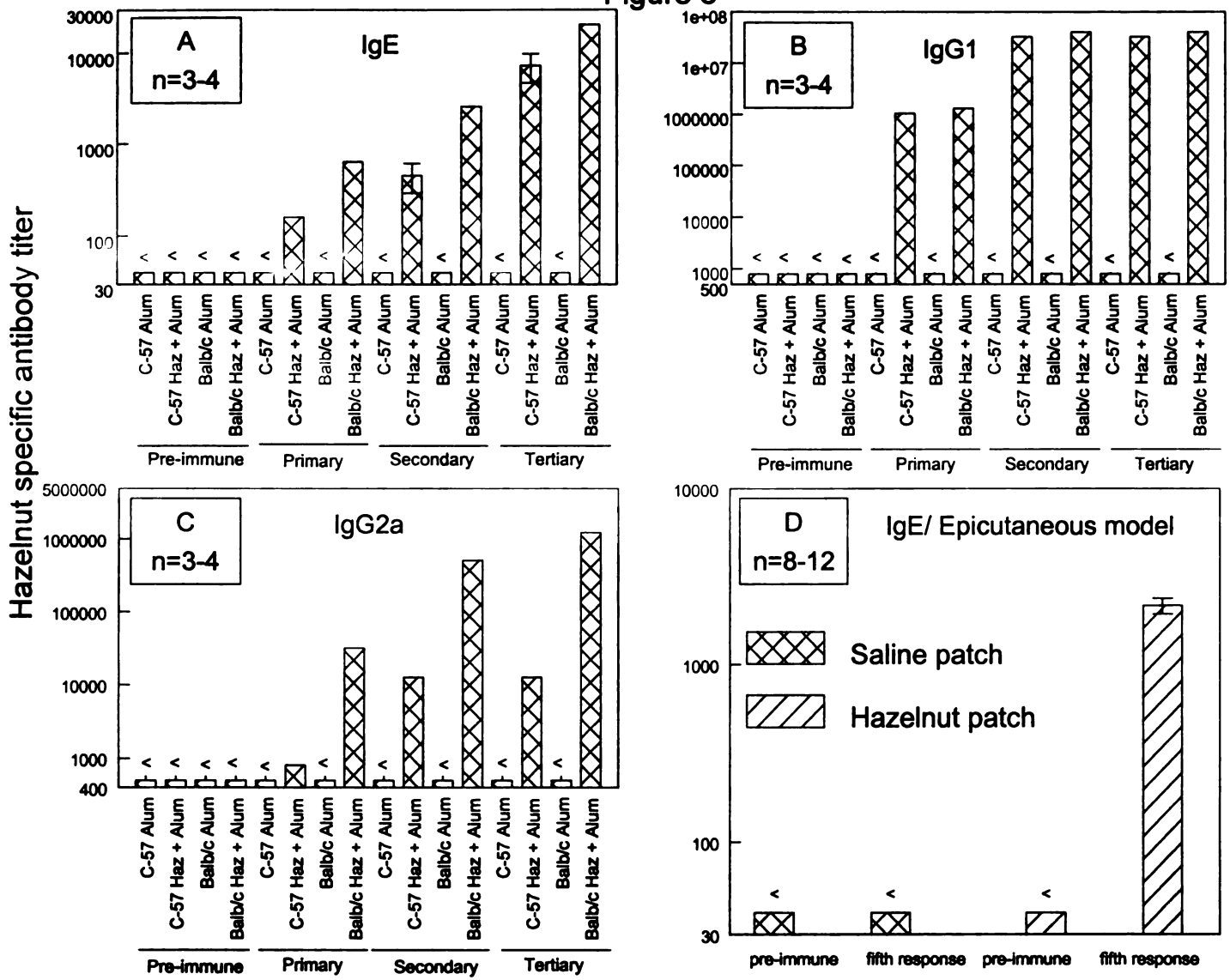
Hazelnut specific antibody titer
A
n=3-4
IgE
B
n=3-4
IgG1
C
n=3-4
IgG2a
D
n=8-12
IgE/ Epicutaneous model
Saline patch
Hazelnut patch
C-57 Alum
C-57 Haz + Alum
Balb/c Alum
Balb/c Haz + Alum
Pre-immune
Primary
Secondary
Tertiary
pre-immune
fifth response

Figure 4.6.3 (A-D): Hazelnut specific antibody responses in mice strains with differing MHC haplotype. (A-C) Groups of C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (n=4) were injected with hazelnut protein extracts plus alum or alum alone and hazelnut binding specific IgE, IgG1 and IgG2a responses were measured before and after three injections of hazelnut extracts. Data shown is geometric mean +/- SE specific antibody titers. (D) Groups of BALB/c mice were epicutaneously exposed to hazelnut (n=12) or saline (n=8) and systemic IgE antibody responses were analyzed. Data shown is geometric mean +/- SE of specific IgE antibody titers in pre-immune plasma vs. plasma collected after five weeks of epicutaneous sensitization. Where titers were identical error bars are not shown.

Figure 4

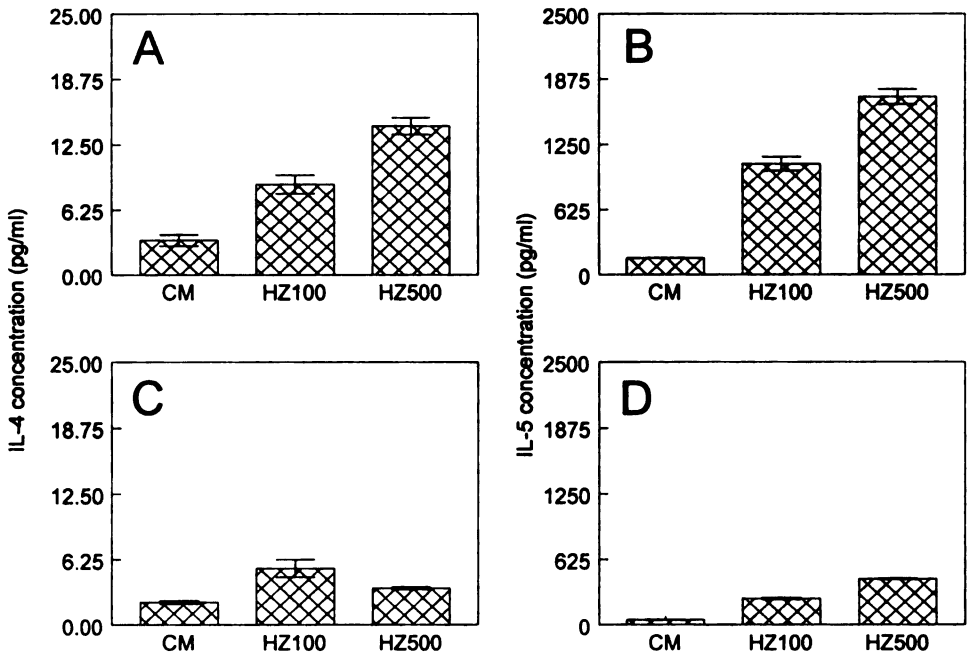

Figure 4.6.4 (A-D): Hazelnut specific Type-2 cytokine (IL-4 and IL-5) responses in mice.

Groups of C57Bl/6 mice (n=4) were sensitized with hazelnut (100 ug per mouse) plus alum or alum alone. Three days following a booster injection with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut or culture medium alone. Cell culture supernatants were harvested at different time points over 3 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from hazelnut plus alum sensitized mice (A, B) and alum alone injected mice (C, D). Student's *t*-test: For IL-4: Hazelnut 100 and 500 μg/mL vs. culture medium alone, $P<0.05$, Fig. A; For IL-5: hazelnut 100 and 500 μg/mL vs. culture medium alone; $p<0.05$, Fig. B; Student's *t*-test. Con-A was used as a positive control (At 24 hr: IL-4: 10.11 +/- 0.42 pg/mL; IL-5: 658.88 +/- 8.13 pg/mL).

Table 4.1 Hazelnut specific IgE antibody response in mice without the use of alum adjuvant*

Mouse Strain	Time Point	Dose of Hazelnut (μg/per mouse)		
		0	**100**	**1000**
BALB/c (n=4/group/dose)	Pre-immune**	≤20	≤20	≤20
	Post***	≤20	2560	160
C-57BL/6 (n=4/group/dose)	Pre-immune	≤20	≤20	≤20
	Post	≤20	≤20	≤20

* Groups of mice were injected i.p., with hazelnut at indicated doses six times over a four week period and antibody response was analyzed at multiple time points

** Antibody levels in pre-immune plasma

*** Peak antibody levels in the plasma noted following six injections of hazelnut protein extract is shown; error bars are not shown because antibody titers were identical

4.7 References

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CHAPTER FIVE

5.0 Characterization of the systemic immune response following transdermal hazelnut protein exposure in BALB/c mice

5.1 Abstract

Background: Previously we found that in BALB/c mice, transdermal exposure to hazelnut was capable of eliciting hazelnut specific (IgE) antibodies without the use of adjuvant.

Hypothesis, objectives, and approach: In this study we tested the hypothesis that transdermal application of hazelnut protein activates Type-2 cytokines, thus promoting hazelnut specific IgE formation. Groups of BALB/c mice were exposed to 4 different doses of hazelnut protein transdermally over a six-week period. Hazelnut specific antibody responses (IgE, IgG2a) were studied. Short-term antigen driven recall cytokine responses were assessed using spleen cells ex vivo.

Results: We confirmed our earlier finding, that exposure of BALB/c mice to LPS-free hazelnut—a model tree nut, without the use of adjuvant, elicits a robust systemic IgE response; the IgE response was dose-dependent and increased with boosting. Hazelnut specific IgG2a was also detectable and increased with boosting. A marked hazelnut protein driven recall IL-4 response by spleenocytes was found. Surprisingly, there was also evidence for a modest induction of IgG2a and IFN-γ responses.

Conclusion: Transdermal exposure to hazelnut protein without the use of adjuvant results in activation of IL-4 response leading to IgE antibody response.

5.2 Introduction

Mechanisms underlying food allergy sensitization are not completely understood. A major question still yet to be answered in food allergy is how are people sensitized to food allergens? Is the gastrointestinal tract the site of primary sensitization, or are other sites seeing allergen first? Since people as well as rodents usually develop oral tolerance to ingested proteins [1], what is causing this breakdown in oral tolerance, or is there one?

A recent study found a significant relationship of peanut allergy with the use of skin preparations for the treatment of diaper rashes, eczema, dry skin, and IFNlammatory skin conditions in IFNancy containing peanut oil (odds ratio, 6.8; 95 percent confidence interval, 1.4 to 32.9) [2]. Previous reports have shown that epicutaneous application of OVA can lead to a Th2 type of response with subsequent OVA-specific IgE [3]. With the skin being a possible being a new, emerging route of exposure and our previous data showing BALB/c mice can make IgE without adjuvant by high number of i.p. injections, we sought out to profile the immune response to transdermal hazelnut exposure.

In this study we tested the hypothesis that transdermal application of hazelnut protein activates IL-4 cytokine, thus promoting hazelnut specific IgE formation.

5.3 Materials and Methods

5.3.1 Materials

The following materials were purchased from sources as indicated in the parenthesis. Hazelnut/filbert protein extract (Greer Labs, Lenoir, NC, USA); hazelnut extract was tested for protein content by Lowry's method; LPS content of hazelnut protein extract was measure by LAL assay (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA); Biotin conjugated Rat anti-mouse IgE antibodies; paired antibodies and recombinant standards for mouse IL-4 and IFN-γ (BD PharMingen, San Diego, CA, USA); *p* nitro phenyl phosphate (Sigma, St Louis, MO, USA); Streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); PMA, inomycin (Sigma, St Louis, MO, USA).

5.3.2 Transdermal sensitization and bleeding

All mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). Only female mice (6-8 weeks age) were used in the study. All animal procedures used were in accordance with the Michigan State University policies. Transdermal exposure experiments were performed using the method described by us before (Chapter 4) [5]. The experimental protocol for the sensitization process is shown in Figure 5.1.

5.3.3 Measurement of hazelnut specific IgE antibodies and total IgE in the plasma

We have previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgE antibody analyses (Chapter 3) [6, 7].

5.3.4 Spleen cell culture and cytokine analyses

Spleen cells were harvested and standard cell cultures were setup essentially as described (Chapter4) [5, 8].

5.3.5 Statistical Analysis

Student's t-test and ANOVA tests were used to evaluate significance using Analyse-It™ software program (Analyse-It software Ltd, Leeds, UK). The statistical significance level was set at 0.05.

5.4 Results

5.4.1 Characterization of systemic hazelnut specific antibody responses following transdermal exposure to hazelnut protein in BALB/c mice

We used LPS-free preparations of hazelnut protein extract (Greer Labs) in this study LPS level as measured by LAL assay was at <0.4 pg/mg of protein. Mice were evaluated for the appearance of hazelnut binding IgE and IgG2a antibodies in the plasma before and after each transdermal exposure. Pre-immune serum had less than detectable hazelnut specific IgE antibodies (mean titer <1/20) as well as less than detectable levels of hazelnut specific IgG2a (mean titer <1/250). Following transdermal exposure with hazelnut (500 μg/mouse) but not saline, elevated levels IgE antibodies appeared after the third exposure (Figure 5.2). Measurable levels of hazelnut specific IgG2a were seen after the third exposure, but extremely modest until the sixth response (Figure 5.3). We next examined the dose-response and time-course of IgE antibody responses during transdermal exposure over a six-week period. As evident, a dose of 5 μg/mouse did not elicit detectable hazelnut specific IgE response, but IgE response was clearly evident with the 50 and 500 μg/mouse dose (Figure 5.4A). Further analysis of antibody titers revealed progressively higher memory IgE antibody responses (response from activation with the same allergen at a later time point). Furthermore, analysis of total serum IgE levels further confirmed elevated, dose and time dependent induction of allergic antibody response (Figure 5.4B).

5.4.2 Characterization of hazelnut driven recall cytokine (IL-4 and IFN-γ) responses following transdermal exposure to hazelnut in BALB/c mice

In order to study the mechanism of IgE response to hazelnuts, we examined both Type-2 cytokine (IL-4) responses and Type-1 cytokine (IFN-γ) responses in hazelnut-sensitized mice. As evident, hazelnut activated a Type-2 cytokine (IL-4) response in spleen cells isolated from hazelnut but not saline exposed mice (Figure 5.5). IL-4 response was dose dependent with spleen cells isolated from hazelnut-sensitized mice responding to 500 μg of hazelnut stronger than to 100 μg hazelnut ($p<0.05$)(Figure 5.5), whereas in spleen cells isolated from control mice no dose response was noted.

There was a background level of IFN-γ being produced by saline sensitized mice spleen cells exposed to hazelnut (Figure 5.6). Further analysis of the cytokine profile was done with determination of the IFN-γ/IL-4 ratio for the hazelnut sensitized mice. There was a push towards IL-4 in animals that were sensitized to hazelnut thus providing a mechanism to favor IgE antibody development (Figure 5.7).

5.5 Discussion

In this study we profiled hallmarks of allergenic immune responses (specific and total IgE, IL-4, and IFN-γ / IL-4 ratio) to transdermal hazelnut protein in BALB/c mice. There are several novel finding of these studies; i) Repeated transdermal exposure of BALB/c mice to hazelnut (in the absence of adjuvant) can activate systemic antibody response characterized by robust Type-2 dependent isotopes (IgE); ii) Transdermal exposure to hazelnut activates memory Type-2 cytokine (IL-4) responses; iii) Transdermal exposure to hazelnut alters the IFN-γ / IL-4 ratio in favor of IL-4, thus promoting Type-2 antibody development.

In a recent study Lack et al. [2] used data from the Avon Longitudinal Study of Parents and Children, a geographically defined cohort study of 13,971 preschool children, to first identify children with history of peanut allergy and then find association within them. From this, they found a significant relationship between peanut allergy and the use of skin emollients (creams) containing peanut oil for the treatment of diaper rashes, eczema, dry skin, and IFNlammatory skin conditions in IFNancy (odds ratio, 6.8; 95 percent confidence interval, 1.4 to 32.9 [2]. With the skin possible being a new, emerging route of exposure, our adjuvant free model of sensitization may be more physiologically relevant than it would have been in the past.

Wang et al. [3] for the first time found that epicutaneous allergen can induce a Th2 response, without the use of allergen. They exposed both C57BL/6J and BALB/c mice to various concentrations of OVA (10mg, 100μg and 1μg) by a patch method of applying a

patch with the allergen and securing it with an elastic bandage. They found that epicutaneous exposure to both strains of mice results in high OVA specific IgE levels. We found our low dose of hazelnut (5μg) in BALB/c failed to elicit a hazelnut specific IgE titer (levels of <10) at our low dose of hazelnut), whereas they had antibody titers of OVA specific IgE in BALB/c mice between 0 and 1800 for 1μg after 5 courses of immunization. Overall specific IgE levels in BALB/c mice were similar between our study and theirs, with our peak hazelnut antibody titer being 5120 at the fifth response in the 500 μg exposed group, whereas they saw a peak IgE antibody titer of around 5000 in their 100 μg exposed group.

In the OVA epicutaneous model a rather weak IgG2a response was noted (peak IgG2a titer of around 2000 at 4^{th} response in 100μg exposed group) from the BALB/c strain with very few animals making even detectable levels. They did not report on the C57BL/6 strain. Similarly, our model had a rather modest IgG2a response, with a peak IgG2a titer of 8000 at 6^{th} response in 500μg exposed BALB/c group.

The OVA epicutaneous model, they report a Th-2 predominant response, but there was only a 4-5picogram increase in IL-4 levels following OVA ex vivo stimulation. Our transdermal model shows a robust IL-4 response following ex vivo stimulation (around a 280picogram increase following hazelnut ex vivo stimulation). One reason for this difference could be due to the cell type, spleen cells in our model vs. lymph node cells in the OVA model. Also in the OVA model cells were collected only 24 hours after stimulation, whereas our culture is done over 5 days and peak cytokine response is

reported. IFN-γ responses in both models were assessed. Two courses of OVA epicutaneous stimulation lead to a barely detectable level of IFN-γ from ex vivo lymph node cell stimulation with OVA, whereas spleen cell stimulation with hazelnut following six courses of transdermal hazelnut exposure gave a more modest hazelnut specific IFN-γ level of around 500picograms. Differences here also could be due to cell type used or a lack of time course analysis in the OVA model in both the collection of cell culture supernatants and when cells were harvested for culture (only after 2 immunizations).

Although groundbreaking work, showing that epicutaneous allergen can sensitize for a Th2 cytokine (IL-4) and IgE response, further analysis of IL-4 to IFN-γ ratio would have been beneficial to assess the effect of the doses of OVA and cytokine profile leading to allergy.

In summary, we found that transdermal sensitization of mice without the use of adjuvant results in activation of hazelnut driven IL-4 response leading to IgE antibody response. Surprisingly, there was also evidence for a modest induction of IgG2a and IFN-γ responses.

5.6 Tables and Figures

Figure 5.1

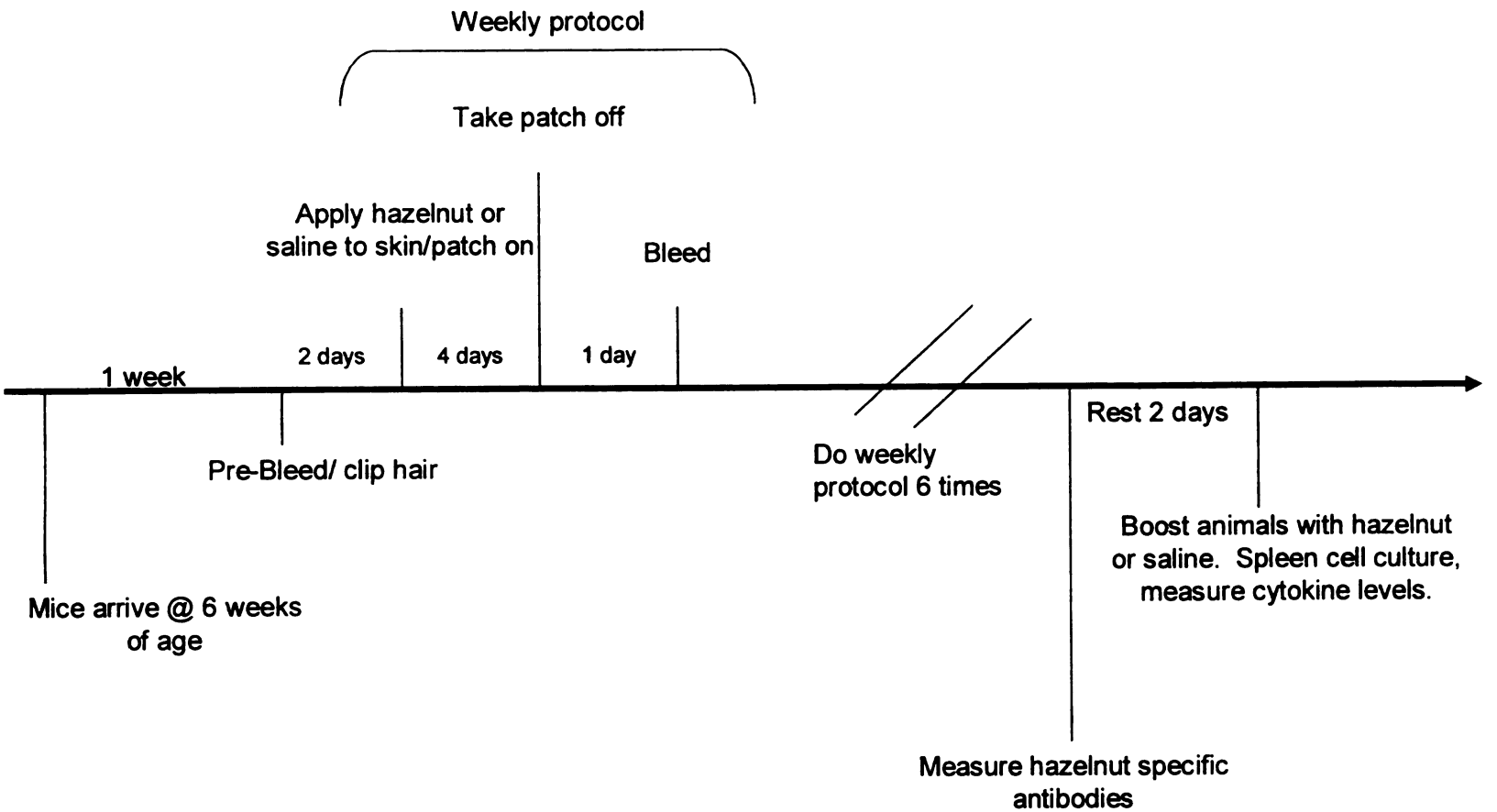

Figure 5.1: Transdermal sensitization protocol: The schedule of cycles of hazelnut sensitization is shown.

Figure 5.2

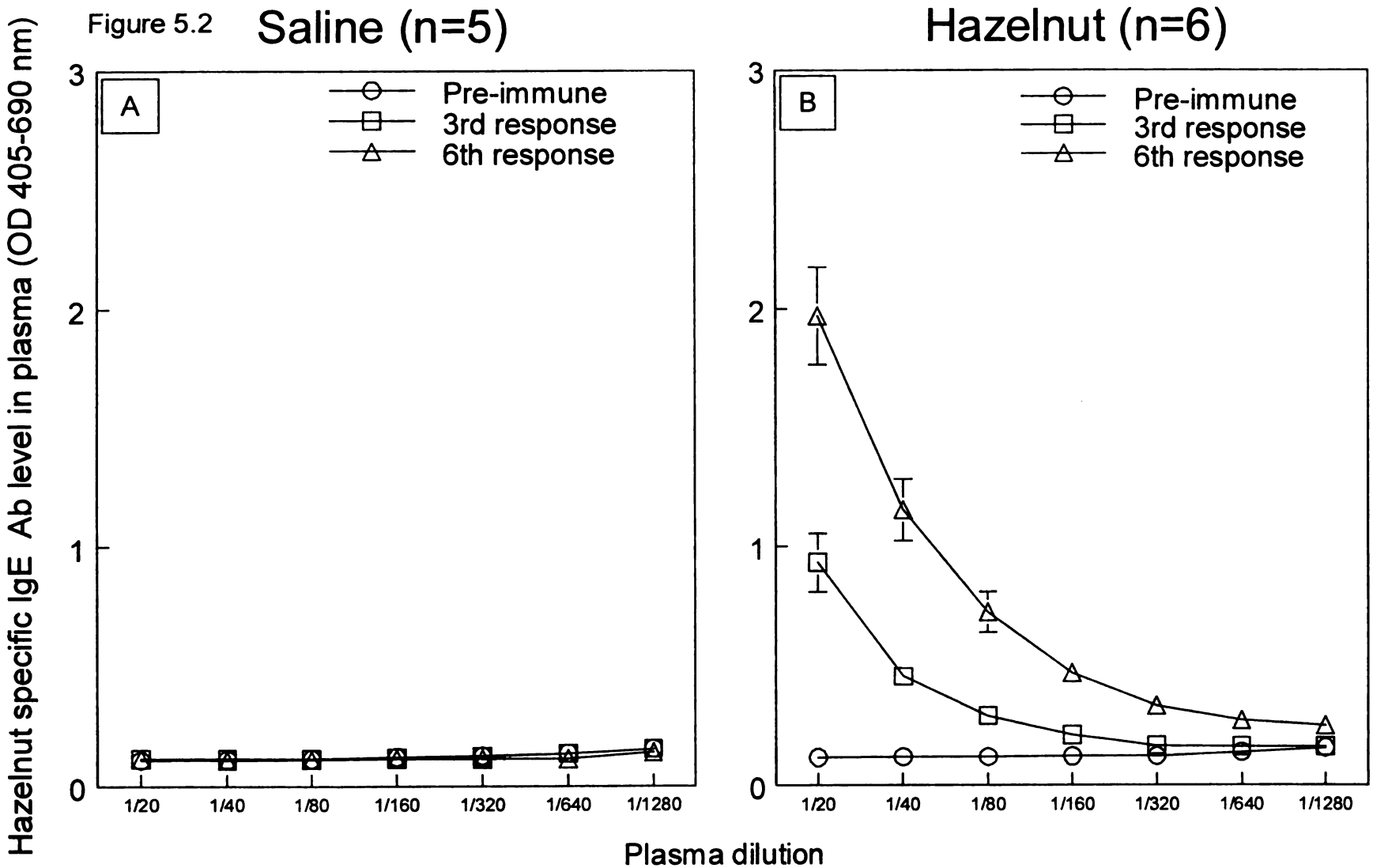
Saline (n=5)
Hazelnut (n=6)
A
B
Pre-immune
3rd response
6th response
Hazelnut specific IgE Ab level in plasma (OD 405-690 nm)
0
1
2
3
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
Plasma dilution

Figure 5.2 (A-B): Characterization of hazelnut specific IgE antibody responses in BALB/c mice following repeated transdermal exposure to hazelnut. Groups of mice (n=5-6/group) were transdermaly exposed to 500 μg/mouse of LPS-free hazelnut protein extracts or saline and hazelnut binding specific IgE (Figs. A, B) antibodies were measured by optimized ELISA. Pre-immune: plasma collected before exposure; 3rd response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures. Data shows hazelnut specific antibody isotype levels as OD (mean +/- SE). At many points error bars are not visible.

Figure 5.3

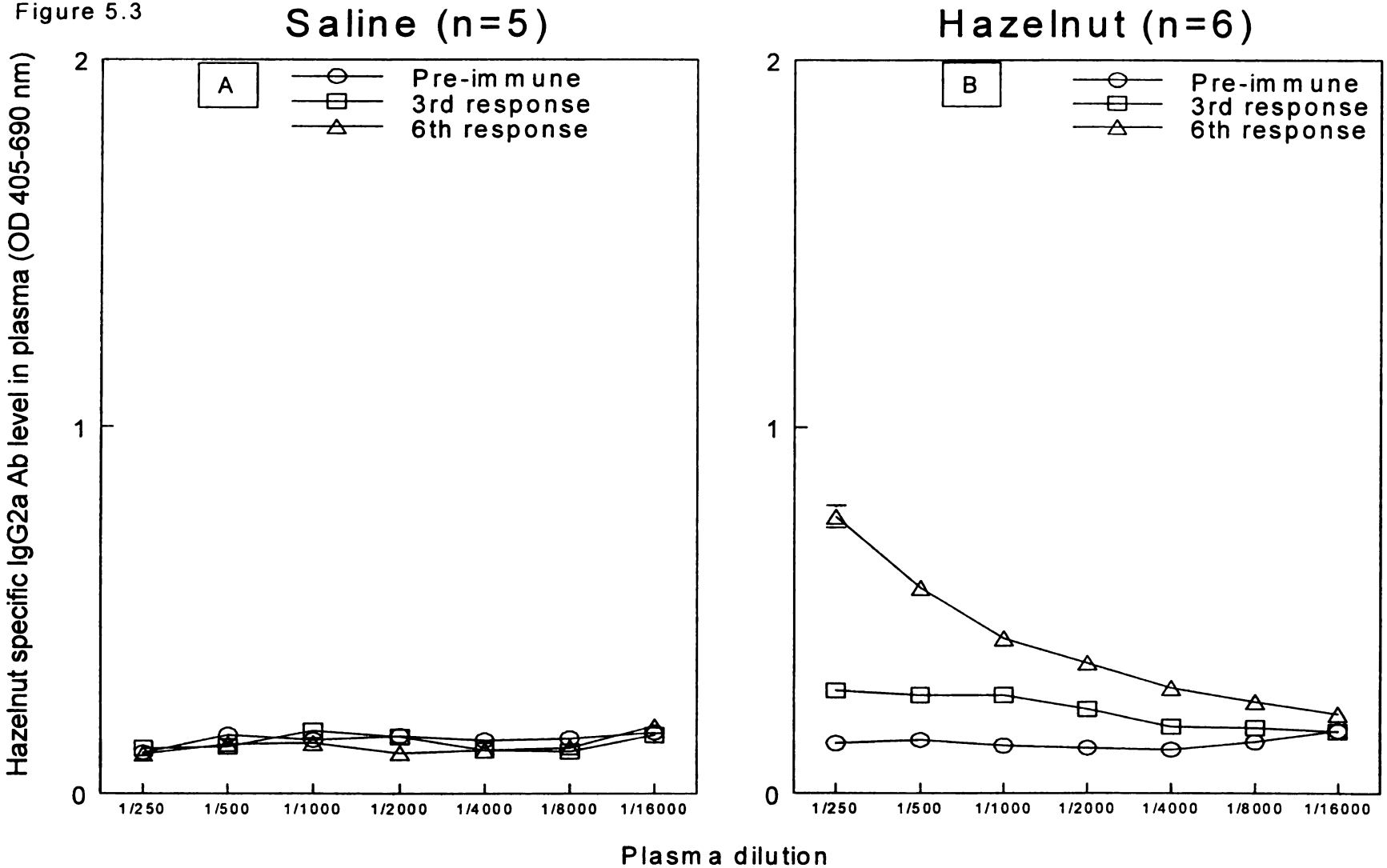
Saline (n=5)
Hazelnut (n=6)
A
B
Pre-immune
3rd response
6th response
Hazelnut specific IgG2a Ab level in plasma (OD 405-690 nm)
2
1
0
1/250
1/500
1/1000
1/2000
1/4000
1/8000
1/16000
Plasma dilution

Figure 5.3 (A-B): Characterization of hazelnut specific antibody IgG2a antibody responses in BALB/c mice following repeated transdermal exposure to hazelnut. Groups of mice (n=5-6/group) were transdermaly exposed to 500 μg/mouse of LPS-free hazelnut protein extracts or saline and hazelnut binding specific IgG2a (Figs. A, B) antibodies were measured by optimized ELISA. Pre-immune: plasma collected before exposure; 3rd response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures. Data shows hazelnut specific antibody isotype levels as OD (mean +/- SE). At many points error bars are not visible.

Figure 5.4

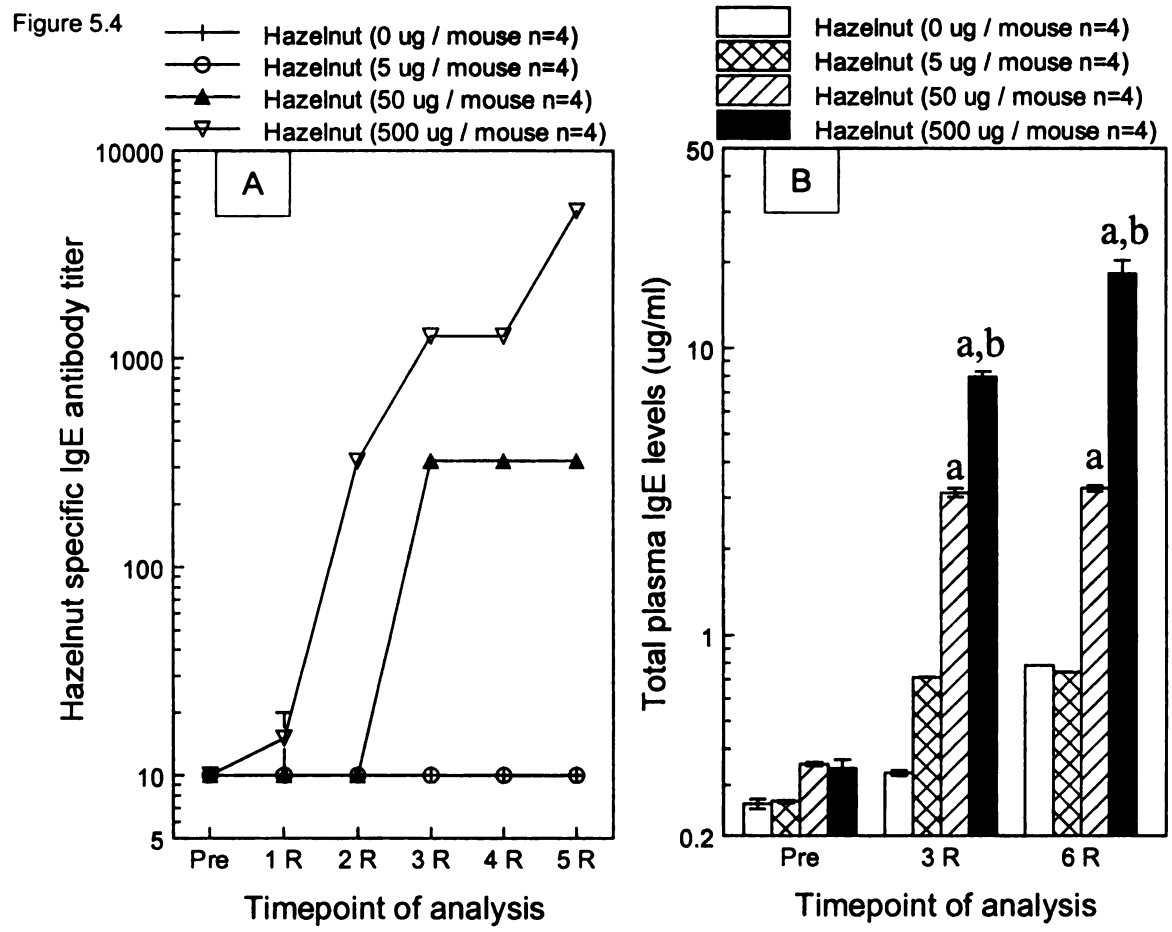

Figure 5.4 (A & B): Dose-response and time-course analyses of IgE antibody response to transdermal hazelnut exposure in BALB/c mice. Groups of mice (n=4/group) were transdermally exposed to indicated dose of hazelnut protein extract (0, 5, 50 or 500 μg/mouse) and hazelnut binding specific IgE antibody titer (Fig. A; Data shown is geometric mean +/- SE) and total plasma IgE (Fig. B) levels were measured over a six week period. Where titers were identical error bars are not shown. AVOVA test results: Panel B, a= $p<0.001$ vs. hazelnut 0 group at same time point; b= $p<0.001$ vs. all other hazelnut doses at the same time point.

Figure 5.5

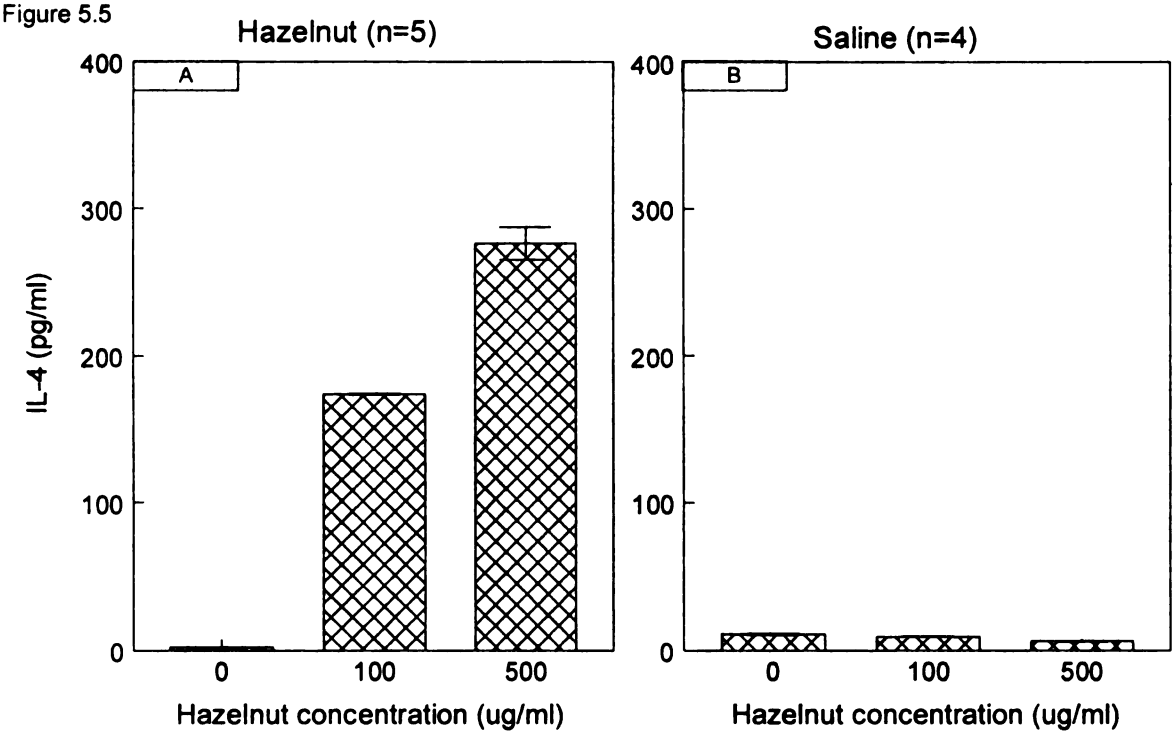

Figure 5.5 (A-B): Hazelnut driven Type-2 cytokine (IL-4) responses in mice transdermally sensitized with hazelnut or saline.

Groups of BALB/c mice (n=4-5/group) were sensitized with hazelnut (500 μg per mouse) (Figs. A) or saline (Figs. B) by transdermal exposure as described in Figure 5.1. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from hazelnut-sensitized mice (A) and saline exposed mice (B). ANOVA test: graph A; for all comparisons between 100 and 0 and 500 and 0: $p<0.01$).

Figure 5.6

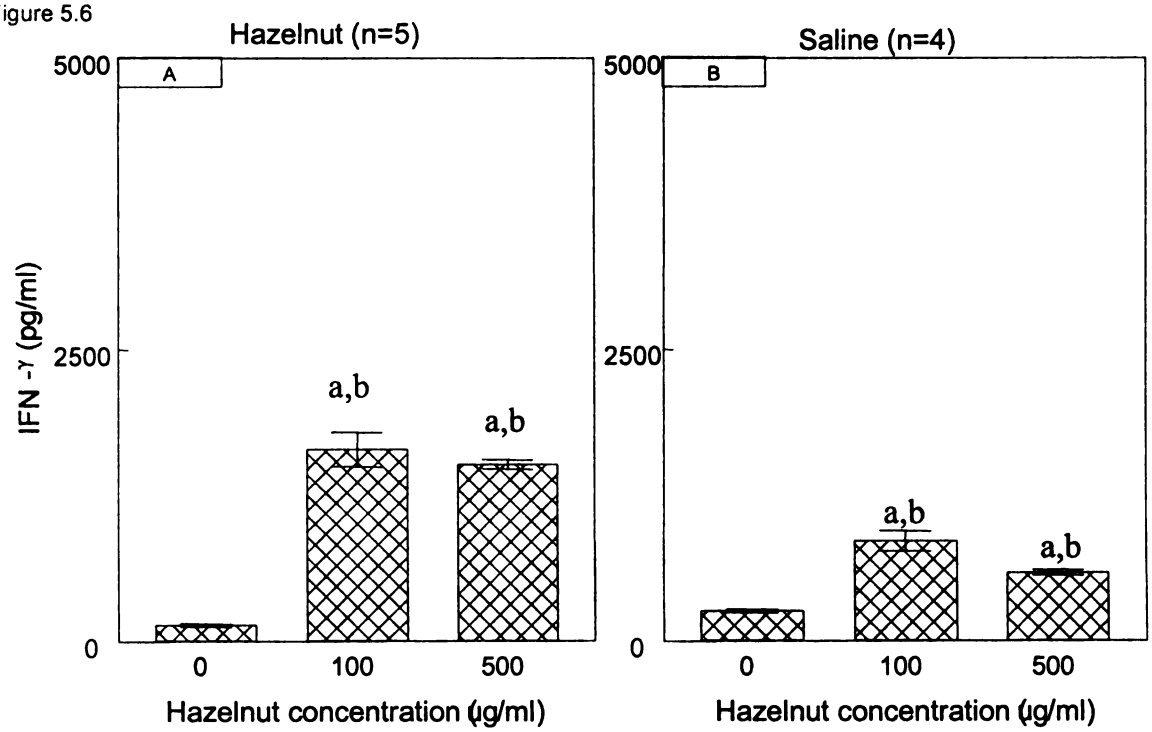

Figure 5.6 (A-B): Hazelnut driven Type-1 cytokine (IFN-γ) in mice transdermally sensitized with hazelnut or saline.

Groups of BALB/c mice (n=4-5/group) were sensitized with hazelnut (500 µg per mouse) (Figs. A) or saline (Figs. B) by transdermal exposure as described in Figure 5.1. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is Day 3 of cell culture (average +/- SE of duplicate analyses of data) from hazelnut-sensitized mice (A) and saline exposed mice (B). ANOVA test: Graph A and B, a=$p<0.01$ vs. 0 hazelnut group at same time point; b=$p<0.01$ vs. same dose of hazelnut in other panel.

Figure 5.7

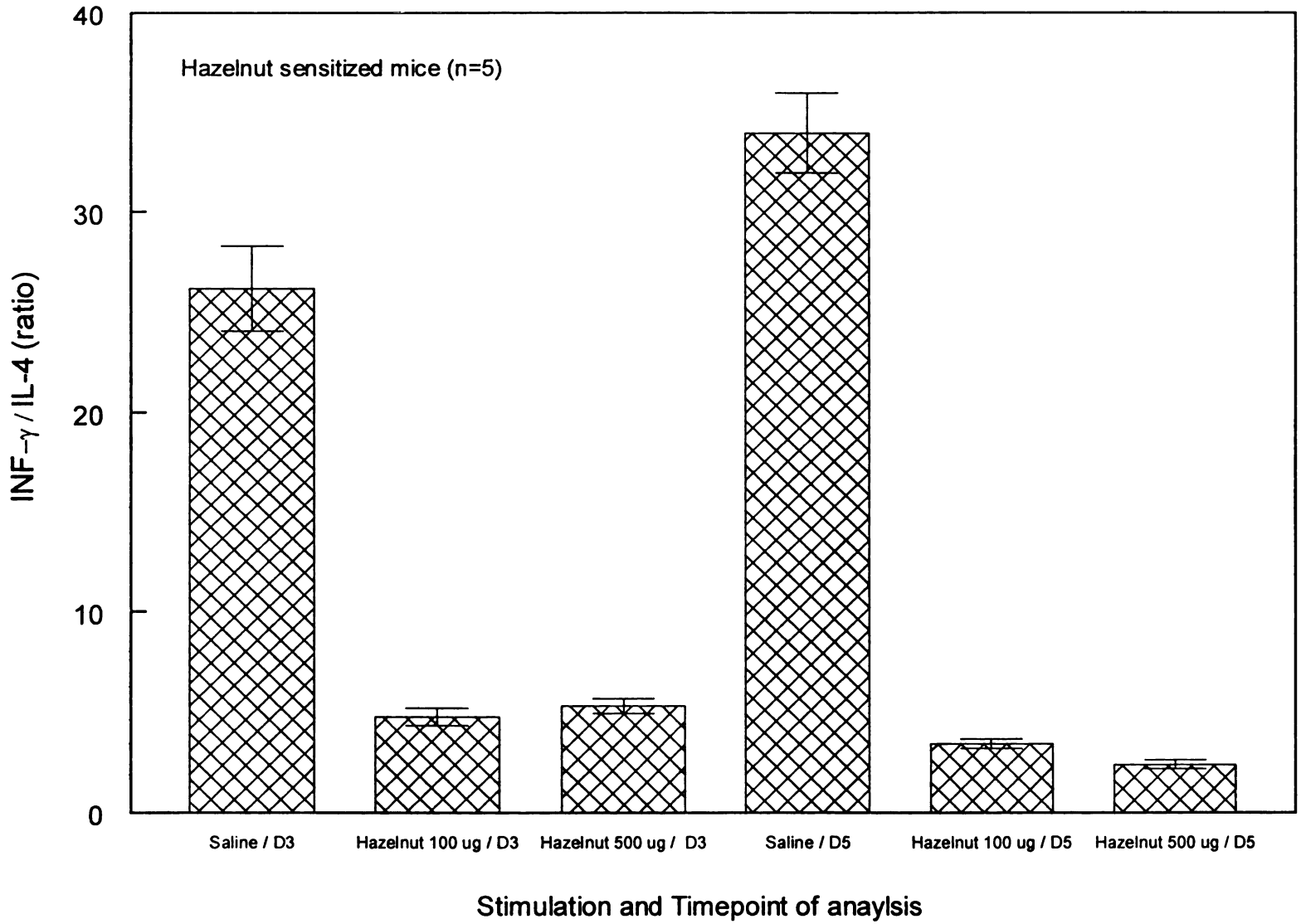
Hazelnut sensitized mice (n=5)
INF-γ / IL-4 (ratio)
40
30
20
10
0
Saline / D3
Hazelnut 100 ug / D3
Hazelnut 500 ug / D3
Saline / D5
Hazelnut 100 ug / D5
Hazelnut 500 ug / D5
Stimulation and Timepoint of anaylsis

Figure 5.7: IFN-γ / IL-4 ratio in mice transdermally sensitized with hazelnut.

Groups of BALB/c mice (n=5/group) were sensitized with hazelnut (500 μg per mouse) by transdermal exposure as described in Figure 5.1. Three days following a booster exposure with hazelnut, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone (saline). Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is D3 of IFN-γ / D3 of IL-4 response and D5 of IFN-γ / D5 of IL-4 for saline, 100 μg and 500 μg hazelnut stimulation (average +/- SE of duplicate analyses of data). ANOVA test results: $p<0.05$ for both 100 and 500 μg hazelnut stimulation vs. its same day saline control.

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7. Birmingham, N., et al., *An ELISA-based method for measurement of food-specific IgE antibody in mouse serum: an alternative to the passive cutaneous anaphylaxis assay.* J Immunol Methods, 2003. **275**(1-2): p. 89-98.

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CHAPTER SIX

6.0 Development of an adjuvant-free model of tree-nut protein induced systemic anaphylaxis

6.1 Abstract

Background: Our previous studies indicated that transdermal application of LPS free hazelnut in the absence of adjuvant can lead to the development of hazelnut specific allergic (IgE) antibodies *via* activating IL-4 cytokine. Food allergy is not only having hallmarks of allergy, but also having an adverse response after challenge (disease). A mouse model of tree nut induced systemic anaphylaxis, the focus of this study, was unavailable.

Hypothesis, objectives, and approach: Here we tested the hypothesis whether transdermal application of hazelnut can prime BALB/c mice for systemic anaphylaxis when challenged either systemically (i.p.) or orally. Further testing to assess histamine release after challenge (i.p.) and pathological changes in the gastrointestinal tract were also done.

Results: Repeated transdermal exposure of BALB/c mice to hazelnut, again, elicited a robust systemic IgE Ab response. Transdermal sensitization also primed for systemic anaphylaxis as evidenced by dose-dependent clinical scores, marked drop in rectal temperature and elevated histamine levels with 0.1 mg/mouse being the threshold dose when challenged by i.p. injection. Following oral challenge (13 mg/mouse) with hazelnut, sensitized mice showed several signs of systemic anaphylaxis (diarrhea,

hypothermia, convulsions). Tissues from the gastrointestinal tract were collected and fixed with 10% formalin in PBS and stained with hematoxylin-eosin. In hazelnut-sensitized mice that underwent systemic anaphylaxis, the small intestine had marked edema, vascular congestion and enterocyte sloughing, whereas in saline control mice, no pathological lesions were noted.

Conclusion: We have developed an adjuvant-free mouse model of tree nut induced systemic anaphylaxis that should serve as a useful tool not only to elucidate further mechanisms of this disease but also to develop improved therapeutic and/or prophylactic methods.

6.2 Introduction

In the past several years, a number of interesting mouse models --often using adjuvants such as cholera toxin in the experimental protocols, to study food allergies have been reported. These include peanut, egg and milk allergies [1-3]. Unlike human food allergy, these models used cholera toxin to break down oral tolerance and achieve their food allergy state. After sensitization, animals are challenged with intra-gastric allergen and systemic anaphylaxis occurs.

Previously, we found that transdermal application of LPS free hazelnut in the absence of adjuvant can lead to the development of hazelnut specific allergic (IgE) antibodies *via* activating Type-2 cytokines. However, a mouse model of tree nut induced systemic anaphylaxis with concurrent pathology was not available. Therefore, we here tested the hypothesis that transdermal sensitization of BALB/c mice to hazelnut primes for systemic anaphylaxis.

In this study we report on systemic anaphylaxis and pathological changes seen in an adjuvant free model of tree nut allergy. Following transdermal sensitization mice undergo systemic anaphylaxis reactions following i.p. or oral challenge with allergen. With the current study, pathological changes were noted in the small intestine after oral challenge. In summary, we have developed and characterized an adjuvant-free mouse model of tree nut induced systemic anaphylaxis that should serve as a useful tool for further mechanistic and applied studies.

6.3 Materials and Methods

6.3.1 Materials

Materials were used as previously described in chapters 4 and 5.

6.3.2 Transdermal sensitization, bleeding, and challenge

Sensitization procedure was done as previously described in chapter 4. The experimental protocol for the sensitization and challenge process is shown in Figure 6.1. Tables 6.1 and 6.2 show the number of animals in each sensitization group. Following challenge animals were bleed for plasma histamine determination, observed for clinical scores of systemic anaphylactic responses and had rectal temperature taken.

Table 6.1 I.P. challenge: study design

Group ID	Number of mice	Sensitized transdermally with	Challenged i.p. with
1	5	Saline	Saline
2	5	Saline	Hazelnut
3	5	Hazelnut	Saline
4	5	Hazelnut	Hazelnut

Table 6.2 Oral gavage challenge: study design

Group ID	Number of mice	Sensitized transdermally with	Challenged orally with
1	16	Saline	Saline
2	16	Saline	Hazelnut
3	16	Hazelnut	Saline
4	16	Hazelnut	Hazelnut

6.3.3 Induction of systemic anaphylaxis, clinical scoring, measurement of rectal temperature

Groups of hazelnut sensitized vs. saline exposed mice challenged i.p. or orally with indicated amount of hazelnut protein or saline. Mice were then observed for signs of systemic anaphylaxis and video-recorded for symptoms during the next 4 hours. Clinical scoring (on a scale of zero to 5) was performed by 3 to 4 individuals in a blinded manner according to the method described previously [1]. Score of 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsion; and 5, death. Rectal temperature was measured using a temperature probe (Yellow Springs Instrument Co., Yellow Springs, OH, USA) at indicated time points before and after challenge.

6.3.4 Histamine measurement

Plasma was collected 30 minutes after the i.p. challenge by cardiac puncture. Samples were stored at -80 $^{\circ}$ C. Histamine content was measured using a Histamine ELISA Kit developed by Neogen Inc. (A kind gift from Dr. Paul Satoh, Neogen Inc., Lansing, MI).

6.3.5 Pathological changes

Histological changes in the gastrointestinal tract were evaluated 1hr after one oral challenge. Mice transdermally sensitized with hazelnut protein and saline control mice challenged with hazelnut were sacrificed 1 hour after oral challenge by CO2 euthanasia. Tissues from the gastrointestinal tract were collected for histological analysis. The gastrointestinal tract was divided into sections (esophagus, stomach, duodenum, jejunum, ileum, and colon) and fixed in 10% neutral buffered formalin for 4 days. Of the sixteen animals per group, six of them had their tissues taken for further analysis. Fixed tissues were embedded in paraffin and cut into 3-5 μm thick sections and mounted on glass slides (done by the Michigan State University Histology Lab). Slides were then stained with hematoxylin-eosin (done by the Michigan State University Histology Lab) and analyzed for histological changes by light microscopy. Each mouse tissue had one slide prepared with both a longitudinal and cross-section. Therefore, there were six slides for each of the six animals per group (esophagus, stomach, duodenum, jejunum, ileum, and colon).

6.3.6 Measurement of hazelnut antibodies in the plasma

We have previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgE antibody analyses (chapter 3) [4, 5].

6.3.7 Statistical Analysis

Student's t-test, ANOVA, Mann Whitney, and the Kruskal-Wallis tests were used to evaluate significance using Analyse-It™ software program (Analyse-It software Ltd, Leeds, UK). The statistical significance level was set at 0.05.

6.4 Results

6.4.1 Conformation of hazelnut specific antibody responses to transdermal exposure in BALB/c mice

Mice were evaluated for the appearance of hazelnut binding allergic (IgE) antibodies before and after each transdermal exposure. We used LPS-free preparations of hazelnut protein extract (Greer Labs) in this study LPS level as measured by LAL assay was at <0.4 pg/mg of protein. As previously seen, pre-immune serum had no detectable hazelnut specific IgE antibodies (mean titer <1/20). Similar to what we had previously seen, following transdermal exposure with hazelnut (500 μg/mouse) but not saline, elevated levels of IgE antibodies were noted (Table 6.3).

Table 6.3 Hazelnut specific IgE antibody titers in BALB/c mice pre-immune and at 6th response.

Challenge	Group	n=	Pre-immune	After 6th response
I.P.	Saline transdermal/ Saline I.P.	5	<20	<20
I.P.	Saline transdermal/ Hazelnut I.P.	5	<20	<20
I.P.	Hazelnut transdermal/ Saline I.P.	5	<20	7500 +/-650
I.P.	Hazelnut transdermal/ Hazelnut I.P.	5	<20	8300+/-280
Oral	Saline transdermal/ Saline oral	16	<20	<20
Oral	Saline transdermal/ Hazelnut oral	16	<20	<20
Oral	Hazelnut transdermal/ Saline oral	16	<20	8600+/-570
Oral	Hazelnut transdermal/ Hazelnut oral	16	<20	8200+/-610

*Titer is shown as geometric mean +/- SE.
** Pre-immune= Plasma from naïve mice prior to sensitization.
*** 6th response is plasma from mice following six courses of transdermal hazelnut exposure.

6.4.2 Transdermal exposure to hazelnut protein sensitizes BALB/c mice for systemic anaphylaxis: Determination of threshold challenge dose

Hazelnut sensitized and saline exposed control mice were challenged systemically (i.p.) with hazelnut or saline in a crisscross manner and observed for clinical signs of systemic anaphylaxis during the next 4 hours. Hazelnut but not saline control mice exhibited signs of systemic anaphylaxis (convulsions, hypothermia, labored breathing) that were scored as described in the methods section (Figure 6.2). As evident, a significant difference in the clinical score was seen between hazelnut sensitized vs. saline control mice both challenged with hazelnut (Figure 6.2; mean score: HN group 3.67 +/-0.21; saline group: 0.0 +/- 0.0). Opposite challenge confirmed the need for prior-sensitization for systemic anaphylaxis. We then examined rectal temperature as an additional indicator of systemic anaphylaxis. As evident, hazelnut sensitized but not control mice exhibited a significant drop in rectal temperature at 30 minutes (Figure 6.3). Furthermore, there was significant elevation of systemic histamine levels at 30 minutes following systemic challenge with hazelnut but not saline (Figure 6.4).

We then conducted a detailed study to determine the threshold of systemic dose of hazelnut required to induce systemic anaphylaxis in previously sensitized mice. As evident, 100 μg/mouse was sufficient to induce signs of systemic anaphylaxis ($p<0.01$; Figure 6.5, 6.6), whereas a dose of 10 μg/mouse failed to induce any signs of systemic anaphylaxis.

6.4.3 Transdermal exposure to hazelnut primes BALB/c mice for systemic anaphylaxis when orally challenged

Hazelnut sensitized mice and saline control mice were challenged orally with hazelnut (13 mg) and observed for clinical signs of systemic anaphylaxis. Following oral challenge with hazelnut, hazelnut sensitized mice underwent systemic anaphylaxis, as evident by convulsions, systemic anaphylactic scores (Figure 6.7) and diarrhea, whereas saline control mice had no signs of anaphylaxis. Similar to systemic anaphylactic scores, hazelnut sensitized mice challenged with hazelnut had a marked drop in rectal temperature when comparing to saline control mice challenged with hazelnut, indicating systemic anaphylaxis (Figure 6.8).

6.4.4 Transdermal exposure to hazelnut protein sensitizes BALB/c mice for pathological changes in the small intestine when challenged orally with hazelnut

Hazelnut and saline exposed control mice were challenged orally one time with 13 mg of hazelnut. Hazelnut sensitized mice underwent systemic anaphylaxis; whereas saline exposed mice had no such effect. Tissues (esophagus, stomach, duodenum, jejunum, ileum, and colon) were collected and fixed for pathological analysis. During tissue collection severe edema of the small intestine was noted in hazelnut-sensitized mice following oral challenge. There were marked pathological changes seen in mice that were sensitized with hazelnut. Severe edema, vascular congestion and enterocyte sloughing was seen in all three areas of the small intestine (duodenum, jejunum, ileum) when compared to saline control mice (Figure 6.11, 6.12, 6.13 A-B). Mild edema was noted in the esophagus of hazelnut-sensitized mice when compared to saline control mice

(Figure 6.9). No lesions were noted in the stomach or colon (Figure 6.10, 6.14 A-B) at this time point.

6.5 Discussion

In this study we attempted to further our model of tree-nut allergy by profiling the systemic anaphylactic responses (symptom scores, rectal temperature, plasma histamine) and pathological changes of the gastrointestinal tract associated with tree-nut allergy. The finding of these studies are i) Repeated transdermal exposure can sensitize mice for systemic anaphylaxis (symptom scores, increased plasma histamine, decreased rectal temperature) to hazelnut when challenged with an i.p. injection; ii) Repeated transdermal exposure can sensitize mice for systemic anaphylaxis (symptom scores, decreased rectal temperature, diarrhea) to hazelnut when challenged orally; and iii) Marked pathological changes are seen in the small intestine at 1 hour after oral challenge with hazelnut in mice transdermally sensitized mice.

To our knowledge, there is currently is not a validated mouse model of tree-nut allergy. In the recent years, parallel with our studies, efforts to develop animal model of tree-nut allergy have been reported. One that has been developed uses the atopic dog as a model for peanut and tree-nut allergy [6]. This model has both strengths and weaknesses. One strength is that the dog is one of the few species other than humans in which allergies develop naturally on normal environmental exposure to a broad spectrum of allergens, including pollens, house dust mites, human dander, fleas, and foods [7-9]. Also, the dog can respond both vomiting and diarrhea in response to oral challenge due to food allergy [6, 10]. A key weakness is the high expense of dog studies, having high enough n numbers in studies, and having knockout strains / molecular reagents available to study mechanisms. Also, in this model animals are injected with an adjuvant (Alum), multiple

allergens are injected at a time (ex. Brazil nut, wheat, and soy all injected together) and requires 2.5 years before animals are challenged to allergen, delaying scientific progress.

The only mouse based model of tree-nut allergy proposed uses anti-ulcer drugs to promote hypersensitivity [11]. Here they report hazelnut specific IgG1, but no detectable levels of IgE when mice were orally fed hazelnut extract with a pretreatment of anti-ulcer drugs. Although oral sensitization is highly sought after, here without adjuvant (anti-ulcer) there is no response and mice develop oral tolerance. They claim that this treatment did sensitize mice for type I skin reactivity to hazelnut extract, as evidenced by passive cutaneous anaphylaxis (PCA) reactions using naïve mice and hazelnut specific IgG1 purified from plasma from the anti-ulcer group of mice. Here they do show that the IgG1 specific to hazelnut is anaphylactogenic in naïve animals when concentrated, but do not show if there is an in vivo consequence after challenge. In mice there are two distinct mechanisms that can induce anaphylaxis [12]. The first is the classical IgE-mast cell mediated pathway that is associated with human allergy and the second is IgE independent, using IgG and macrophages. Although a novel and important finding, this model does not follow classical allergy mechanisms, showing allergen specific IgE, therefore it is not a validated tree-nut allergy model. Therefore a model having the classical immune markers seen in human allergy (IgE) is desirable.

Limitations of our study are that only a single dose of allergen was used in the oral challenge study. A further profile of other doses could show a threshold dose with milder

symptoms. Plasma histamine could not be studied in the oral challenge model because of difficulty in bleeding mice following oral challenge.

In summary, this method of sensitization can prime BALB/c mice for systemic anaphylaxis when challenged systemically (i.p.) or orally with hazelnut protein. This should serve as a useful tool not only to study further mechanisms of tree nut induced systemic anaphylaxis but also to develop improved methods of treating/preventing this immune mediated, potentially fatal, disorder.

Figures 6.6

Figure 6.1

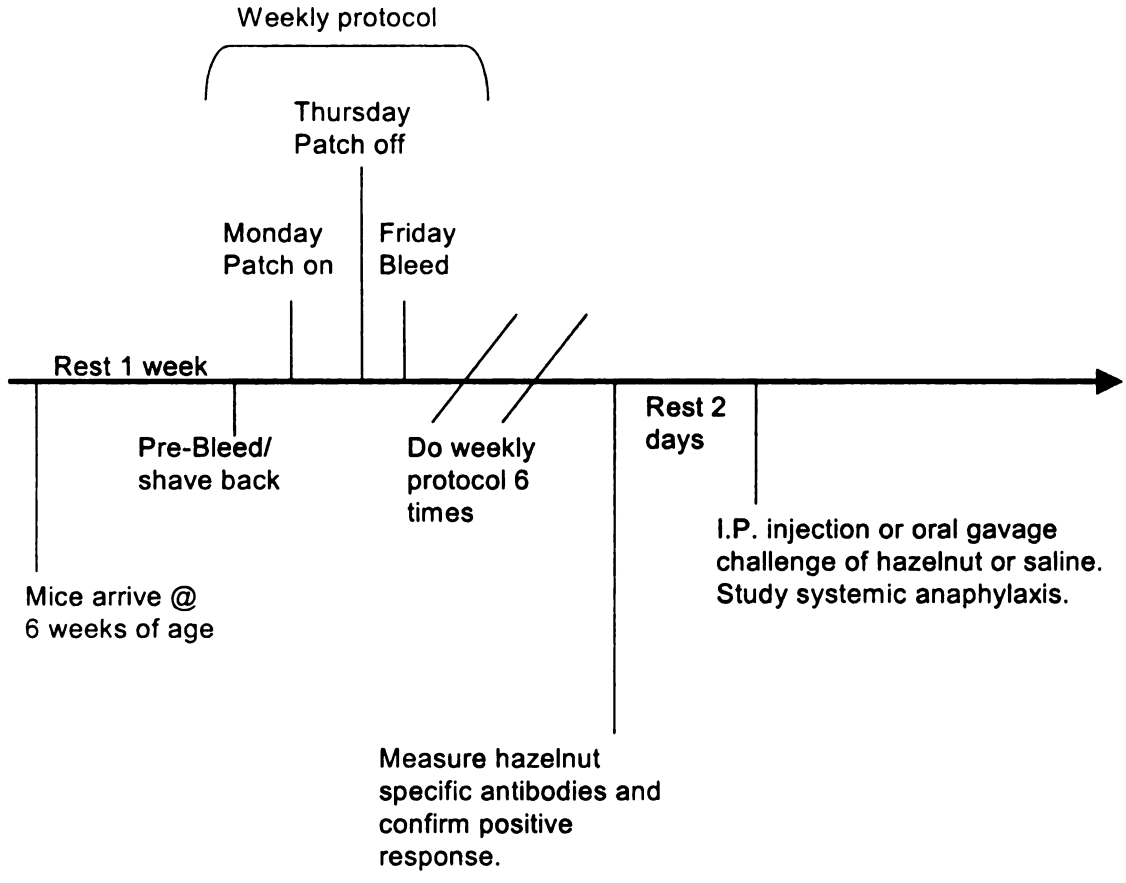

Figure 6.1: Transdermal sensitization protocol for systemic anaphylaxis to hazelnut.

The schedule of cycles of hazelnut sensitization and testing for systemic anaphylaxis are shown.

Figure 6.2

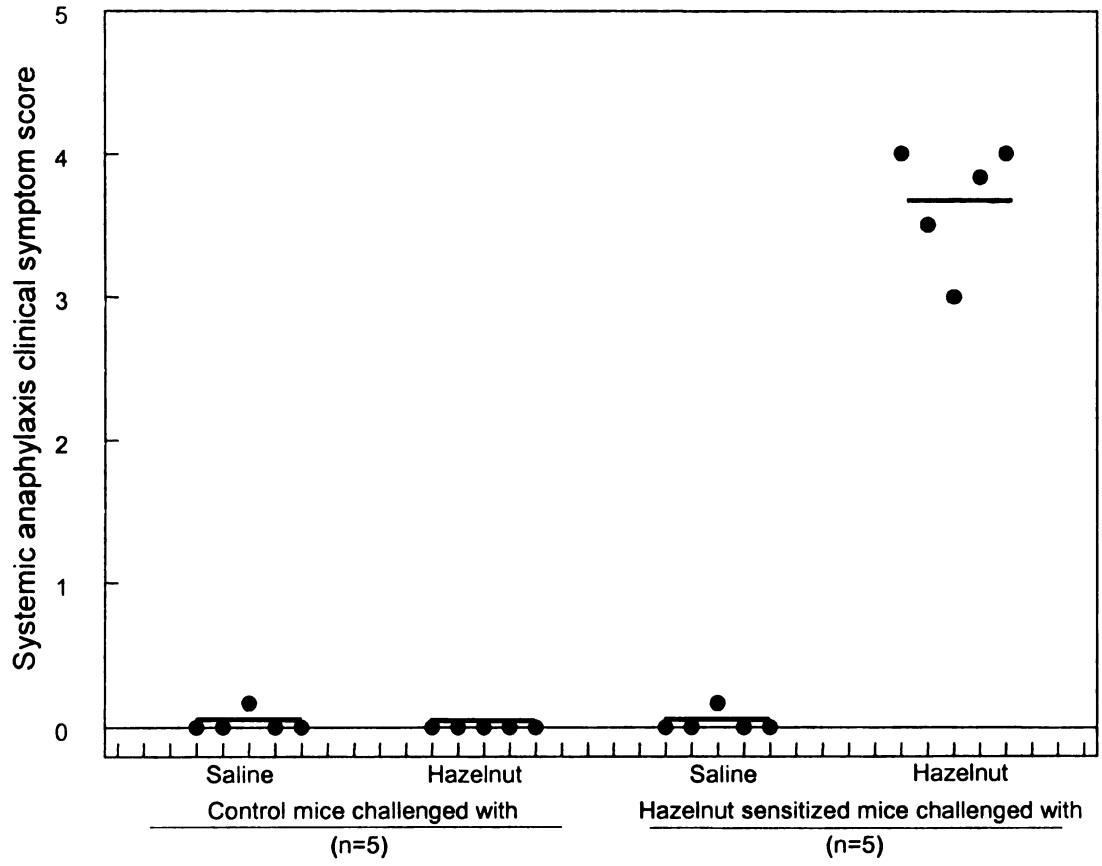

Figure 6.2 Transdermal exposure to hazelnut sensitizes BALB/c mice for systemic anaphylaxis when challenged by i.p. injection: Clinical symptom scores

Groups of BALB/c mice (n=5/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then exposed to hazelnut (1,000 μg/mouse) or saline systemically (i.p. injections) and examined for indicators of systemic anaphylaxis. Clinical symptoms are shown as a scatter plot with each symbol representing one mouse. Mann-Whitney test results: hazelnut sensitized mice with hazelnut challenge $p<0.05$ vs. all other groups.

Figure 6.3

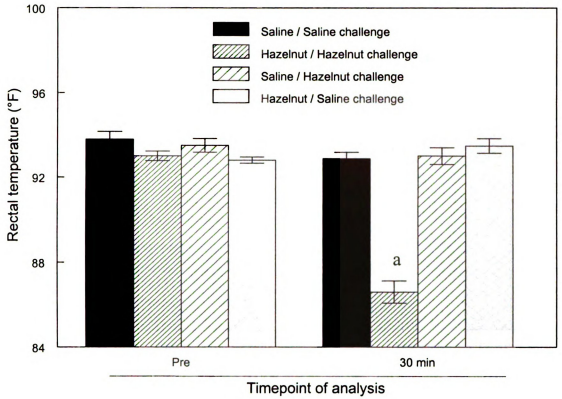

Figure 6.3 Transdermal exposure to hazelnut sensitizes BALB/c mice for systemic anaphylaxis when challenged by i.p. injection: Rectal temperature

Groups of BALB/c mice (n=5/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then exposed to hazelnut (1,000 μg/mouse) or saline systemically (i.p. injections) and examined for indicators of systemic anaphylaxis. Changes in rectal temperature are shown. Data shown is mean +/- SE. Kruskal-Wallis test results: $P<0.001$ vs. all other groups.

Figure 6.4

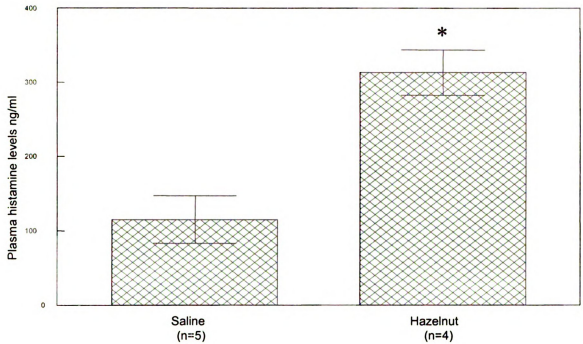

Figure 6.4 Transdermal exposure to hazelnut sensitizes BALB/c mice for systemic anaphylaxis when challenged by i.p. injection: Plasma histamine levels

Groups of BALB/c mice (n=5/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then exposed to hazelnut (1,000 µg/mouse) or saline systemically (i.p. injections) and examined for indicators of systemic anaphylaxis. Plasma histamine profile at 30 minutes following systemic exposure to hazelnut or saline is shown as mean +/- SE. Student's t-test results: * = $p<0.001$ for saline vs. hazelnut.

Figure 6.5

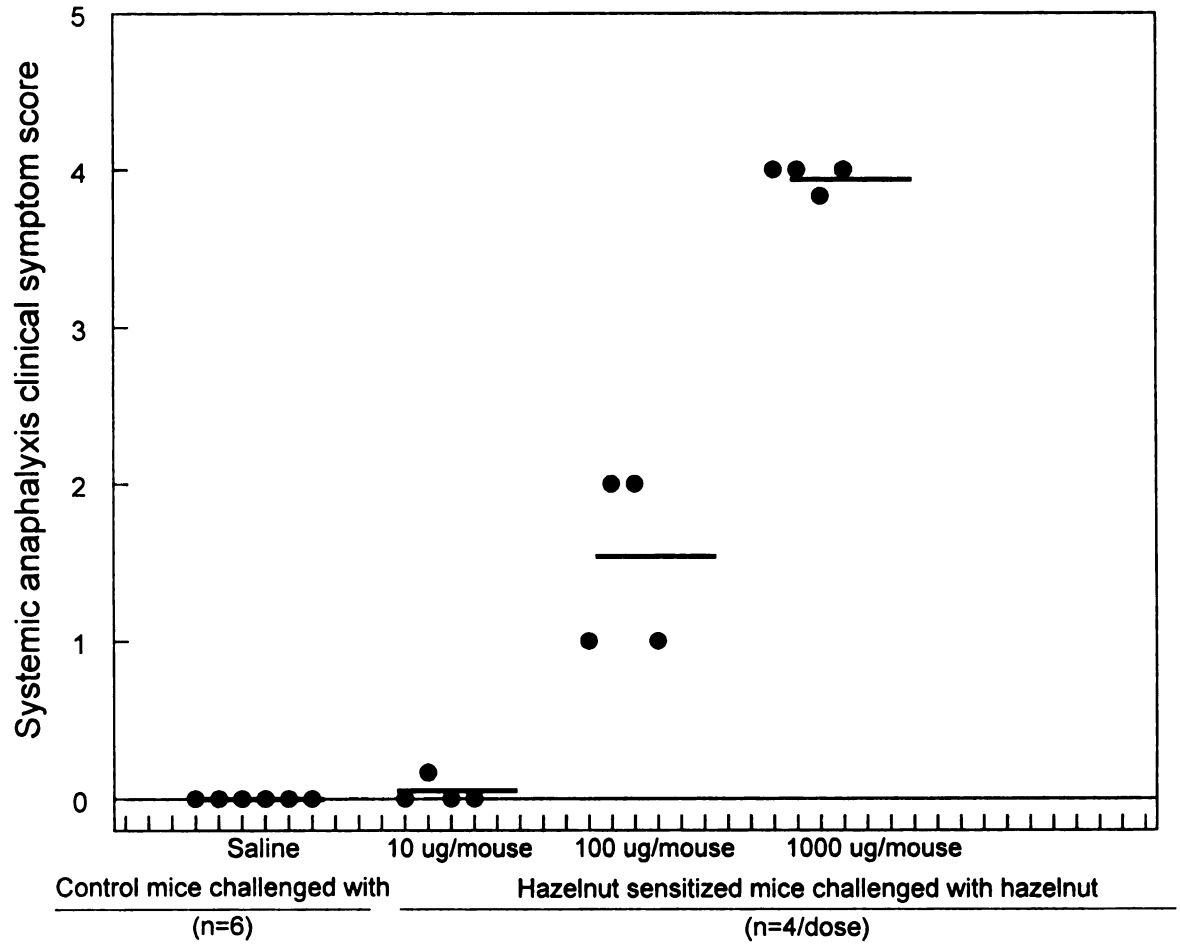

Figure 6.5 Determination of threshold challenge dose of hazelnut required to induce systemic anaphylaxis in hazelnut sensitized BALB/c mice: Clinical symptom scores

Groups of BALB/c mice (n=4-6/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then exposed to indicated challenge doses (10, 100, and 1,000 μg/mouse) or saline systemically (i.p. injections) and examined for indicators of systemic anaphylaxis. Clinical symptoms are shown as a scatter plot with each symbol representing one mouse. Kruskal-Wallis test results; hazelnut sensitized mice with hazelnut 1,000-μg/ mouse challenge $p<0.05$ vs. all other groups.

Figure 6.6

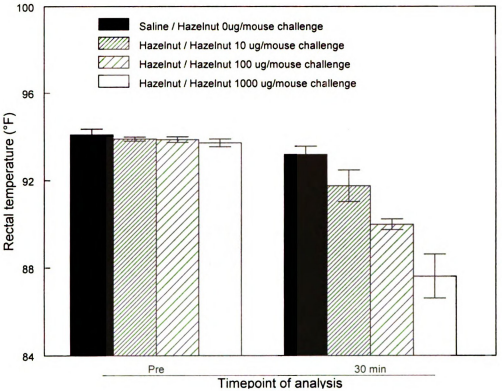

Figure 6.6 Determination of threshold challenge dose of hazelnut required to induce systemic anaphylaxis in hazelnut sensitized BALB/c mice: Changes in rectal temperature

Groups of BALB/c mice (n=4-6/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After [illegible]cles of exposure, IgE induction was confirmed and then exposed to indicated challenge dose [illegible], 100, and 1,000 µg/mouse) or saline systemically (i.p. injections) and examined for indicators of systemic anaphylaxis. Changes in rectal temperature are shown as mean +/- SE. ANOVA test results: * = $p<0.01$ vs. all other groups.

Figure 6.7

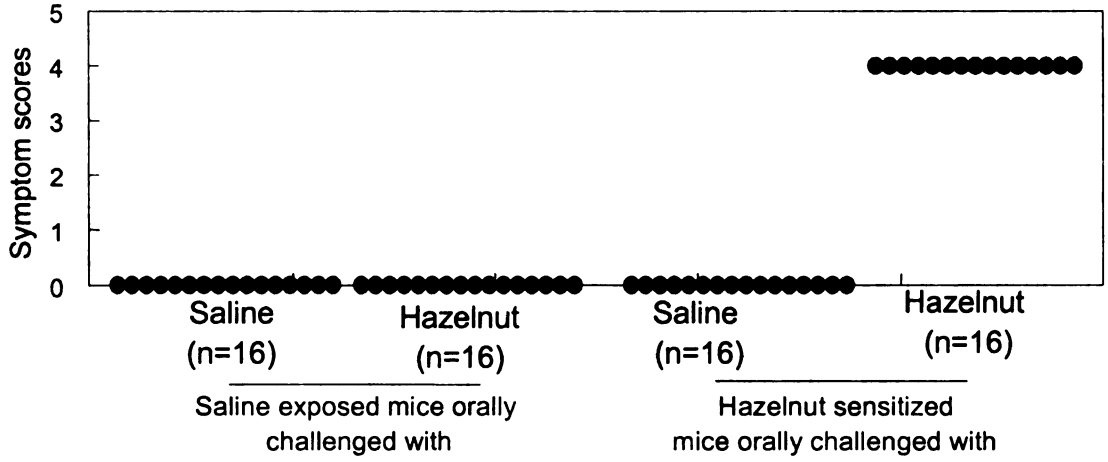

Figure 6.7 Transdermal exposure to hazelnut sensitizes BALB/c mice for systemic anaphylaxis when challenged by oral gavage: Clinical symptom scores

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then exposed to hazelnut (13 mg/mouse) or saline by oral gavage and examined for indicators of systemic anaphylaxis. Clinical symptoms at 30 minutes post challenge are shown as a scatter plot with each symbol representing one mouse. Mann-Whitney test results: hazelnut sensitized mice with hazelnut challenge $p<0.05$ vs. all other groups.

Figure 6.8

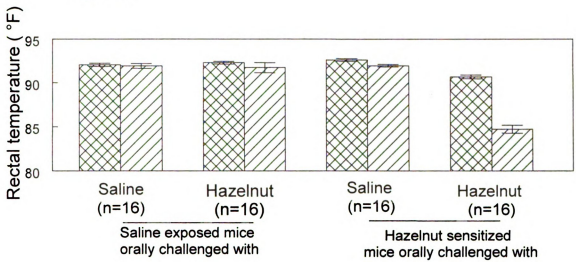

Figure 6.8 Transdermal exposure to hazelnut sensitizes BALB/c mice for systemic anaphylaxis when challenged by oral gavage: Changes in rectal temperature

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then exposed to hazelnut (13 mg/mouse) or saline by oral gavage and examined for indicators of systemic anaphylaxis. Change in rectal temperature 30 minutes after oral challenge is shown as mean +/- SE. Before hazelnut challenge (crossed lines) and 30 minutes after hazelnut challenge (single lines). ANOVA test results: hazelnut sensitized mice challenged with hazelnut $p<0.001$ at 30 min vs. all other groups.

Figure 6.9

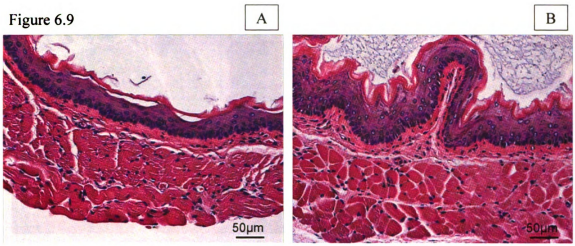

Figure 6.9 (A-B) Transdermal exposure to hazelnut sensitizes BALB/c mice for pathological changes in the esophagus when challenged by oral gavage

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then mice were exposed to hazelnut (13 mg/mouse) by oral gavage. The esophagus was taken 1 hr after hazelnut oral gavage and fixed for 4 days in 10% formalin in PBS. Fixed tissue was embedded in paraffin, cut into 3-5 µm thick sections and mounted on slides. Slides were then stained with hematoxylin-eosin and analyzed with a light microscope for histological changes. A) Example of an esophagus from saline sensitized mice (n=16) orally gavaged with hazelnut; B) Example of an esophagus from hazelnut sensitized mice (n=16) orally gavaged with hazelnut.

Figure 6.10

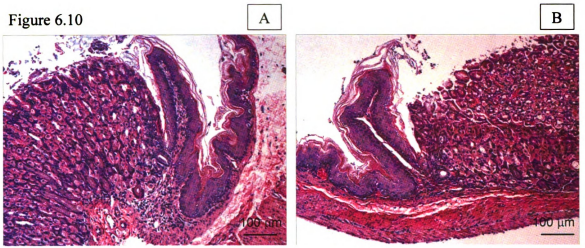

Figure 6.10 (A-B) Transdermal exposure to hazelnut sensitizes BALB/c mice for pathological changes in the stomach when challenged by oral gavage

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then mice were exposed to hazelnut (13 mg/mouse) by oral gavage. The stomach was taken 1 hr after hazelnut oral gavage and fixed for 4 days in 10% formalin in PBS. Fixed tissues were embedded in paraffin, cut into 3-5 µm thick sections and mounted on slides. Slides were then stained with hematoxylin-eosin and analyzed with a light microscope for histological changes. A) Example of a stomach from saline sensitized mice (n=16) orally gavaged with hazelnut; B) Example of a stomach from hazelnut sensitized mice (n=16) orally gavaged with hazelnut.

Figure 6.11

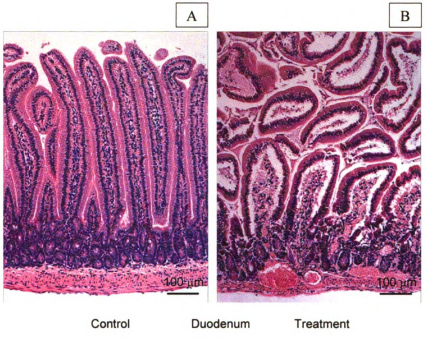

Figure 6.11 (A-B) Transdermal exposure to hazelnut sensitizes BALB/c mice for pathological changes in the duodenum when challenged by oral gavage

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then mice were exposed to hazelnut (13 mg/mouse) by oral gavage. The duodenum was taken 1 hr after hazelnut oral gavage and fixed for 4 days in 10% formalin in PBS. Fixed tissues were embedded in paraffin, cut into 3-5 µm thick sections and mounted on slides. Slides were then stained with hematoxylin-eosin and analyzed with a light microscope for histological changes. A) Example of a duodenum from saline sensitized mice (n=16) orally gavaged with hazelnut; B) Example of a duodenum from hazelnut sensitized mice (n=16) orally gavaged with hazelnut.

Figure 6.12

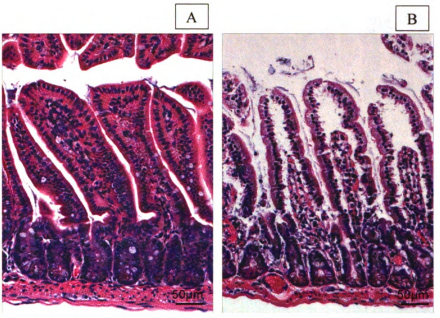

Figure 6.12 (A-B) Transdermal exposure to hazelnut sensitizes BALB/c mice for pathological changes in the jejunum when challenged by oral gavage

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then mice were exposed to hazelnut (13 mg/mouse) by oral gavage. The jejunum was taken 1 hr after hazelnut oral gavage and fixed for 4 days in 10% formalin in PBS. Fixed tissues were embedded in paraffin, cut into 3-5 µm thick sections and mounted on slides. Slides were then stained with hematoxylin-eosin and analyzed with a light microscope for histological changes. A) Example of a jejunum from saline sensitized mice (n=16) orally gavaged with hazelnut; B) Example of a jejunum from hazelnut sensitized mice (n=16) orally gavaged with hazelnut.

Figure 6.13

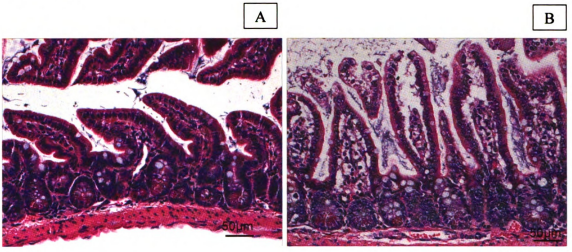

Figure 6.13 (A-B) Transdermal exposure to hazelnut sensitizes BALB/c mice for pathological changes in the ileum when challenged by oral gavage

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then mice were exposed to hazelnut (13 mg/mouse) by oral gavage. The ileum was taken 1 hr after hazelnut oral gavage and fixed for 4 days in 10% formalin in PBS. Fixed tissues were embedded in paraffin, cut into 3-5 μm thick sections and mounted on slides. Slides were then stained with hematoxylin-eosin and analyzed with a light microscope for histological changes. A) Example of an ileum from saline sensitized mice (n=16) orally gavaged with hazelnut; B) Example of an ileum from hazelnut sensitized mice (n=16) orally gavaged with hazelnut.

Figure 6.14

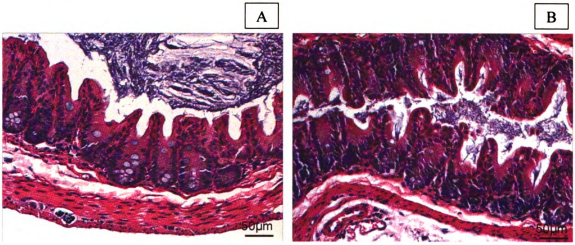

Figure 6.14 (A-B) Transdermal exposure to hazelnut sensitizes BALB/c mice for pathological changes in the colon when challenged by oral gavage

Groups of BALB/c mice (n=16/group) were sensitized with hazelnut (500 µg per mouse) or saline by transdermal exposure as described in Figure 6.1. After 6 cycles of exposure, IgE induction was confirmed and then mice were exposed to hazelnut (13 mg/mouse) by oral gavage. The colon was taken 1 hr after hazelnut oral gavage and fixed for 4 days in 10% formalin in PBS. Fixed tissues were embedded in paraffin, cut into 3-5 µm thick sections and mounted on slides. Slides were then stained with hematoxylin-eosin and analyzed with a light microscope for histological changes. A) Example of a colon from saline sensitized mice (n=16) orally gavaged with hazelnut; B) Example of a colon from hazelnut sensitized mice (n=16) orally gavaged with hazelnut.

6.7 References

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CHAPTER SEVEN

7.0 Effect of a diet rich in EPA and DHA on systemic immune responses to hazelnut: A pilot study

7.1 Abstract

Background: Previously, we found that in BALB/c mice, transdermal hazelnut is capable of directly eliciting hazelnut specific binding allergenic (IgE) antibodies *via* activating Type-2 cytokine. EPA and DHA, the main n-3 polyunsaturated fatty acids of fish oil, have a controversial role in several allergic disorders, with several groups showing a beneficial role in asthma. Currently it is not known whether n-3 fatty acids from fish (EPA, DHA) may have either therapeutic or preventative effects in food allergy.

Hypothesis, objective and approach: Here we tested if EPA + DHA together can have an effect on systemic immune responses to transdermal hazelnut sensitization in either a preventative or therapeutic manner in this model.

Results: With the addition of EPA and DHA in the diet in a preventative manner, hazelnut specific IgE responses were increased ($p<0.05$) in comparison to the control diet. EPA and DHA supplementation also altered the IFN-γ / IL-4 ratio in favor of IL-4 dominance, thus providing a mechanism of enhancement of IgE production seen here. In the therapeutic study no significant alteration in specific IgE response was noted.

Conclusion: EPA and DHA supplementation seems to enhance hazelnut specific IgE responses via reducing the IFN-γ / IL-4 ratio in a preventative model. Further studies

(e.g. repeating with a larger number of mice per group (i.e. n=10), dose response, and disease model studies) are suggested to test the impact of fish oil on tree-nut allergy.

7.2 Introduction

Currently, our modern diets differ in many aspects from our past traditional diets, with more complex, processed foods coming to the forefront, replacing fruits and vegetables. One change that has been identified is increased consumption of n-6 polyunsaturated fatty acids and decreased consumption of n-3 polyunsaturated fatty acids [1]. It has been estimated that the ratio of n-6 to n-3 fatty acids in the typical western diet now ranges from approximately 20-30:1 instead of the traditional range of 1-2:1 [2]. This alteration in fatty acid consumption is a major candidate factor, as both epidemiological and experimental evidence suggest a link between the declining consumption of anti-IFNlammatory n-3 polyunsaturated fatty acids with the rise in allergenic diseases [3]. Two of these n-3 polyunsaturated fatty acids EPA and DHA have had controversial outcomes in allergenic disorders. Hodge et al have shown positive observations, observing that children who regularly ate oily fish were significantly less likely to develop asthma, odds ratio 0.26 [4]. While others such as Woods et al in the Cochrane review 2003 conclude that there is no evidence of consistent benefit of n-3 PUFAs in the management of asthma. Currently it is not known weather EPA and DHA could benefit, harm, or have no impact on food allergy sufferers.

A recent study published in the *New England Journal of Medicine* showed that monthly injections of humanized recombinant anti-IgE antibodies could increase the amount of peanut that is tolerable in peanut-sensitive subjects [5], showing IgE as a target mechanism for food allergy. Although a great find, monthly injections are needed to achieve this increased tolerance. Studies like this highlight the importance that therapies

targeted at IgE in food allergy therapies. Therefore new, non-invasive therapies and strategies (e.g. dietary supplementation, pro-biotics) need to be developed / studied to alter the immune response to food allergens.

Previously we characterized the systemic immune response to transdermal hazelnut protein in BALB/c mice as having robust specific IgE, total IgE, hazelnut driven IL-4, and an IFN-γ/IL-4 ratio in favor of IL-4.

Here we test if EPA + DHA together can have an effect on systemic immune responses to transdermal hazelnut sensitization in either a preventative or therapeutic manner in this model. EPA and DHA supplementation seems to enhance hazelnut specific IgE responses via reducing the IFN-γ / IL-4 ratio in a preventative model. Further studies (e.g. repeating with a larger number of mice per group (i.e. n=10), dose response, and disease model studies) are suggested to test the impact of fish oil on tree-nut allergy.

7.3 Materials and Methods

7.3.1 Materials

Materials used here were as listed earlier in Chapter 5.

7.3.2 Transdermal sensitization and bleeding

All mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). Only adult animals (6-8 weeks age) were used in the study. All animal procedures used were in accordance with the Michigan State University policies. Transdermal exposure experiments were performed using the method described by us before (Chapter 4) [6].

7.3.3 Diets and experimental design

Profiling the immune response to transdermal hazelnut was done using a commercially available diet, Harlan Teklad 22/5 Rodent diet. Experimental diets were based on the purified AIN-93G formulation [7], and had the following ingredients (per kg): 10g AIN-93G mineral mix, 10g AIN 93G vitamin mix, 200g casein, 397.5g cornstarch, 132 g Dyetrose (dextrinized cornstarch), 50g cellulose, 3g L-cystine, 2.5g choline bitartrate, 14 mg *tert*-butylhydroquinine, and 100g sucrose (Dyets). Oils were then added at 70g/kg described below. Corn oil (Dyets), oleic acid (Dyets) DHA-enriched fish oil (containing 604g/kg DHA, 71g/kg EPA) (Ocean Nutrition) and EPA-enriched fish oil (containing 540g/kg EPA, 71g/kg DHA) (Ocean Nutrition) were added to the basal diet to achieve two experimental diets. Diet formulation was previously described by Jia Q. et al. [8]. Table 7.1 shows the experimental diets, AIN-93G diets containing 10 g/kg corn oil plus 60 g/kg oleic acid (control), 10 g/kg corn oil plus 37 g/kg oleic acid and 23 g/kg DHA + EPA enriched fish oil (402 g/kg DHA, 341 g/kg EPA) (Ocean Nutrition). Dry ingredients

were added to a clean, Kitchen Aid mixer and mixed for five minutes. Oils were then added and mixed for an additional thirty minutes to ensure even distribution of contents. Diets were prepared fresh every 2 weeks, stored in aliquots at -20°C, and was provided fresh daily to the mice. The final lipid profiles of the experimental diets are shown in (Table 7.2). In the preventative study the respective diets were feed to their respective groups for 4 weeks prior to hazelnut sensitization and continued throughout the study (Figure 7.7). In the therapeutic study animals were on a chow diet during hazelnut sensitization and then put on their respective diets after an established IgE response was seen (Figure 7.14). Comparison of the immune response to the chow diet (Harlan Teklad 22/5) and the control diet for the fish oil experiment were done.

7.3.4 Measurement of hazelnut specific IgE antibodies and total IgE in the plasma

We have previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgE antibody analyses (Chapter 3) [9, 10].

7.3.5 Spleen cell culture and cytokine analyses

Spleen cells were harvested and standard cell cultures were setup essentially as described (Chapter4) [11].

7.3.6 Lipid extraction and fatty acid analysis

To confirm tissue incorporation of (n-3) PUFA after experiment is complete, spleen phospholipid content was be measured by a modification of the method of Hasler et al

[12]. Briefly, pooled mouse spleens were homogenized with a chloroform: methanol (2:1) solution. Total phospholipids were extracted, separated, and collected using a silica column. Phospholipid samples were dried and esterified with methanol: acetonitrile: boron tri-fluoride (11:4:5, by vol). The resulting FAME was then extracted with hexane. After centrifugation at 1200 x g for 5 min, the hexane supernatant was decanted, dried, and re-dissolved in chloroform and then analyzed by GC utilizing a Varian 3700 GLC. Fatty acid profiles were then identified by comparing the retention times with those of appropriate standard FAME (Nu-Check-Prep). Samples were run twice on the Varian 3700 GLC so that a standard error could be determined. Table 7.3 and 7.4 shows the spleen phospholipid fatty acid profile for the preventative and therapeutic studies respectively.

7.3.7 Statistical Analysis

Student's t-test and ANOVA tests were used to evaluate significance using Analyse-It^{TM} software program (Analyse-It software Ltd, Leeds, UK). The statistical significance level was set at 0.05.

7.4 Results

7.4.1 Immune responses in mice following transdermal exposure to hazelnut protein in BALB/c mice: comparison of different control diets

In order to study dietary changes in out model of hazelnut allergy, we first needed to compare the immune responses in the chow diet (Harlen Teklad 22/5) and the AIN-93 based control diet to be used in the dietary modification studies. Hazelnut specific IgE (Figure 7.1), total plasma IgE levels (Figure 7.2), hazelnut specific IgG2a (Figure 7.3), hazelnut driven IL-4 recall response (Figure 7.4), hazelnut driven IFN-γ (Figure 7.5), and IFN-γ/IL-4 ratio (Figure 7.6) were compared with no major alterations being noted.

7.4.2 EPA and DHA incorporation into the phospholipid fatty acid profile in mouse spleens

Following completion of the experiment, spleens were collected from all of the animals in the various groups. One half of the spleen was used for cell culture and the other half for phospholipid fatty acid analysis to confirm EPA and DHA incorporation into the membranes. Both EPA [C20:5(n-3)] and DHA [C22:6(n-3)] were increased in the fish oil groups ($p<0.05$) after feeding, whereas in control animals there was very little EPA and DHA found (Table 7.3 preventative study, Table 7.4 therapeutic study).

7.4.3 Characterization of hazelnut specific antibody responses to transdermal exposure in BALB/c mice

Mice were evaluated for the presence of hazelnut binding specific antibodies (IgE) in the plasma before and after each transdermal exposure. We used LPS-free preparations of hazelnut protein extract (Greer Labs) in this study (LPS level as measured by LAL assay: <0.4 pg/mg of protein). Pre-immune serum did not have detectable hazelnut specific IgE antibodies (mean titer <1/20) in both the preventative and therapeutic studies (Figure 7.8A, 7.15A). Following transdermal exposure with hazelnut (500 μg/mouse), both in the test diet as well as the control diet, but not saline, elevated levels of hazelnut specific IgE antibodies appeared (Figure 7.8B, 7.15B). In the test diet + HAZ group (preventative study) animals had a significantly higher level ($p<0.05$) of hazelnut specific IgE (Figure 7.8B, C, D) when compared to the control diet counterpart. In the therapeutic study there was no significant effect seen on hazelnut specific IgE levels (Figure 7.15B, C, D). Mice were also evaluated for the presence of hazelnut specific IgG2a before and after transdermal exposure to hazelnut or saline. In the preventative study no effect of diet was seen on hazelnut specific IgG2a (Figure 7.9), whereas in the therapeutic study there was an increased presence of hazelnut specific IgG2a seen at several time points (Figure 7.16).

7.4.4 Measurement of total IgE levels in plasma of BALB/c

Mice were evaluated for total plasma IgE before and after transdermal exposure to hazelnut (500 μg/mouse) or saline alone. As evident, pre-immune plasma had very little total IgE (Figure 7.10, 7.17). Following transdermal application, in the hazelnut sensitized groups but not saline alone, total IgE levels were increased. Total plasma IgE was significantly higher ($p<0.05$) in the test diet groups when compared the control diet

hazelnut sensitized group in both the preventative and therapeutic studies (Figure 7.10, 7.17).

7.4.5 Characterization of hazelnut driven cytokine responses (IL-4, IFN-γ) following transdermal exposure to hazelnut protein in BALB/c mice

In order to study the mechanism of IgE response to hazelnuts, we examined the IL-4 cytokine responses in hazelnut-sensitized mice. As evident, hazelnut significantly activated the Type-2 cytokine, IL-4, in spleen cells from hazelnut but not saline exposed mice (Figure 7.11, 7.18). Spleen cells isolated from the test diet fed groups elicited significantly less IL-4 in response to hazelnut ($p<0.05$) (both 100 and 500 μg stimulation) in both the preventative as well as the therapeutic study (Figure 7.11, 7.18).

In spleen cells isolated from the test diet fed group, IFN-γ levels were significantly lower ($p<0.05$) than the control diet fed group (preventative study) (Figure 7.12). In the therapeutic study a significant effect ($p<0.05$) was also evident (Figure 7.19).

7.4.6 Characterization of IFN-γ / IL-4 ratio in BALB/c mice following transdermal exposure to hazelnut protein: effect of dietary fatty acid

In order to further study the mechanism of IgE response to hazelnuts, we determined the IFN-γ / IL-4 ratio in mice fed both the control diet and also the DHA + EPA diet. In the preventative study, the IFN-γ / IL-4 ratio was significantly lower ($p<0.05$) in the fish oil diet in both the 100 and 500 μg hazelnut stimulation, whereas in the therapeutic study no such trend was seen (Figure 7.13, 7.20).

7.5 Discussion

In this study we profiled hallmarks of allergenic immune responses (specific and total IgE, IL-4, and IFN-γ / IL-4 ratio) to transdermal hazelnut protein in BALB/c mice that were supplemented with a diet rich in EPA and DHA. Here, specific and total IgE are used as markers of Type-I hypersensitivity. Cytokine profile and ratio is used as showing the relative dominance of either T-helper 1 or T-helper 2 cytokines. A T-helper 2 dominated response would favor the development of Type I hypersensitivity. There are several novel finding of these studies; i) EPA + DHA supplementation together can lead to a increased specific IgE response when given in a preventative manner; ii) both IL-4 and IFN-γ hazelnut specific recall responses are significantly lower with EPA + DHA supplementation; iii) the IFN-γ / IL-4 ratio is swayed towards IL-4 with EPA + DHA supplementation, showing a mechanism driving the increase in IgE responses seen in the preventative study.

To our knowledge, there is only one other study using fish oil as a therapy for food allergy. Prickett et al. [13] showed an enhancement in both IgE and IgG antibodies to egg albumin in Sprague-Dawley rats in fish fat fed animals in comparison to beef fat. This study used alum as an adjuvant to elicit a response by I.P. injection. Therefore, in both species (rat or mouse) fish oil seems to drive an enhancement of IgE responses. Whereas they did not address the mechanism of enhancement, we concluded that the alteration of the IFN-γ / IL-4 ratio is driving this increase in IgE levels.

Further analysis of molecules involved in the IFNlammatory response need to be looked at in this type of therapy for food allergy. Ecosinoids of the n-3 variety tend to be less IFNlammatory than their counterparts derived from arachadonic acid (n-6). Interestingly is the fact is arachadonic acid in both the therapeutic study and the preventative study was found to be higher in the fish oil groups than in the control diet groups. Normally in diets rich in n-3 fatty acids, arachadonic acid is partially replaced by the n-3 fatty acids in the diet.

Since activation of Type-2 cytokine response (IL-4) is critical to initiate an allergic response, we tested the hypothesis that a diet rich in fish oil can alter the classical type-2 (IL-4) responses seen in food allergy. Transdermal exposure to hazelnut is capable of activating these cytokines. Our data confirmed the hypothesis. Our data argue that hazelnut activates Type-2 cytokine (IL-4) in a dose dependent manner and is significantly lowered ($p<0.05$) by using a diet rich in fish oil. IL-4 is the key class switch factor allowing IgE to be made; therefore we assessed both total and specific IgE. EPA and DHA supplementation together had a significant effect on hazelnut specific IgE in the preventative study. With IL-4 being lowered and hazelnut specific IgE increasing, we did further analysis of the IFN-γ / IL-4 ratio, EPA + DHA supplementation significantly drove this ratio in favor of IL-4, thus explaining the rise in hazelnut specific IgE seen in the preventative study.

Others have used this same type of dietary approach in battling food allergy. Their approaches have been to use supplement like compounds in pharmaceutical ways in order

to help battle food allergy. These compounds include an edible-mushroom derived protein and food allergy herbal formula-1 [14, 15].

Hsiesh et al. [14] investigated the use of an immunomodulatory protein (FIP-*fve*) isolated from the edible mushroom *Flammulina velutipes*, in a mouse model (BALB/c) of OVA allergy. They used this protein (FIP-*fve* 200 ug/mouse) orally before and during OVA i.p. sensitization using alum as an adjuvant. Following sensitization, OVA-specific antibodies, cytokine profile, anaphylaxis symptoms following oral challenge, plasma histamine and histology of the intestines were examined. They report that mice receiving oral FIP-*fve* treatment during sensitization had an impaired OVA-specific IgE response with a T-helper 1 dominated cytokine response. Further, mice were protected from signs of anaphylaxis after oral challenge with OVA with significant changes seen in symptom scores, plasma histamine levels and very mild mucosal edema and epithelial damage when compared the severe edema, vascular congestion and damage to the intestinal lining. They then report that this protein then may have immunoprophylaxis properties for food allergy. Although very interesting, how this protein can induce a T-helper 1 skewing response, the use of adjuvant here skews the results. It is unknown if FIP-*fve* is altering the adjuvant response or the hypersensitivity response. Therefore our model of adjuvant free sensitization is more useful to elucidate potential therapies for use in altering immune responses to food allergens.

Li et al. [15] has investigated using a traditional Chinese medicine (Food Allergy Herbal Formula-1, FAHF-1) as a therapy for peanut induced anaphylaxis in a mouse model of

peanut allergy. This model uses C3H/HeJ mice sensitized to freshly ground whole peanut in the presence of cholera toxin. One week after the final sensitization dose, mice received either 21 mg of FAHF-1 or water (sham) treatment by intragastric gavage twice daily for seven weeks. Mice were then challenged with 10mg of crude peanut extract. Anaphylaxis symptom scores, body temperatures, plasma histamine, IgE levels, and cytokine profiles were analyzed. They report that FAHF-1 completely blocked peanut-induced anaphylaxis symptom scores and significantly reduced histamine release. Further, peanut specific IgE levels were significantly lower after 2 weeks of treatment and stayed lower even after 4 weeks after discontinuation of treatment. T-helper 2-cytokine synthesis (IL-4, IL-5, IL-13) was lower, but no alteration of IFN-γ was seen. From this they concluded that FAHF-1 protected peanut-sensitized mice from anaphylactic reactions and reversed established IgE-mediated peanut allergy. This therefore may serve as a valuable treatment for peanut allergy. Again exciting results, although the use of cholera toxin as an adjuvant, even though treatment was after sensitization, may alter the immune response to this therapy. The continuing adjuvant effect may be altered and not the actual Type-1 hypersensitivity, therefore an adjuvant free model would be more representative to study dietary therapies.

Treatments such as these, while showing benefits require a constant need to take the supplement, whereas in a dietary lifestyle change such as eating more fish does not require anything else besides a commitment. Therefore more research needs to be done in these non-invasive ways to fight disease.

In this pilot study we assessed the potential use of EPA and DHA as a prevention and/or therapy for genetically prone populations for food allergy. Since this was a pilot study and not actually looking at disease it cannot be concluded if a benefit or harm is evident. After finishing this study our model of tree-nut allergy has progressed to include anaphylactic responses (clinical scores, rectal temperature, plasma histamine and gut pathology). With this we have also assessed dose of allergen needed to cause systemic anaphylaxis in an I.P. challenge. Using this enhanced model, encompassing both hallmarks of allergy as well as clinical responses, a better understanding of how and why EPA and DHA affect food allergy responses could be determined.

Limitations of this study include the relative low number of animals per group. Here we only used one dose of DHA+EPA, a dose study would be desirable to see if an effect could be achieved at a lower dose. We also only used one strain of mouse; further studies on other strains could show different results. We only assessed immune response to transdermal hazelnut protein; a further assessment of disease would transform responses to actual disease state.

In summary, with the changes in the modern diet to a more processed and n-6 rich one and the rise in allergenic disorders seeming to coincide, we looked at hallmarks of food allergy (food specific IgE, total IgE and IL-4) after feeding a diet rich in EPA and DHA in a mouse model of tree-nut allergy. We show here that EPA + DHA can have altering effects with regards to immune responses to transdermal hazelnut protein in BALB/c mice, but more research needs to be done to assess the implications of these effects.

7.6 Figures

Figure 7.1

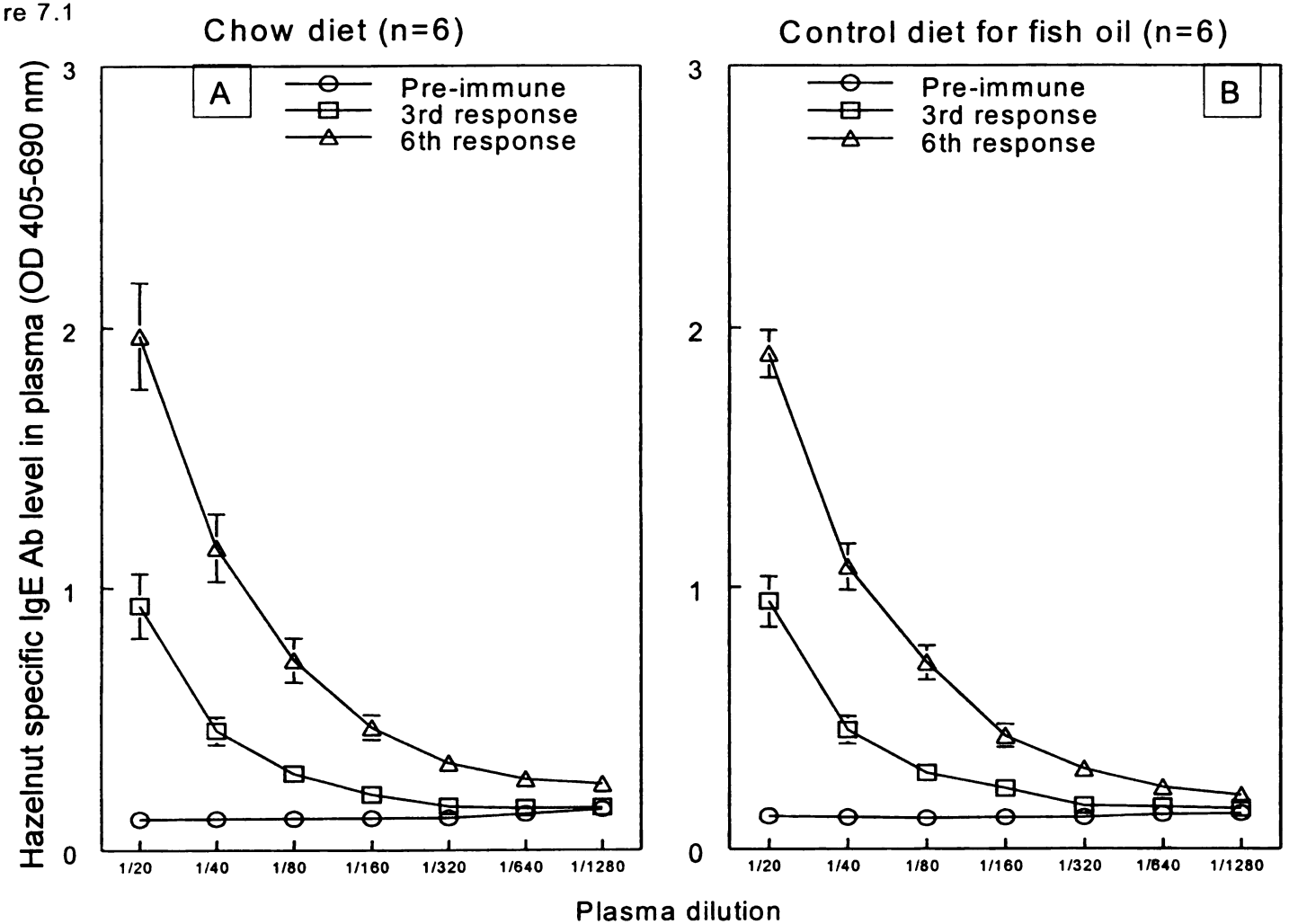


Figure 7.1 (A-B): Comparison of hazelnut specific IgE antibody responses in BALB/c mice following repeated transdermal exposure to hazelnut protein in different control diets. Groups of mice (n=6/group) were transdermaly exposed to 500 μg/mouse of LPS-free hazelnut protein extracts and hazelnut binding specific IgE antibodies (Figs. A, B) were measured by optimized ELISA. Chow diet is the Harlan Teklad 22/5 diet used in all other studies and the control diet for fish oil is an AIN-93 based diet. Pre-immune: plasma collected before exposure; 3rd response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures. Data shows hazelnut specific antibody isotype levels as OD (mean +/- SE). At some points error bars are not visible.

Figure 7.2

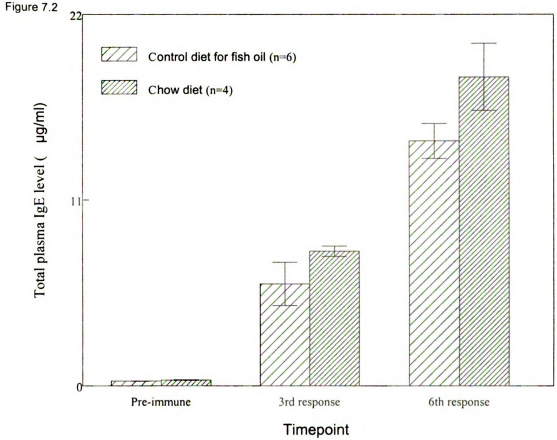


Figure 7.2: Comparison of total IgE in BALB/c mice following repeated transdermal exposure to hazelnut protein in different control diets. Groups of mice (n=4-6/group) were transdermaly exposed to 500 µg/mouse of LPS-free hazelnut protein extracts and total IgE levels were measured by optimized ELISA. Chow diet is the Harlan Teklad diet used in all other studies and the control diet for fish oil is an AIN-93 based diet. Pre-immune: plasma collected before exposure; 3rd response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures. Data shows total plasma IgE levels as µg/ml (mean +/- SE). At some points error bars are not visible.

Figure 7.3

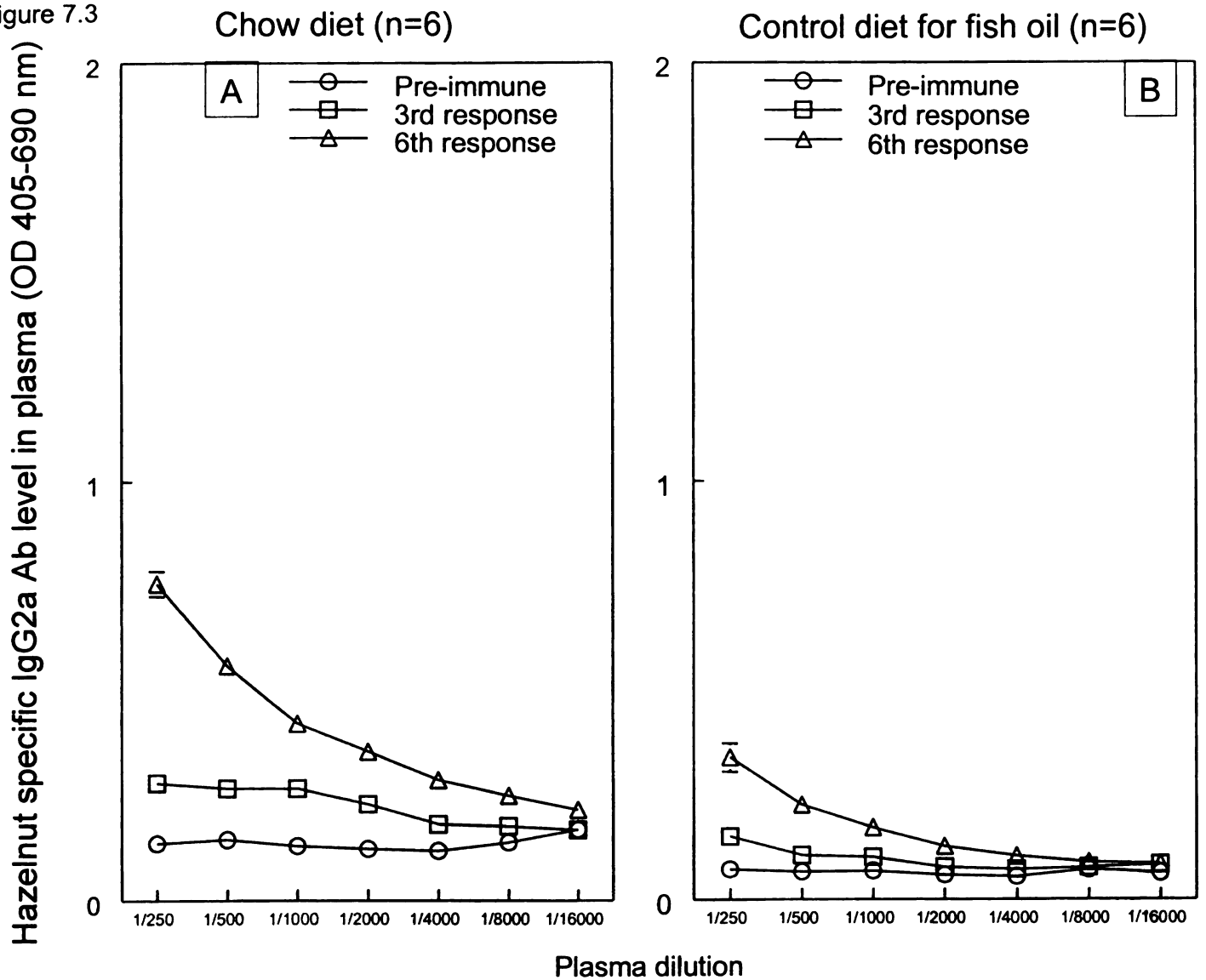

Chow diet (n=6)
Control diet for fish oil (n=6)
A
B
Pre-immune
3rd response
6th response
Hazelnut specific IgG2a Ab level in plasma (OD 405-690 nm)
0
1
2
1/250
1/500
1/1000
1/2000
1/4000
1/8000
1/16000
Plasma dilution

Figure 7.3 (A-B): Comparison of hazelnut specific IgG2a antibody responses in BALB/c mice following repeated transdermal exposure to hazelnut protein in different control diets. Groups of mice (n=6/group) were transdermaly exposed to 500 μg/mouse of LPS-free hazelnut protein extracts and hazelnut binding specific IgG2a antibodies (Figs. A, B) were measured by optimized ELISA. Chow diet is the Harlan Teklad diet 22/5 used in all other studies and the control diet for fish oil is an AIN-93 based diet. Pre-immune: plasma collected before exposure; 3rd response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures. Data shows hazelnut specific antibody isotype levels as OD (mean +/- SE). At some points error bars are not visible. ANOVA test results $p<0.05$ for 6R chow diet vs. 6R control for fish oil diet.

Figure 7.4

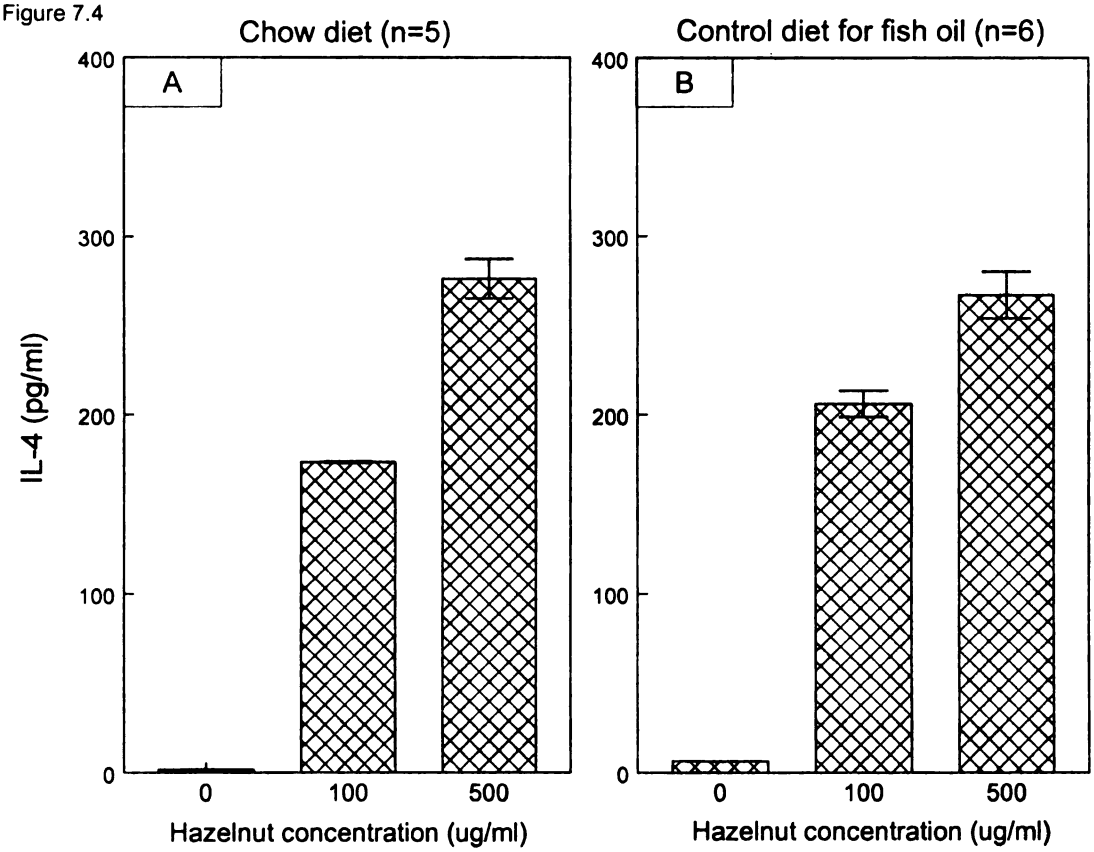


Figure 7.4: Comparison of different control diets: Hazelnut driven Type-2 cytokine (IL-4) responses in mice transdermally sensitized with hazelnut protein

Groups of BALB/c mice (n=5-6/group) were sensitized with hazelnut (500 μg per mouse) by transdermal exposure. Mice were on either a commercially available rodent chow diet (Harlan Teklad 22/5) or an AIN-93 based diet. Three days following a booster exposure with hazelnut, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from hazelnut-sensitized mice. (ANOVA test results: $p<0.05$ 100 μg hazelnut stimulation in chow diet vs. 100 μg hazelnut stimulation in control diet for fish oil).

Figure 7.5

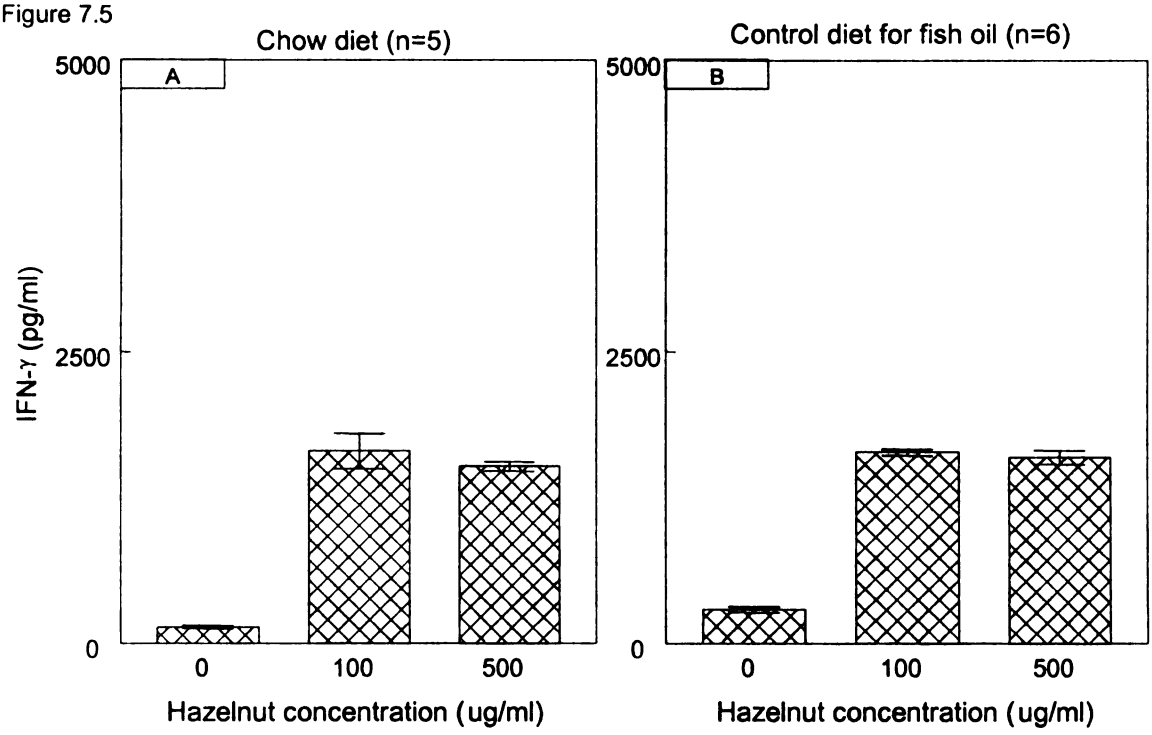


Figure 7.5: Comparison of different control diets: Hazelnut driven Type-1 cytokine (IFN-γ) responses in mice transdermally sensitized with hazelnut protein

Groups of BALB/c mice (n=5-6/group) were sensitized with hazelnut (500 μg per mouse) by transdermal exposure. Mice were on either a commercially available rodent chow diet (Harlan Teklad 22/5) or an AIN-93 based diet. Three days following a booster exposure with hazelnut, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is D3 of cell culture (average +/- SE of duplicate analyses of data) from hazelnut-sensitized mice. ANOVA test results: $p<0.05$ for 100 and 500 ug stimulation vs. 0 ug hazelnut stimulation for both diets.

Figure 7.6

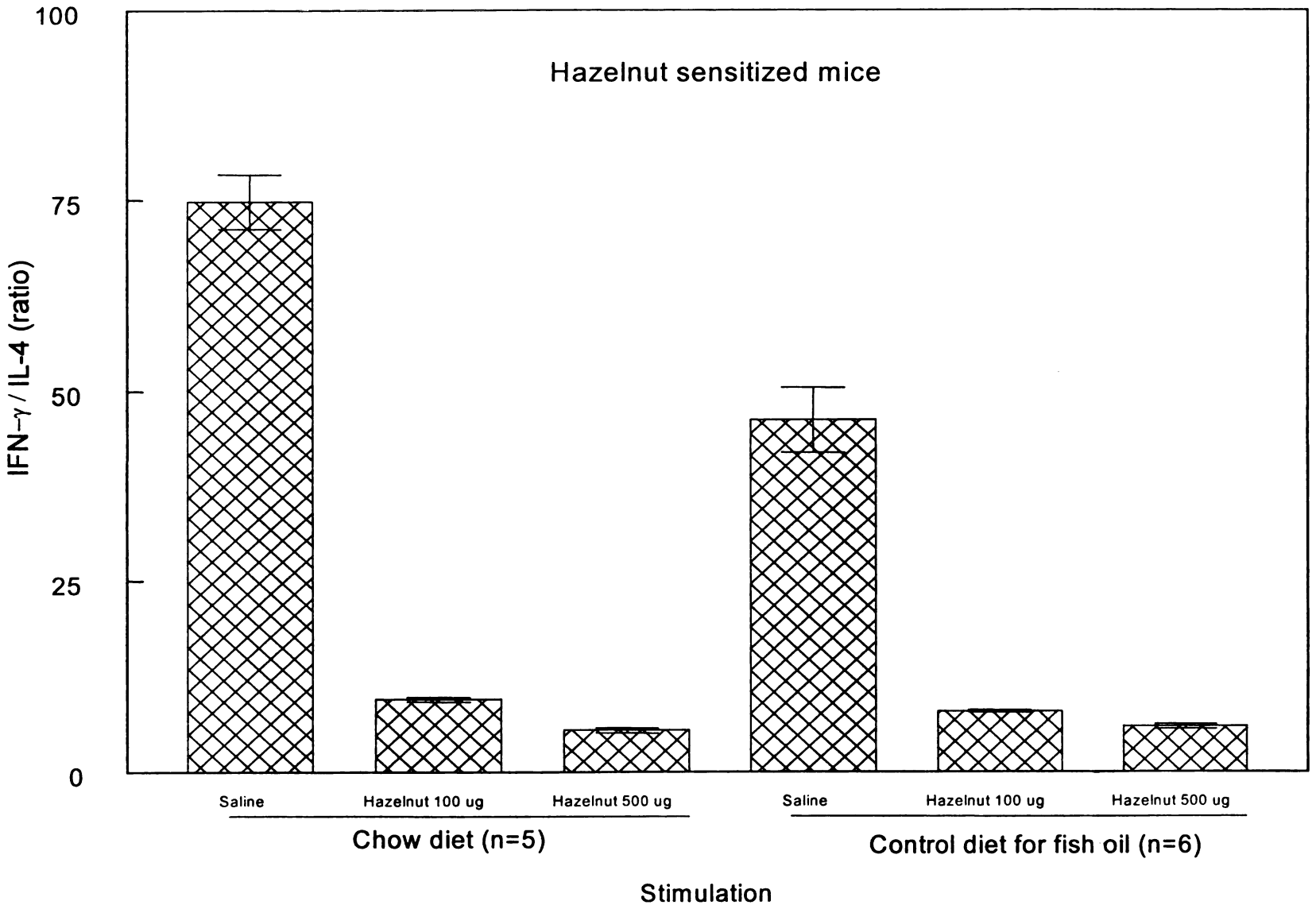
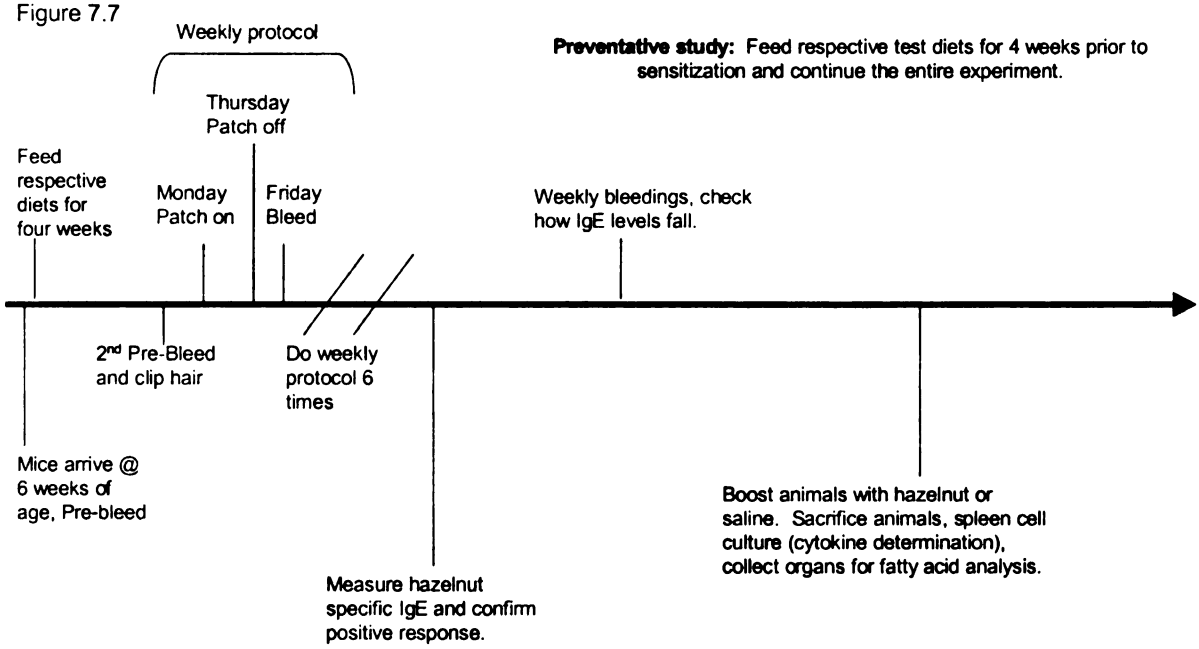

Hazelnut sensitized mice
IFN-γ / IL-4 (ratio)
100
75
50
25
0
Saline
Hazelnut 100 ug
Hazelnut 500 ug
Saline
Hazelnut 100 ug
Hazelnut 500 ug
Chow diet (n=5)
Control diet for fish oil (n=6)
Stimulation

Figure 7.6: IFN-γ / IL-4 ratio in mice transdermally sensitized with hazelnut protein: Comparison of control diets

Groups of BALB/c mice (n=5-6/group) were sensitized with hazelnut (500 μg per mouse) by transdermal exposure. Mice were on either a commercially available rodent chow diet (Harlan Teklad 22/5) or an AIN-93 based diet. Three days following a booster exposure with hazelnut, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone (saline). Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is D3 of IFN-γ / D3 of IL-4 response for saline, 100 μg and 500 μg hazelnut stimulation (average +/- SE of duplicate analyses of data). AVOVA test results: $p<0.05$ for saline chow diet vs. saline control diet for fish oil.

Figure 7.7



Group	n=	Sensitized with	Diet fed prior to and during sensitization	Diet fed after sensitization
1	5	Hazelnut	Test diet	Test diet
2	6	Hazelnut	Control diet	Control diet
3	5	Saline	Control diet	Control diet

Figure 7.7: Transdermal sensitization protocol to hazelnut protein: preventative study. The schedule of cycles of hazelnut sensitization and testing are shown for the preventative study.

Table 7.1

Experimental diets used to assess DHA + EPA enriched oil on tree-nut allergy[1]

Diet	Corn oil	Oleic acid (g/kg diet)	(n-3) enriched oil[2]
Control diet	10	60	0
Test diet (EPA + DHA)	10	37	23

1 All diets were adjusted with oleic acid to have final oil concentrations of 70g/kg.
2 Concentrated oils from fish that are enriched for DHA and EPA. The DHA:EPA ratio was 1:1.

Table 7.2

Fatty acid composition of experimental diets

	Control diet (g/kg diet)	Test diet (DHA + EPA enriched) (g/kg diet)
Fatty acid		
14:0	0.29	0.31
16:0	6.45	5.75
16:1	0.16	0.21
18:0	4.41	3.29
18:1	72.5	50.1
18:1	<0.10	0.85
18:2(n-6)	13.6	12.1
20:0	0.34	0.44
18:3(n-6)	<0.10	<0.10
20:1	0.2	0.82
18:3(n-3)	0.32	0.53
20:2(n-6)	<0.10	0.15
22:0	0.89	0.64
22:1	<0.10	<0.10
20:3(n-3)	<0.10	0.11
20:4(n-6)	<0.10	0.44
24:0	0.22	<0.10
20:5(n-3)	0.28	9.31
24:1	<0.10	<0.10
22:5(n-3)	<0.10	1.64
22:6(n-3)	0.26	10.9
Total (n-6)	13.6	12.7
Total (n-3)	0.86	22.5
(n-6):(n-3)	16:1	1:1.8

* From Jia, Q., et al., J Nutr, 2004. 134(6): p. 1353-61.

Table 7.3

Spleen phospholipid fatty acid composition in mice fed various PUFA[1,2]

	Control diet + hazelnut	Control diet + hazelnut	Test diet + Hazelnut
		g/100g total phospholipids fatty acid	
Preventative study			
16:0	20.25 +/- 2.21	18.89 +/- 1.98	16.07 +/- 1.84
18:0	7.52 +/- 1.38	9.09 +/- 1.11	8.89 +/- 1.01
18:1	3.80 +/- 0.31	3.64 +/- 0.21	3.43 +/- 0.15
18:2 (n:6)	4.07 +/- 0.11	3.83 +/- 0.18	4.29 +/- 0.21
20:4 (n:6)	6.65 +/- 0.13	6.54 +/- 0.23	8.26 +/- 0.31 *
20:5 (n:3)	ND	ND	3.14 +/- 0.22 *
22:6 (n:3)	1.18 +/- 0.15	1.48 +/- 0.09	1.99 +/- 0.12 *
Total n:6	10.72	10.37	12.55
Total n:3	1.18	1.48	5.13
n:6/n:3 ratio	9:1	7:1	2.5:1

1 Only the major fatty acids are shown.
2 ND, not detectable.
3 ANOVA test results, *=$p<0.05$ vs. control diet fed animals.

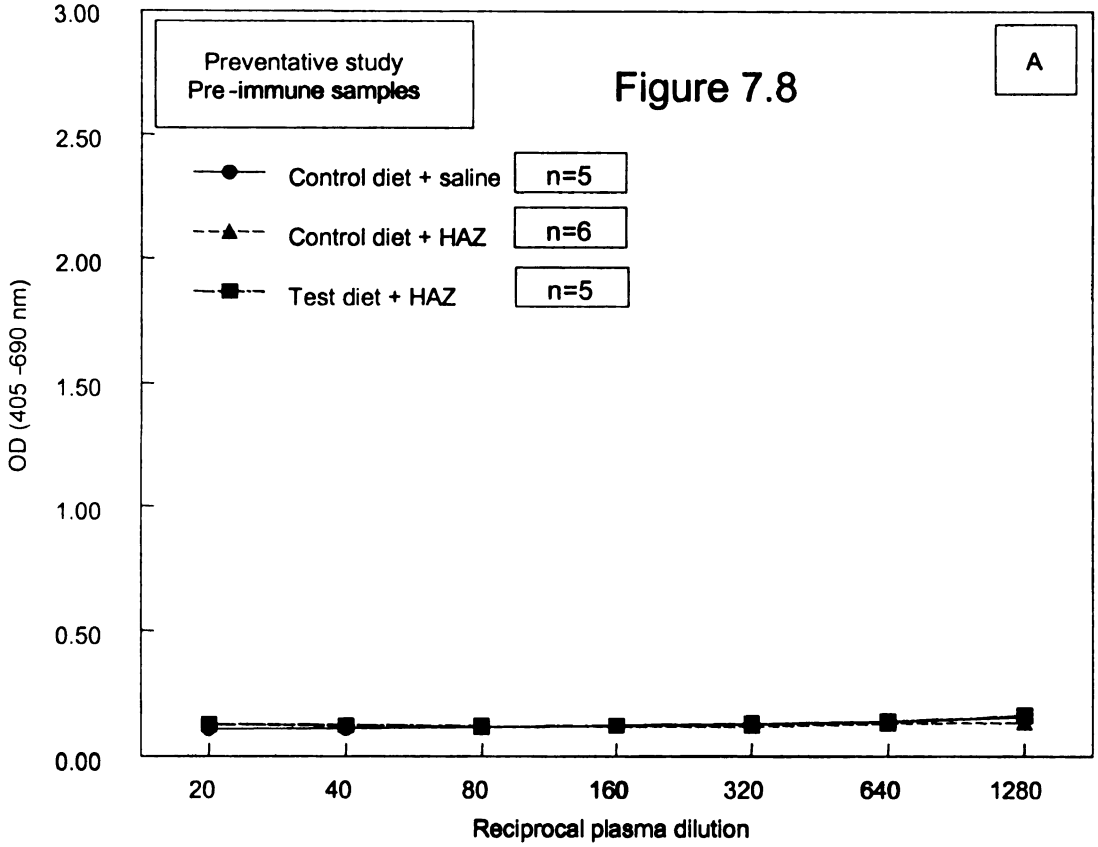

Preventative study
Pre-immune samples
A
Control diet + saline
n=5
Control diet + HAZ
n=6
Test diet + HAZ
n=5
OD (405 -690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.8

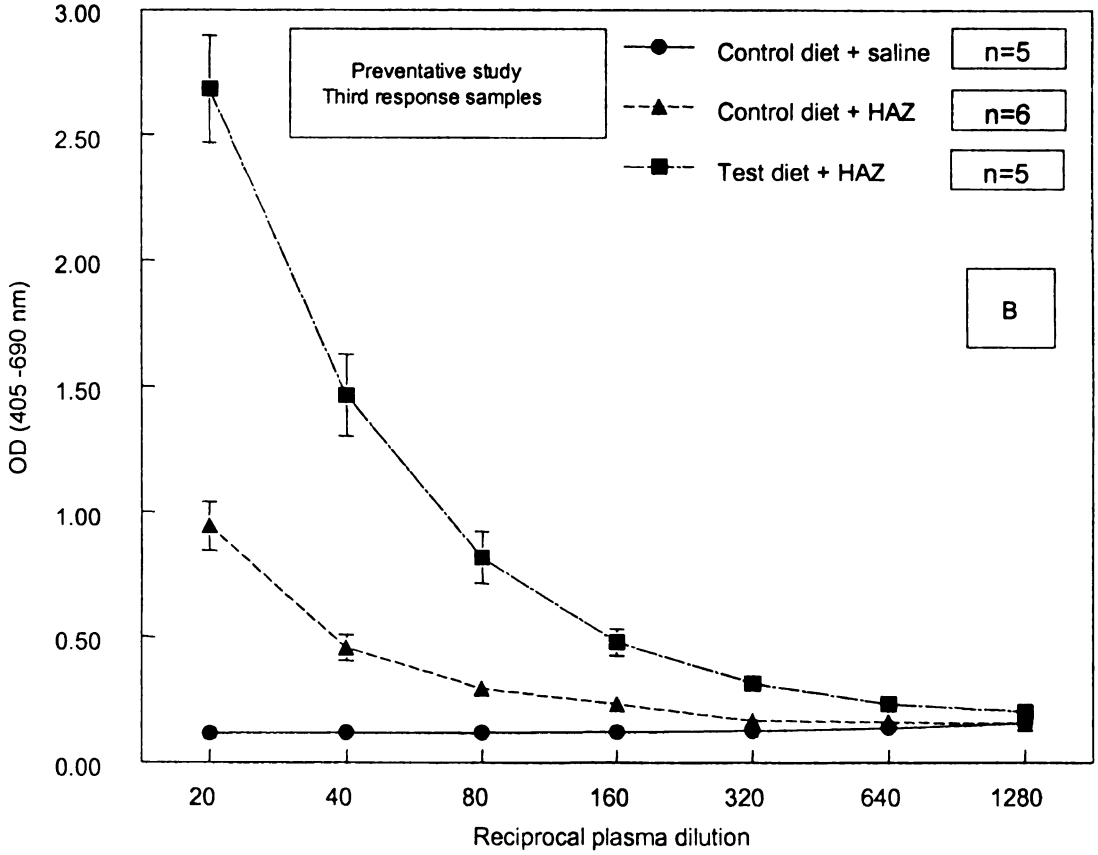

Preventative study
Third response samples
Control diet + saline
n=5
Control diet + HAZ
n=6
Test diet + HAZ
n=5
B
OD (405 -690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.8 (A-B): Characterization of hazelnut specific IgE antibody responses in plasma from BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (preventative study).

Groups of mice (n=5-6/group) were started on their respective diets and feed for 4 weeks prior to being transdermaly exposed to LPS-free hazelnut protein extracts or saline as shown in figure 7.7. Optimized ELISA measured hazelnut binding specific IgE antibodies. Pre-immune: plasma collected before exposure; 3^{rd} response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures; 7^{th} week of rest: plasma samples after seven weeks of rest following the 6 weeks of transdermal exposure. Data shows hazelnut specific IgE antibody levels as OD (mean +/- SE). At some points error bars are not visible. These animals were on their respective diets over the entire experiment (-4weeks, until end of experiment). ANOVA test results: $p<0.05$ for graphs B, C, and D for test diet vs. control diet, $p<0.05$ for graphs B, C, and D for both the hazelnut sensitized groups vs. control (saline) sensitized group.

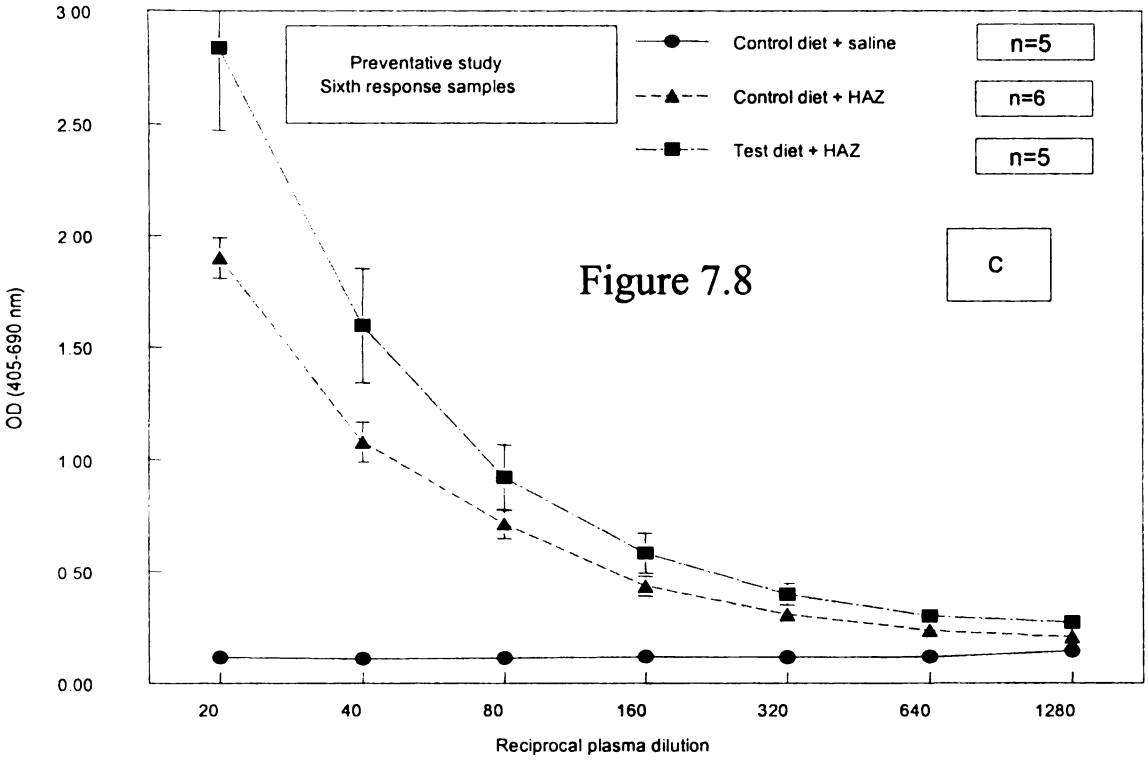

Preventative study
Sixth response samples
Control diet + saline
Control diet + HAZ
Test diet + HAZ
n=5
n=6
n=5
C
OD (405-690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.8

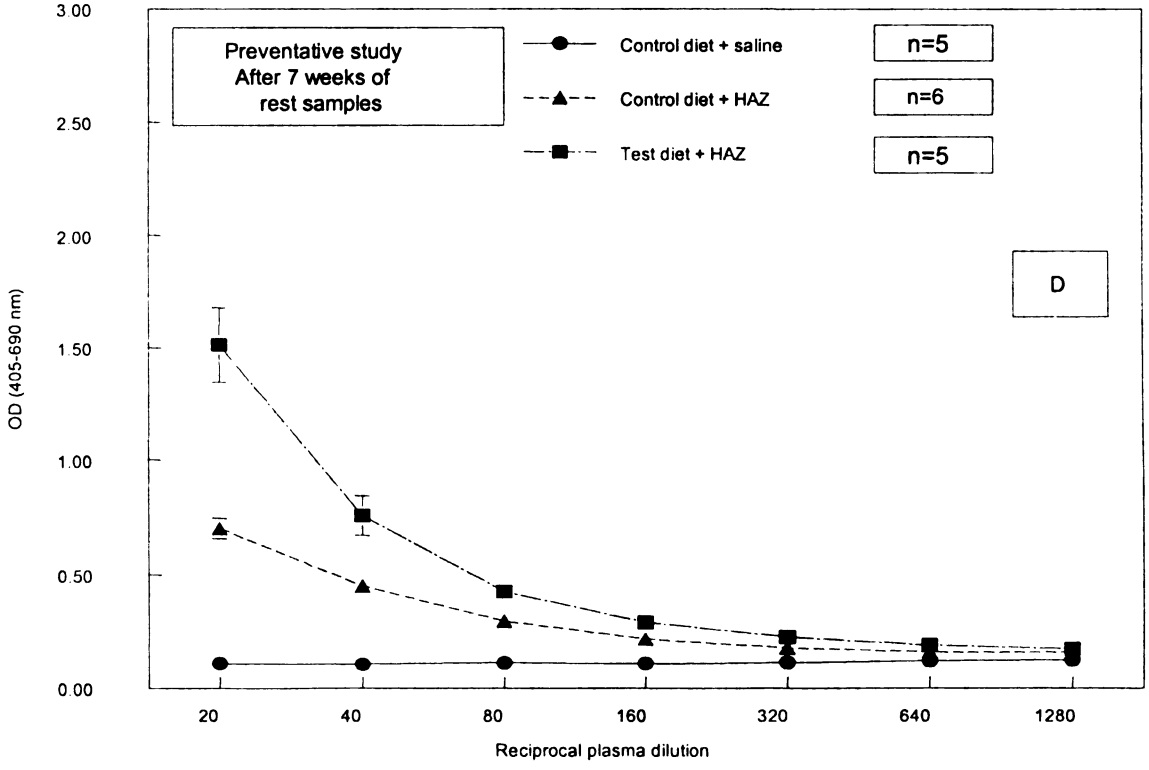

Preventative study
After 7 weeks of
rest samples
Control diet + saline
Control diet + HAZ
Test diet + HAZ
n=5
n=6
n=5
D
OD (405-690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.8 (C-D): Characterization of hazelnut specific IgE antibody responses in plasma from BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (preventative study).

Groups of mice (n=5-6/group) were started on their respective diets and feed for 4 weeks prior to being transdermaly exposed to LPS-free hazelnut protein extracts or saline as shown in figure 7.7. Optimized ELISA measured hazelnut binding specific IgE antibodies. Pre-immune: plasma collected before exposure; 3^{rd} response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures; 7^{th} week of rest: plasma samples after seven weeks of rest following the 6 weeks of transdermal exposure. Data shows hazelnut specific IgE antibody levels as OD (mean +/- SE). At some points error bars are not visible. These animals were on their respective diets over the entire experiment (-4weeks, until end of experiment). ANOVA test results: $p<0.05$ for graphs B, C, and D for test diet vs. control diet, $p<0.05$ for graphs B, C, and D for both the hazelnut sensitized groups vs. control (saline) sensitized group.

Figure 7.9

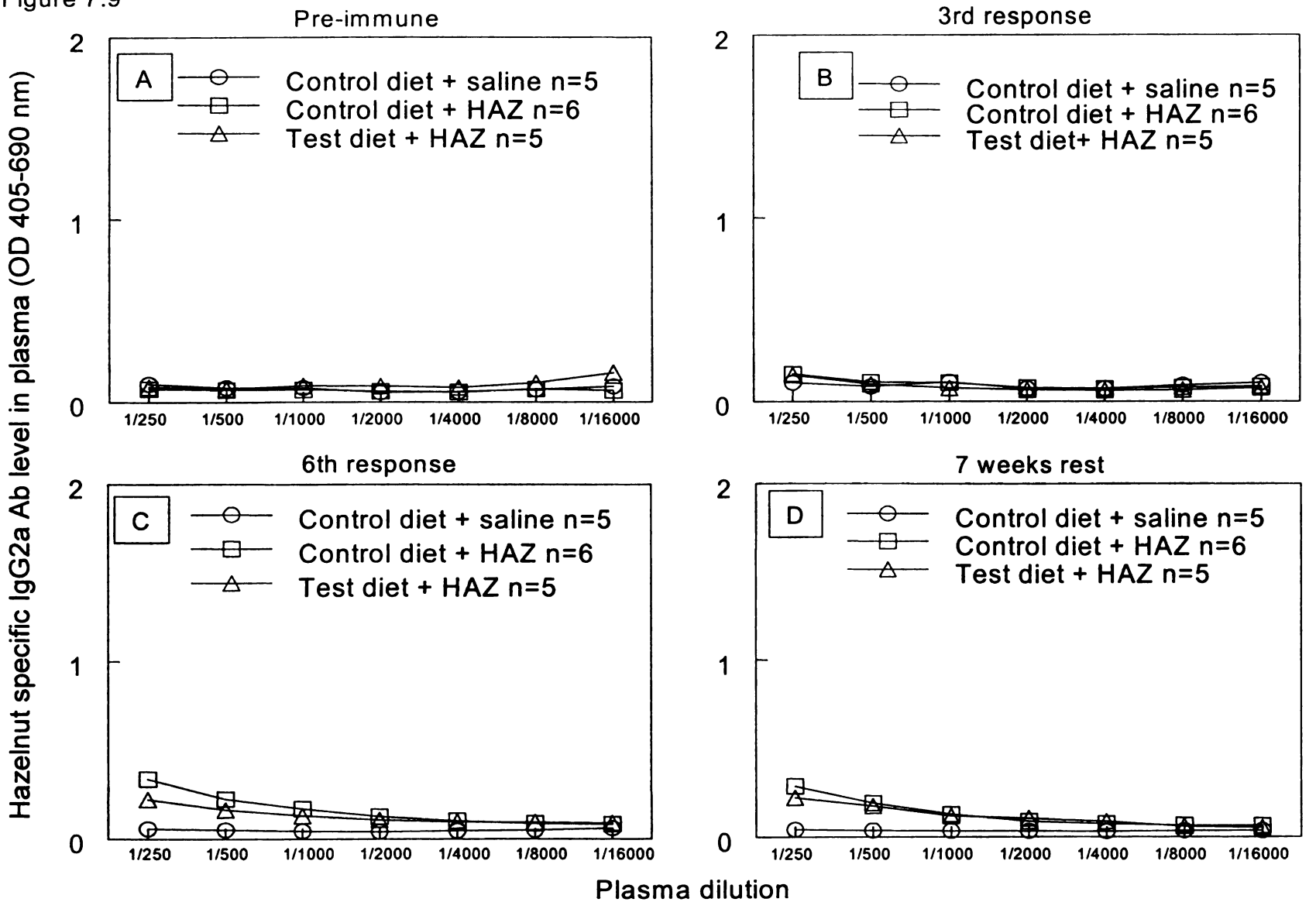

Pre-immune
3rd response
6th response
7 weeks rest
A
B
C
D
Control diet + saline n=5
Control diet + HAZ n=6
Test diet + HAZ n=5
Test diet+ HAZ n=5
Hazelnut specific IgG2a Ab level in plasma (OD 405-690 nm)
Plasma dilution
0
1
2
1/250
1/500
1/1000
1/2000
1/4000
1/8000
1/16000

Figure 7.9 (A-D): Characterization of hazelnut specific IgG2a antibody responses in plasma from BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (preventative study).

Groups of mice (n=5-6/group) were started on their respective diets and feed for 4 weeks prior to being transdermaly exposed to LPS-free hazelnut protein extracts or saline as shown in figure 7.7. Optimized ELISA measured hazelnut binding specific IgG2a antibodies. Pre-immune: plasma collected before exposure; 3^{rd} response: plasma collected after 3 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures; 7^{th} week of rest: plasma samples after seven weeks of rest following the 6 weeks of transdermal exposure. Data shows hazelnut specific IgE antibody levels as OD (mean +/- SE). At some points error bars are not visible. These animals were on their respective diets over the entire experiment (-4weeks until end). ANOVA test results: $p<0.05$ for graphs C and D for both the hazelnut-sensitized groups vs. control (saline) sensitized group.

Figure 7.10

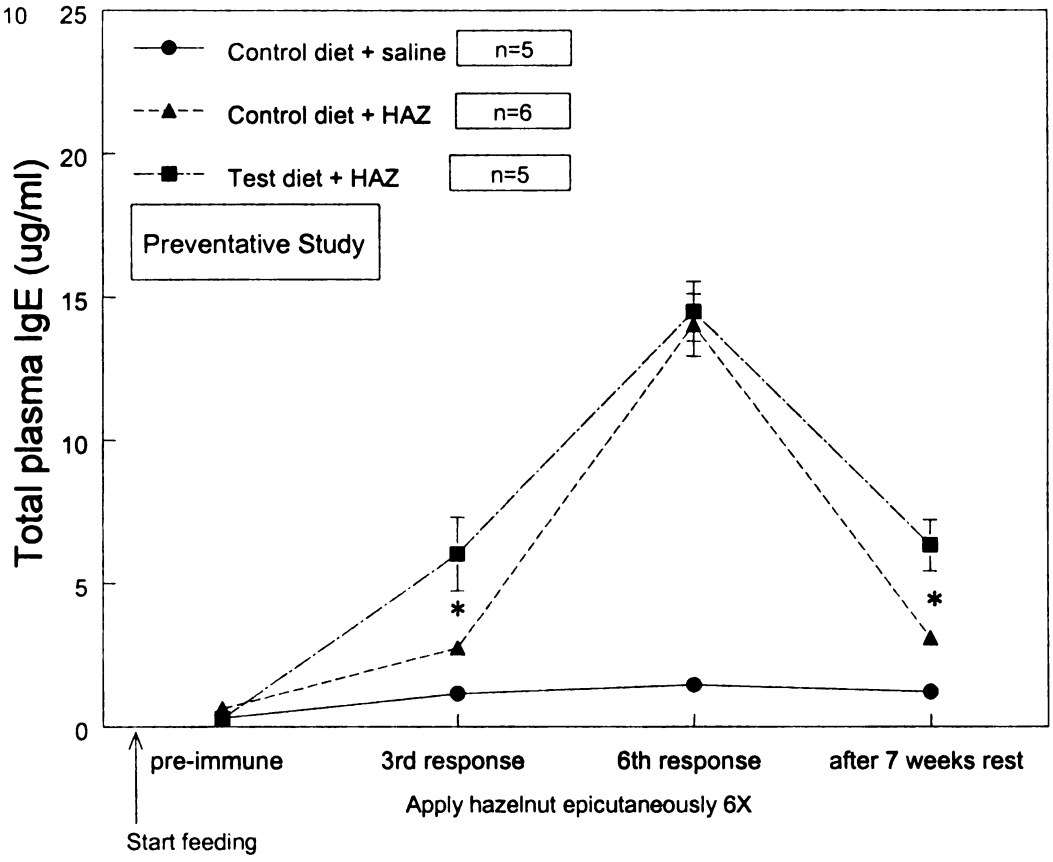


Figure 7.10: Characterization of Total IgE responses in BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet. Samples from the preventative study were analyzed for total IgE. Total IgE is expressed in μg/ml of plasma. ANOVA test results: *= $p<0.05$ for test diet vs. control diet at 3rd response and after 7 weeks of rest.

Figure 7.11

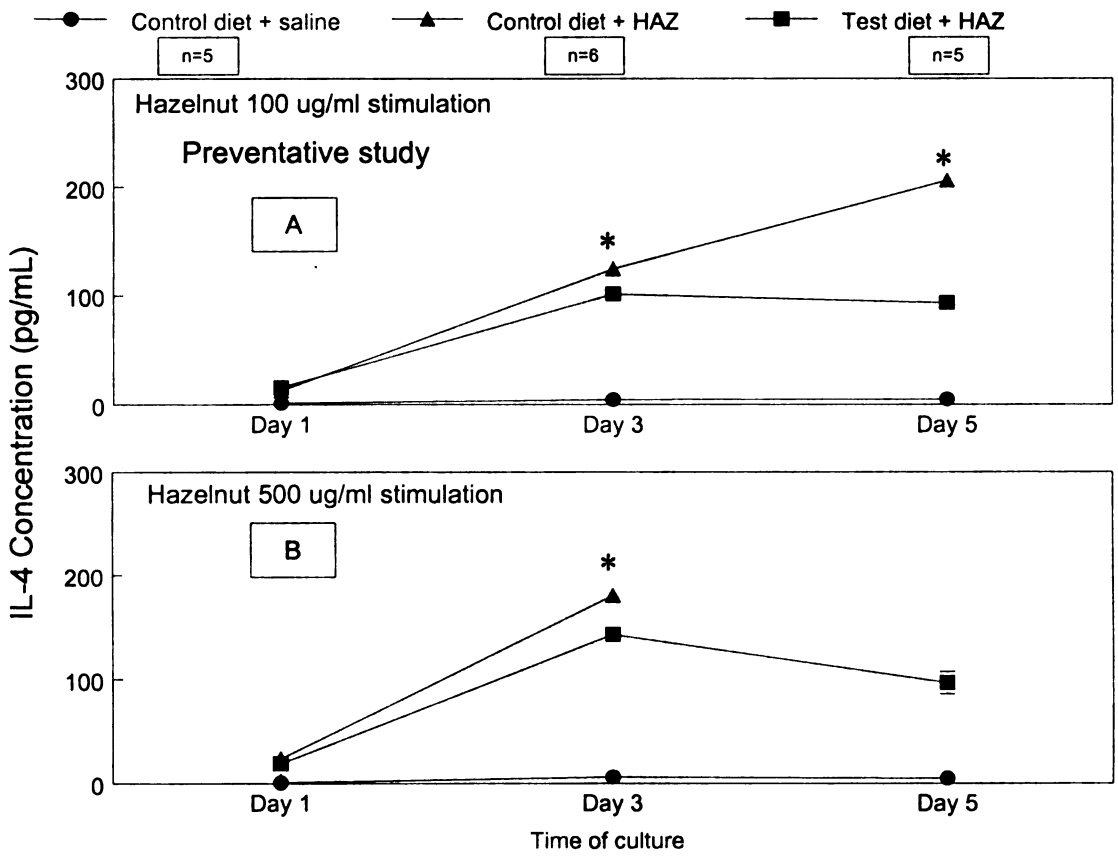


Figure 7.11 (A-B) Hazelnut driven Type-2 cytokine (IL-4) responses in mice transdermally sensitized with hazelnut protein: effect of experimental diets

Groups of BALB/c mice (n=5-6/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 7.7. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from the fish oil preventative study mice (A, B). ANOVA test results: for all comparisons of hazelnut sensitized mice vs. saline control $p<0.05$. *= $p<0.05$ for test diet vs. control diet at marked time points.

Figure 7.12

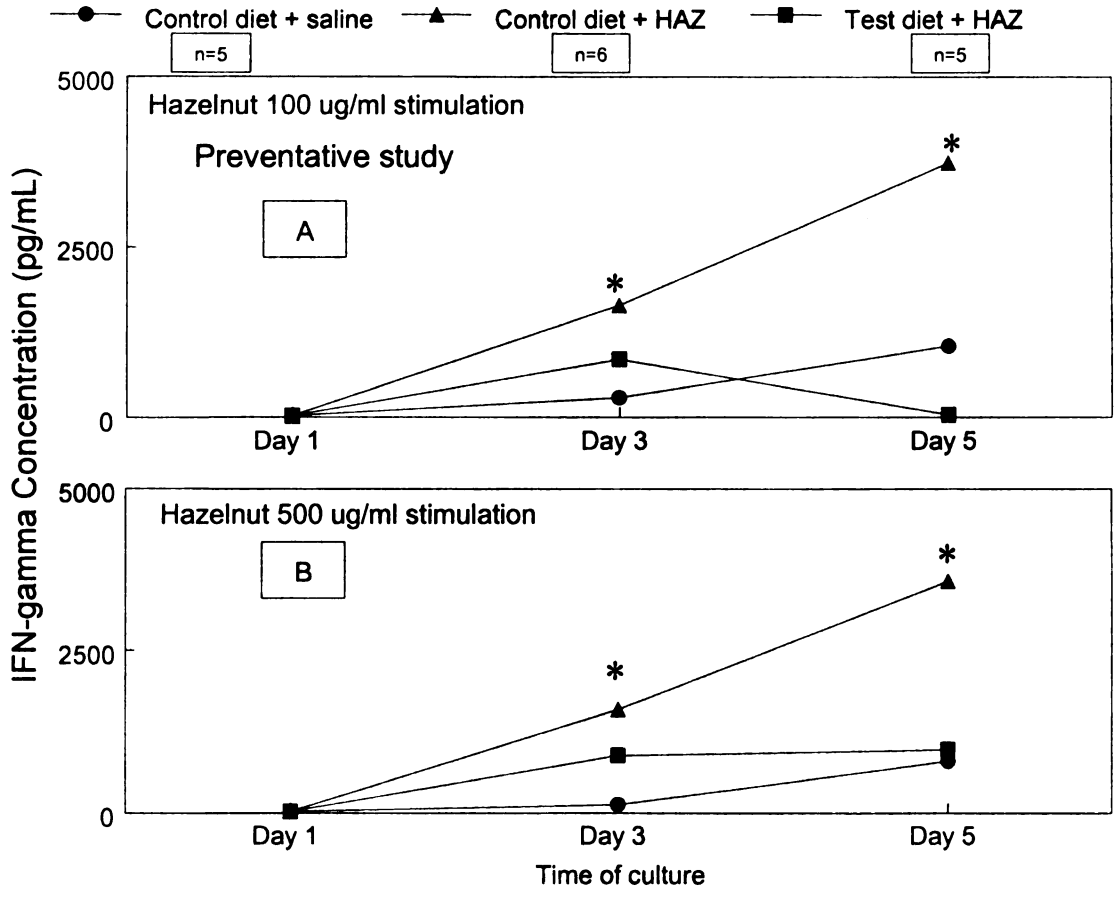


Figure 7.12 (A-B) Hazelnut driven Type-1 cytokine (IFN-γ) responses in mice transdermally sensitized with hazelnut protein: effect of experimental diets

Groups of BALB/c mice (n=5-6/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 7.7. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from the fish oil preventative study mice (A, B). ANOVA test results: *= $p<0.05$ for D3 and D5 test diet + HAZ vs. control diet + HAZ.

Figure 7.13

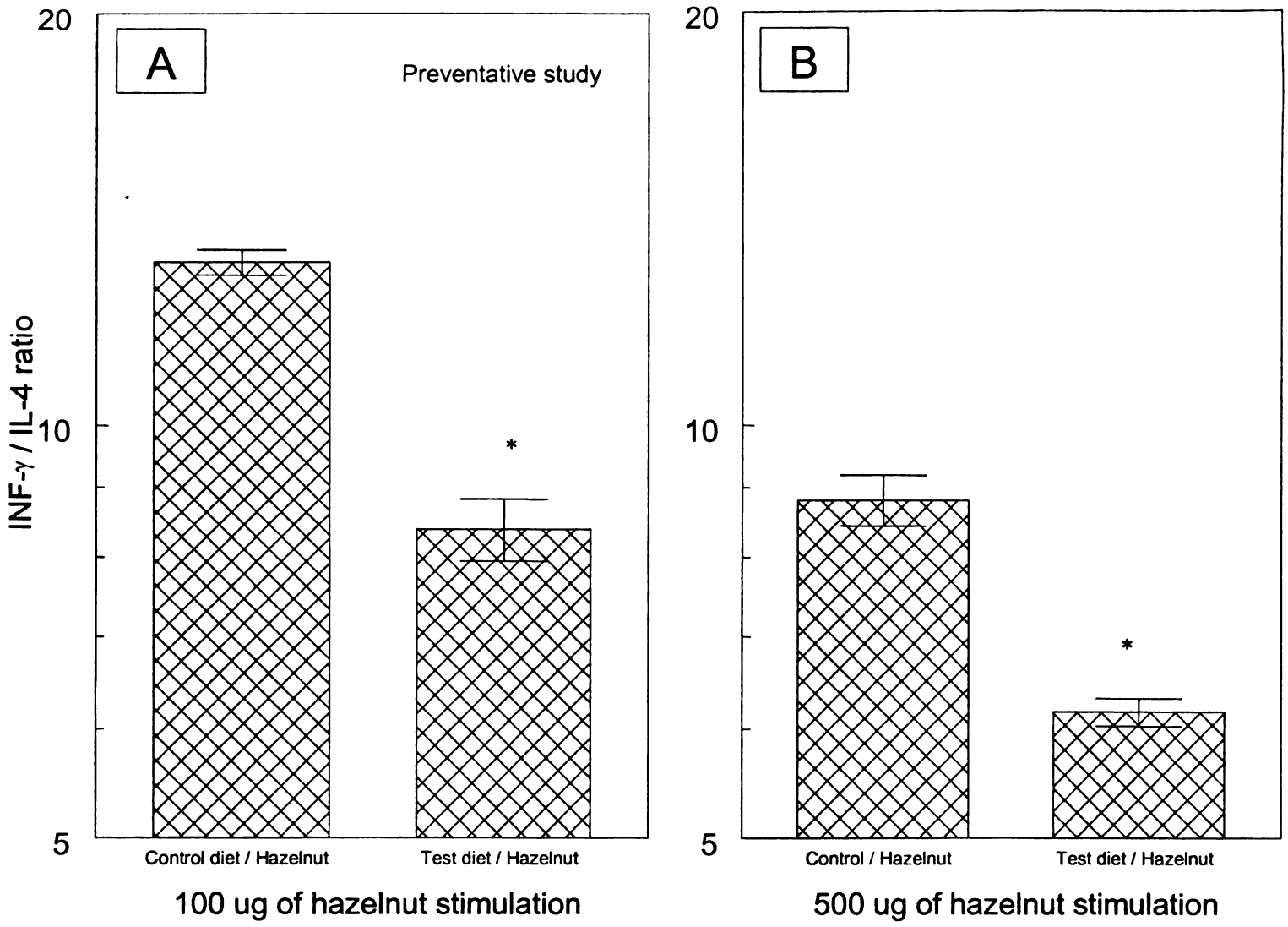
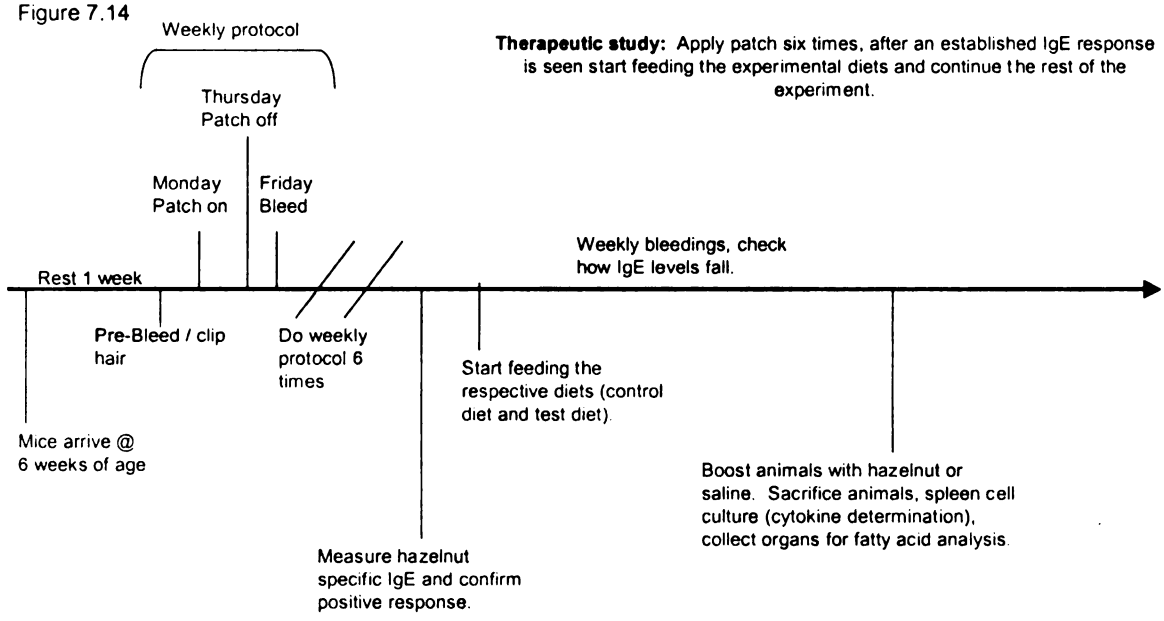

A
Preventative study
B
20
10
5
INF-γ / IL-4 ratio
*
*
Control diet / Hazelnut
Test diet / Hazelnut
Control / Hazelnut
Test diet / Hazelnut
100 ug of hazelnut stimulation
500 ug of hazelnut stimulation

Figure 7.13: IFN-γ / IL-4 ratio in mice transdermally sensitized with hazelnut protein: Effect of experimental diets.

Groups of BALB/c mice (n=5-6/group) were sensitized with hazelnut (500 μg per mouse) by transdermal exposure as described in Figure 7.7. Three days following a booster exposure with hazelnut, spleen cells were harvested and cultured with indicated dose of hazelnut protein. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is D3 of IFN-γ / D3 of IL-4 response for both 100 μg and 500 μg hazelnut stimulation (average +/- SE of duplicate analyses of data) from the fish oil preventative study mice. T-test results: * = $p<0.05$ for test diet vs. control diet for both 100 and 500 μg hazelnut stimulation.

Figure 7.14



Group	n=	Sensitized with	Diet fed prior to and during sensitization	Diet fed after sensitization
1	4	Hazelnut	Chow	Test diet
2	4	Hazelnut	Chow	Control diet
3	4	Saline	Chow	Control diet

Figure 7.14: Transdermal sensitization protocol to hazelnut protein: therapeutic study. The schedule of cycles of hazelnut sensitization and testing are shown for the therapeutic study.

Table 7.4

Spleen phospholipid fatty acid composition in mice fed various PUFA[1,2]

	Control diet + hazelnut	Control diet + hazelnut	Test diet + Hazelnut
		g/100g total phospholipids fatty acid	
Therapeutic study			
16:0	16.29 +/- 2.98	11.09 +/- 2.38	22.22 +/- 1.82
18:0	6.56 +/- 1.14	7.41 +/- 0.79	8.83 +/- 0.92
18:1	2.94 +/- 0.23	4.68 +/- 0.49	4.57 +/- 0.28
18:2 (n:6)	2.92 +/- 0.15	4.07 +/- 0.21	5.01 +/- 0.31
20:4 (n:6)	4.48 +/- 0.21	4.07 +/- 0.12	9.23 +/- 0.42 *
20:5 (n:3)	ND	ND	4.62 +/- 0.34 *
22:6 (n:3)	0.94 +/- 0.08	0.99 +/- 0.11	1.92 +/- 0.14 *
Total n:6	7.4	8.14	14.24
Total n:3	0.94	0.99	6.54
n:6/n:3 ratio	8:1	8:1	2.2:1

1 Only the major fatty acids are shown.
2 ND, not detectable.
3 ANOVA test results, *=$p<0.05$ vs. control diet fed animals.

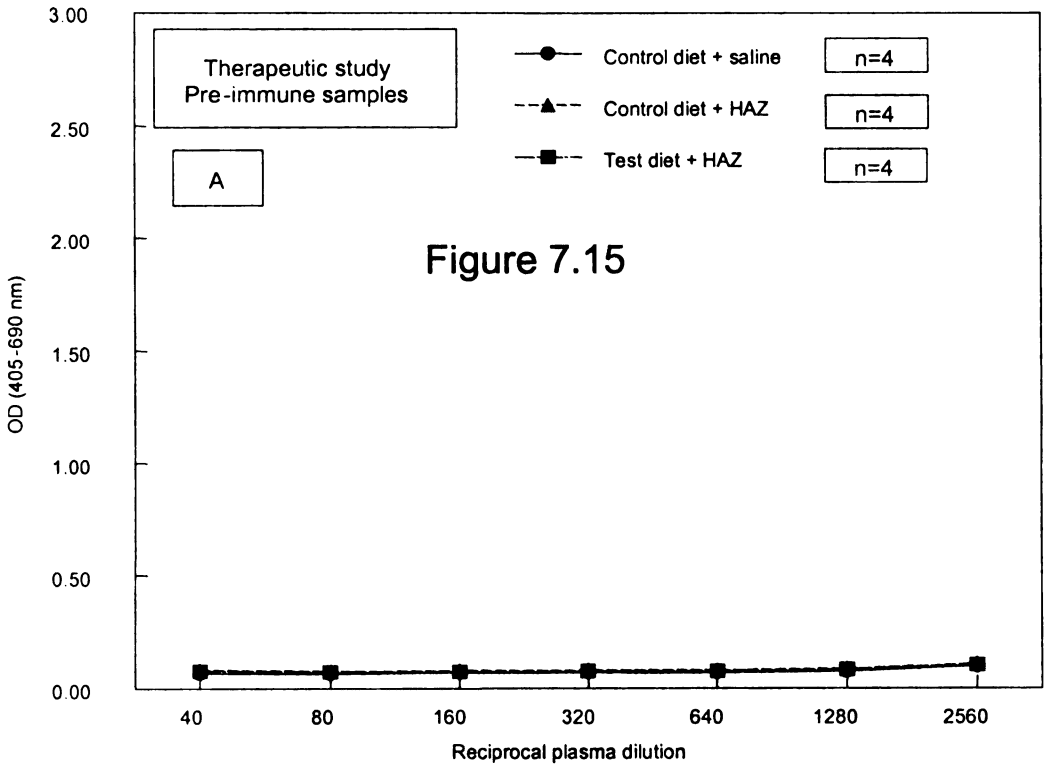

Therapeutic study
Pre-immune samples
A
Control diet + saline
n=4
Control diet + HAZ
n=4
Test diet + HAZ
n=4
OD (405-690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
40
80
160
320
640
1280
2560
Reciprocal plasma dilution

Figure 7.15

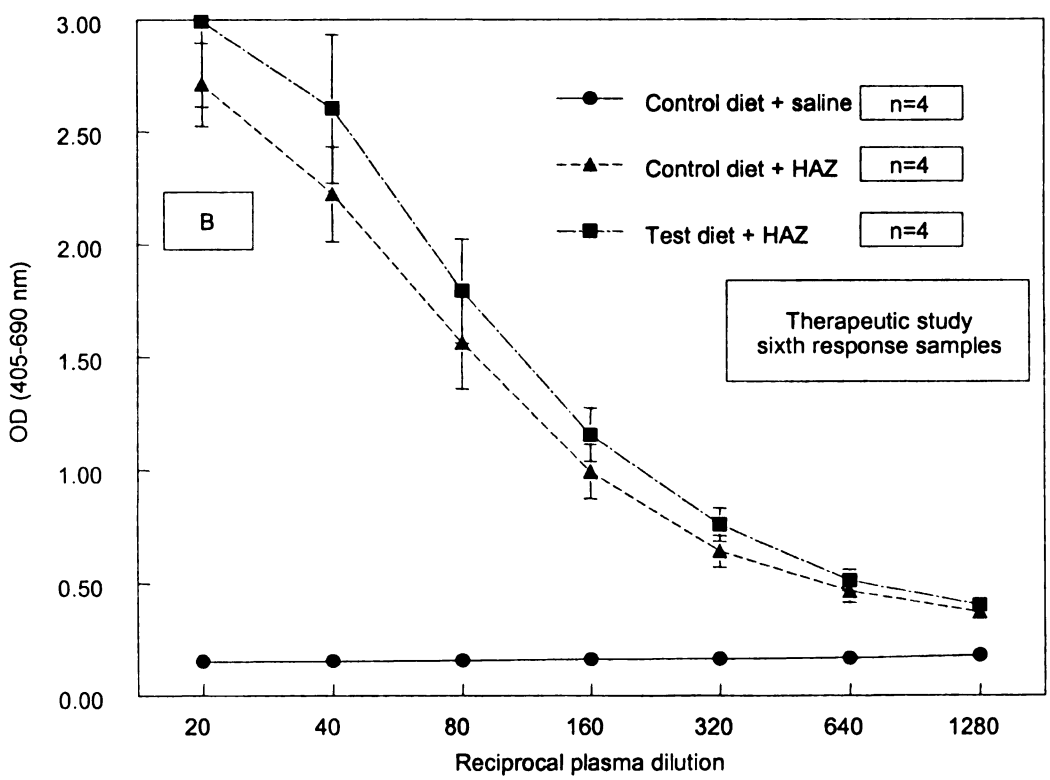

B
Control diet + saline
n=4
Control diet + HAZ
n=4
Test diet + HAZ
n=4
Therapeutic study
sixth response samples
OD (405-690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.15 (A-B): Characterization of hazelnut specific IgE antibody responses in plasma from BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (therapeutic study).

Groups of mice (n=4/group) were transdermaly exposed to LPS-free hazelnut protein extracts or saline as shown in figure 7.14. Optimized ELISA measured hazelnut binding specific IgE antibodies. Following transdermal exposure groups of animals were given their respective diets. Pre-immune: plasma collected before exposure; 6^{th} response: plasma collected after 6 transdermal exposures; 4^{th} week of rest: plasma samples after four weeks of rest following the 6 weeks of transdermal exposure; 7^{th} week of rest: plasma samples after seven weeks of rest following the 6 weeks of transdermal exposure. Data shows hazelnut specific IgE antibody levels as OD (mean +/- SE). At some points error bars are not visible. These animals were on their respective diets only after the sensitization process was over.

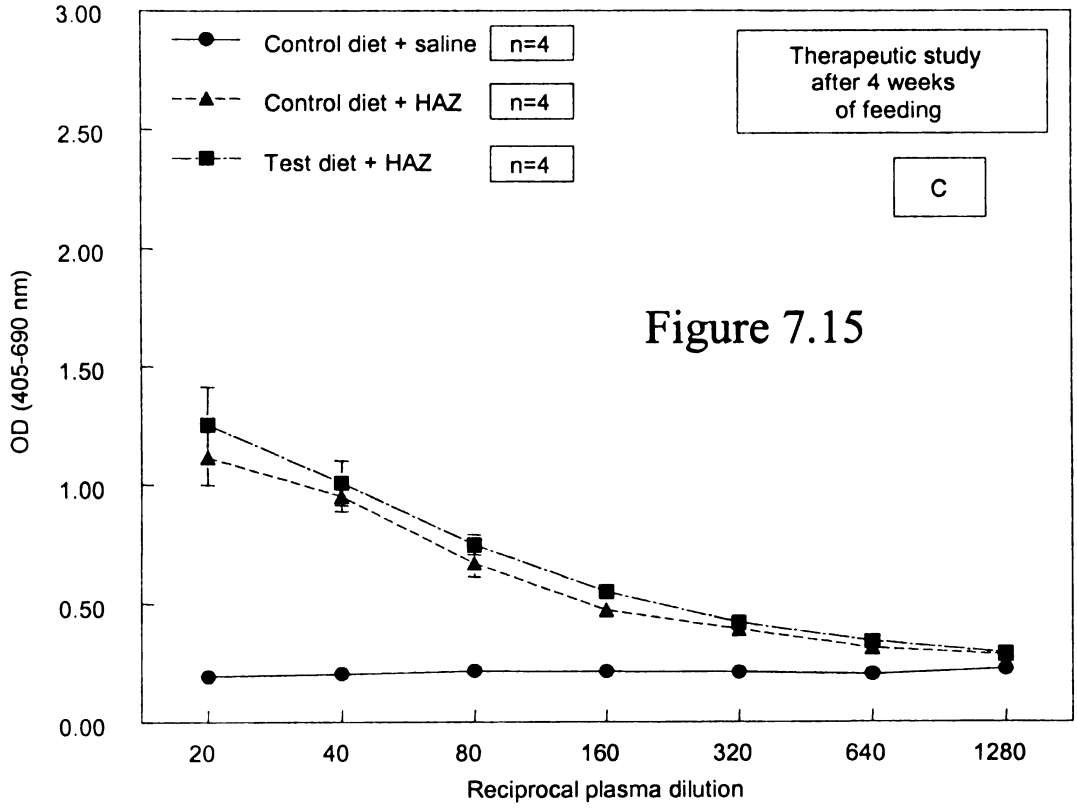

Control diet + saline
n=4
Control diet + HAZ
n=4
Test diet + HAZ
n=4
Therapeutic study after 4 weeks of feeding
C
OD (405-690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.15

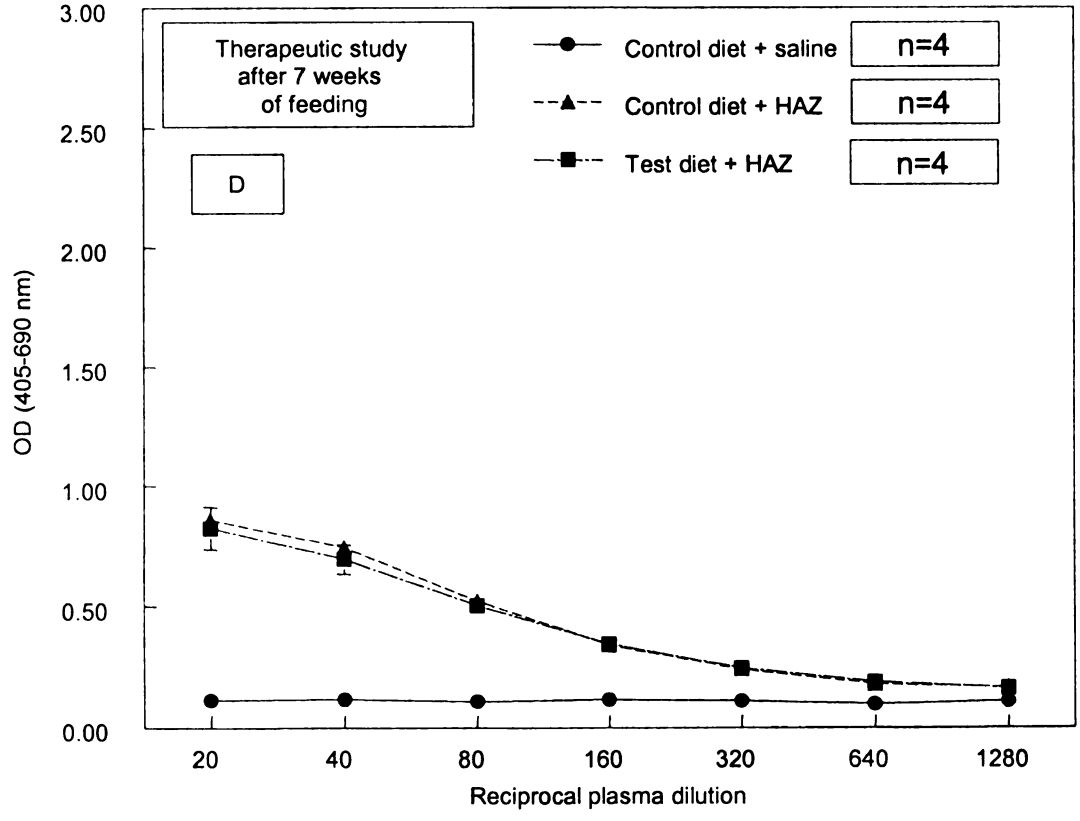

Therapeutic study after 7 weeks of feeding
D
Control diet + saline
n=4
Control diet + HAZ
n=4
Test diet + HAZ
n=4
OD (405-690 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
40
80
160
320
640
1280
Reciprocal plasma dilution

Figure 7.15 (C-D): Characterization of hazelnut specific IgE antibody responses in plasma from BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (therapeutic study).

Groups of mice (n=4/group) were transdermaly exposed to LPS-free hazelnut protein extracts or saline as shown in figure 7.14. Optimized ELISA measured hazelnut binding specific IgE antibodies. Following transdermal exposure groups of animals were given their respective diets. Pre-immune: plasma collected before exposure; 6^{th} response: plasma collected after 6 transdermal exposures; 4^{th} week of rest: plasma samples after four weeks of rest following the 6 weeks of transdermal exposure; 7^{th} week of rest: plasma samples after seven weeks of rest following the 6 weeks of transdermal exposure. Data shows hazelnut specific IgE antibody levels as OD (mean +/- SE). At some points error bars are not visible. These animals were on their respective diets only after the sensitization process was over.

Figure 7.16

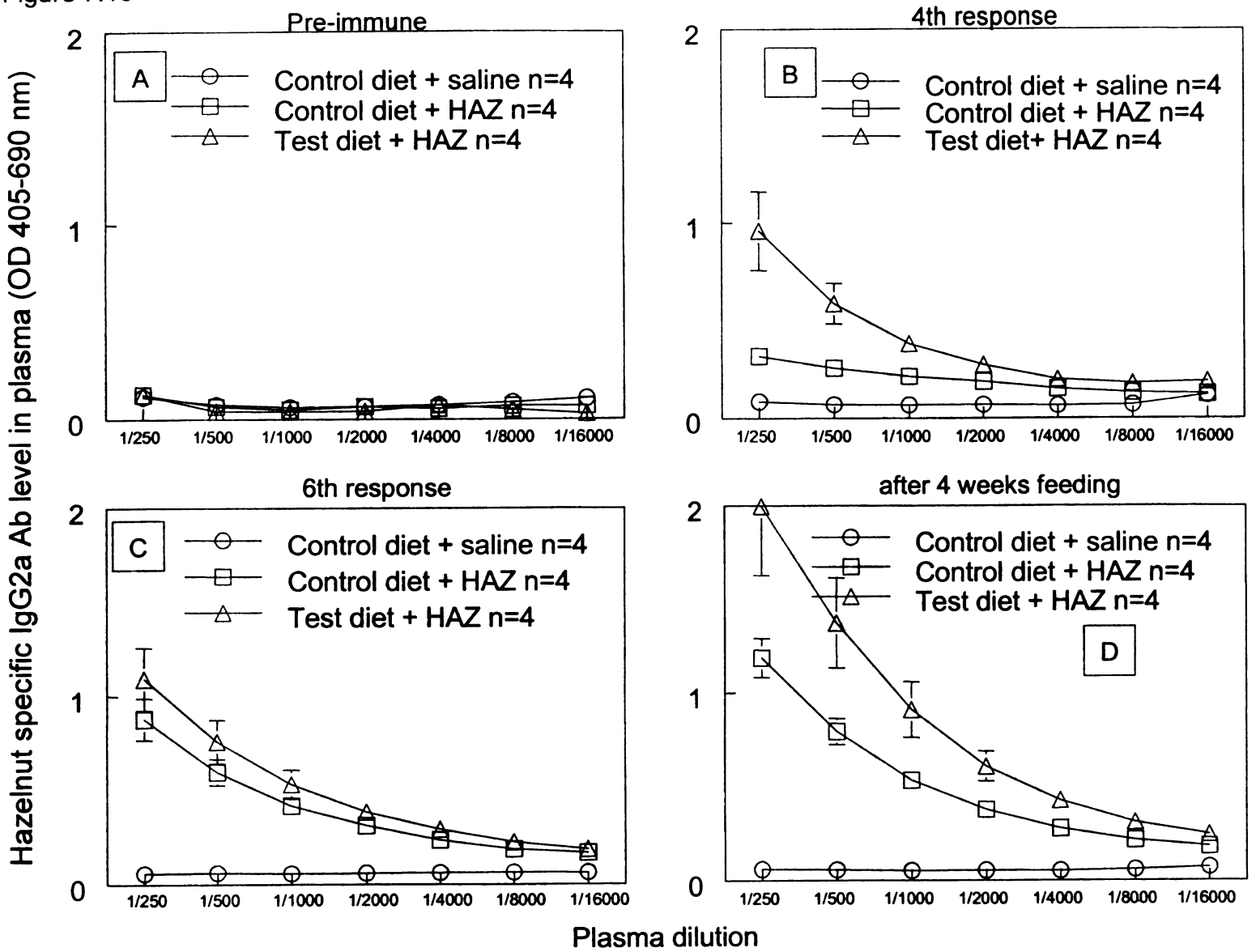

Pre-immune
A
Control diet + saline n=4
Control diet + HAZ n=4
Test diet + HAZ n=4
4th response
B
Control diet + saline n=4
Control diet + HAZ n=4
Test diet+ HAZ n=4
6th response
C
Control diet + saline n=4
Control diet + HAZ n=4
Test diet + HAZ n=4
after 4 weeks feeding
D
Control diet + saline n=4
Control diet + HAZ n=4
Test diet + HAZ n=4
Hazelnut specific IgG2a Ab level in plasma (OD 405-690 nm)
0
1
2
1/250
1/500
1/1000
1/2000
1/4000
1/8000
1/16000
Plasma dilution

Figure 7.16 (A-D): Characterization of hazelnut specific IgG2a antibody responses in plasma from BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (therapeutic study).

Groups of mice (n=4/group) were started on their respective diets and feed for 4 weeks prior to being transdermaly exposed to LPS-free hazelnut protein extracts or saline as shown in figure 7.14. Optimized ELISA measured hazelnut binding specific IgG2a antibodies. Pre-immune: plasma collected before exposure; 4^{th} response: plasma collected after 4 transdermal exposures; 6^{th} response: plasma collected after 6 transdermal exposures; after 4 weeks of feeding: plasma samples after four weeks of feeding experimental diets following the 6 weeks of transdermal exposure. Data shows hazelnut specific IgG2a antibody levels as OD (mean +/- SE). At some points error bars are not visible. These animals were on their respective diets only after the sensitization process was over.

Figure 7.17

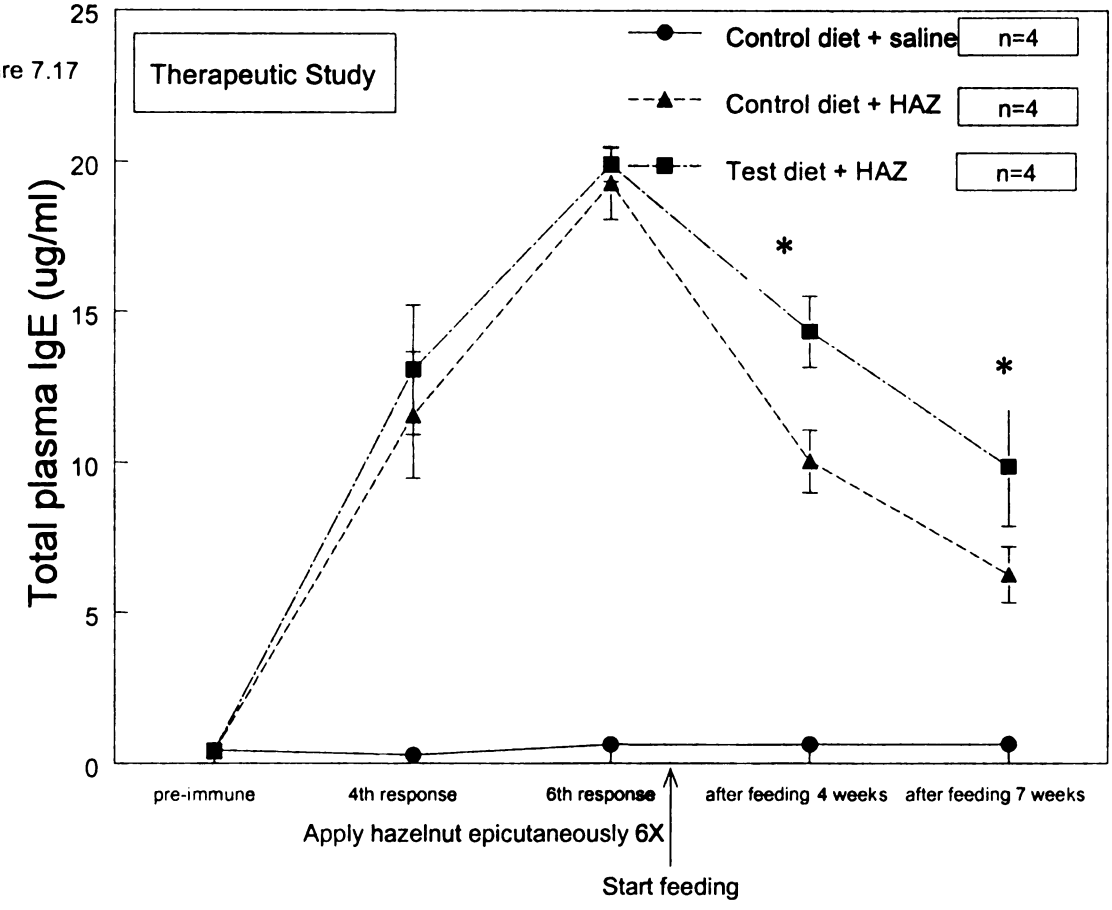


Figure 7.17: Characterization of Total IgE responses in BALB/c mice fed the test diet enriched with EPA and DHA vs. control diet (therapeutic study). Samples from the therapeutic study were analyzed for total IgE. Total IgE is expressed in μg/ml of plasma. ANOVA test results: * = $p<0.05$ for test diet + HAZ vs. control diet + HAZ for the after 4 weeks of feeding and after 7 weeks of feeding samples.

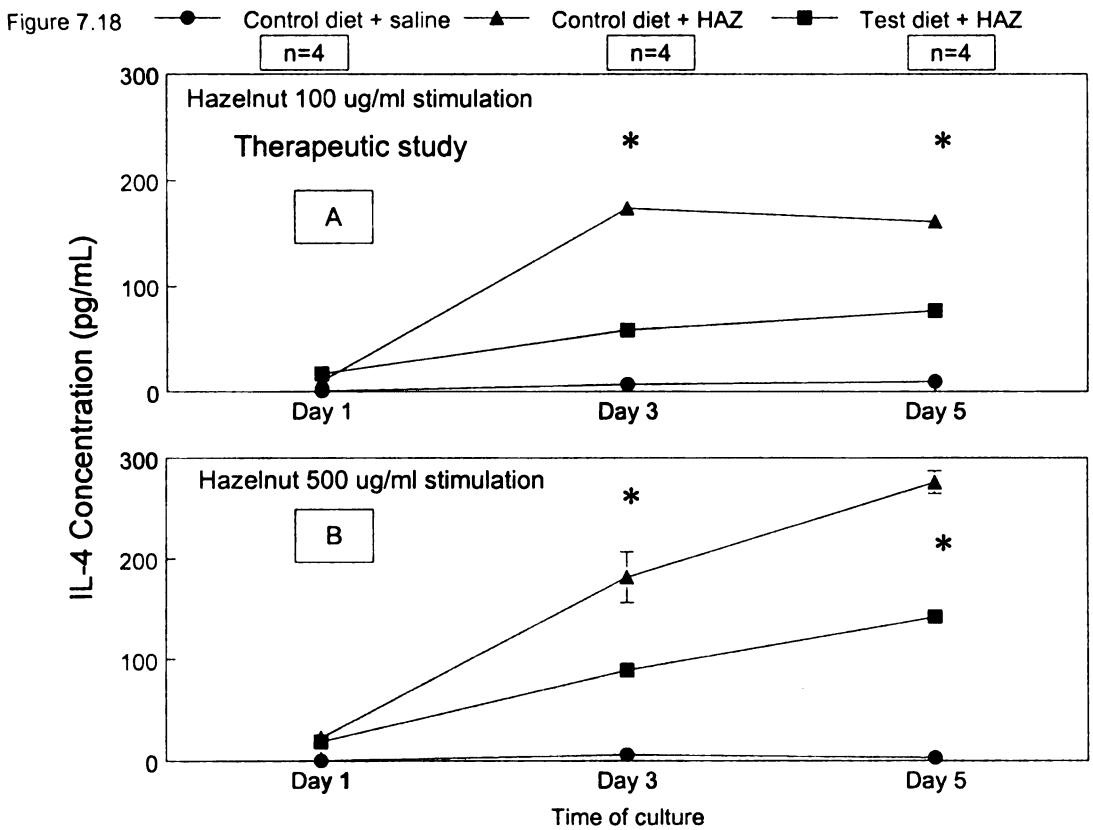


Figure 7.18 (A-B) Hazelnut driven Type-2 cytokine (IL-4) responses in mice transdermally sensitized with hazelnut protein: Effect of experimental diets. Groups of BALB/c mice (n=4/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 7.14. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from the fish oil therapeutic study mice (A, B). ANOVA test results: for all comparisons of hazelnut sensitized mice vs. saline control $p<0.05$. Also, * = $p<0.05$ for comparisons of test diet + HAZ vs. Control diet + HAZ for D3 and D5.

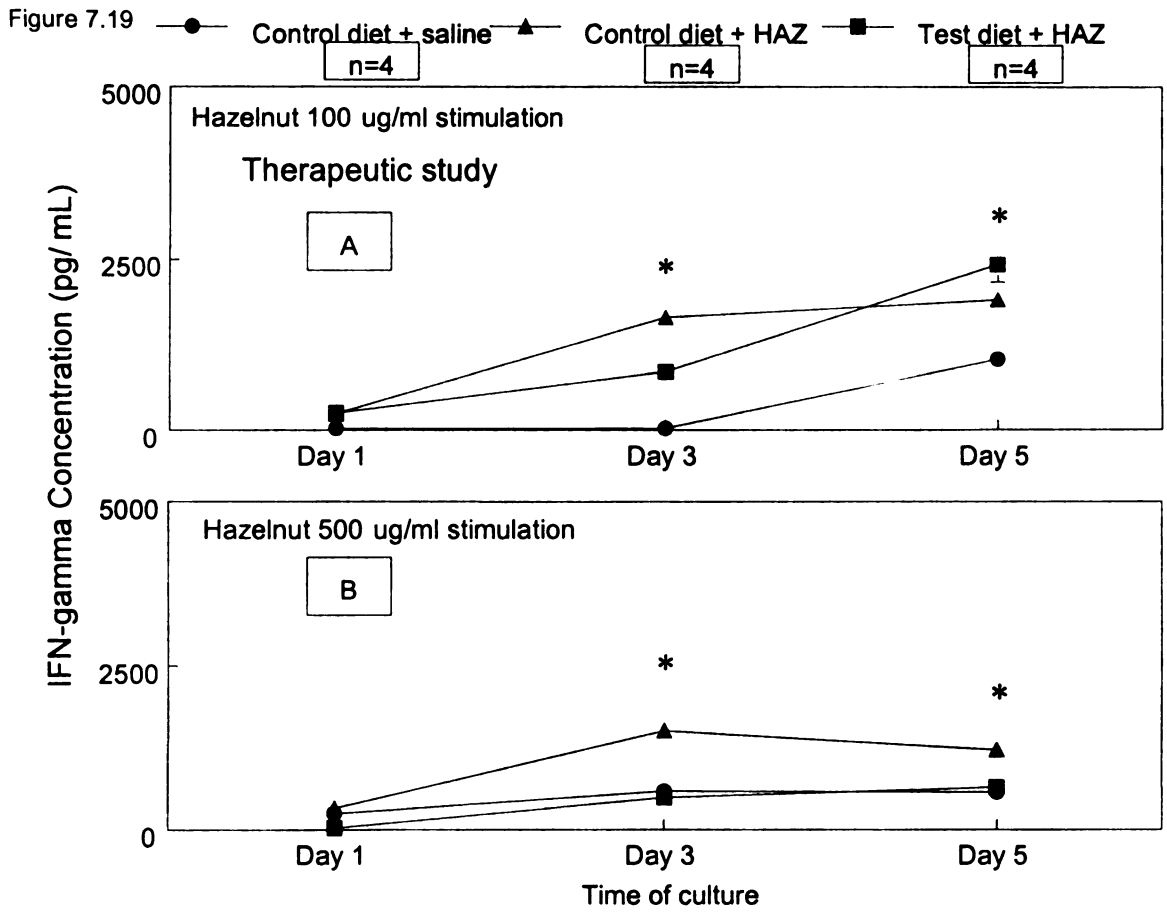


Figure 7.19 (A-B) Hazelnut driven Type-1 cytokine (IFN-γ) responses in mice transdermally sensitized with hazelnut protein: Effect of experimental diets.

Groups of BALB/c mice (n=4/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 7.14. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is peak cytokine response (average +/- SE of duplicate analyses of data) from the fish oil therapeutic study mice (A, B). ANOVA test results: for comparisons of test diet + HAZ vs. control diet + HAZ, * = $p<0.05$ for D3 and D5 for 500 μg stimulation and D3 for 100 μg stimulation.

Figure 7.20

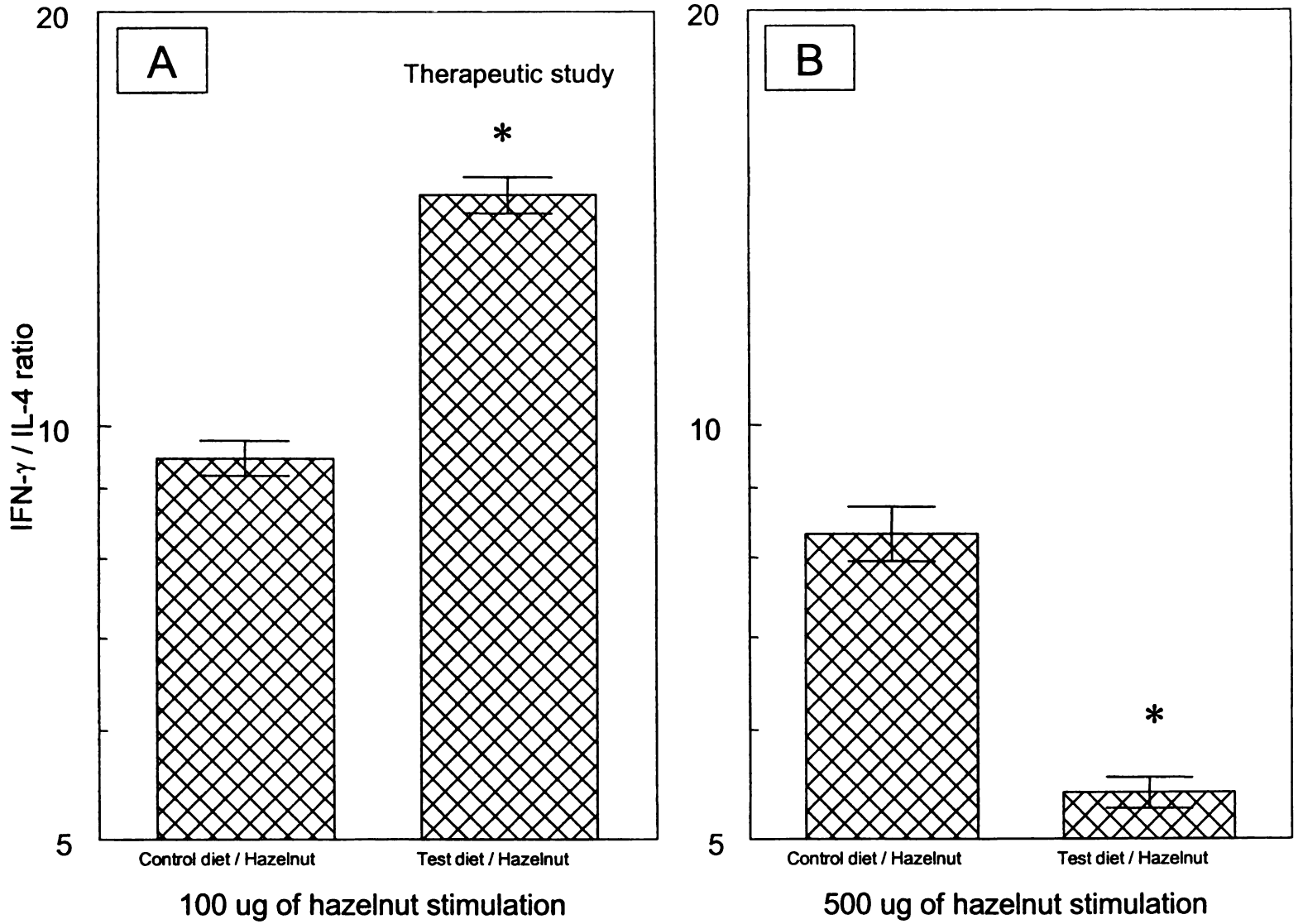

A
Therapeutic study
*
20
10
5
IFN-γ / IL-4 ratio
Control diet / Hazelnut
Test diet / Hazelnut
100 ug of hazelnut stimulation
B
20
10
5
*
Control diet / Hazelnut
Test diet / Hazelnut
500 ug of hazelnut stimulation

Figure 7.20: IFN-γ / IL-4 ratio in mice transdermally sensitized with hazelnut protein.

Groups of BALB/c mice (n=4/group) were sensitized with hazelnut (500 μg per mouse) or saline by transdermal exposure as described in Figure 7.14. Three days following a booster exposure with hazelnut or saline, spleen cells were harvested and cultured with indicated dose of hazelnut protein or culture medium alone. Cell culture supernatants were harvested at different time points over 5 days and analyzed for cytokines using optimized ELISA. Data shown is D3 of IFN-γ / D3 of IL-4 response for both 100 μg and 500 μg hazelnut stimulation (average +/- SE of duplicate analyses of data) from the fish oil therapeutic study mice. T-test results: Test diet + HAZ vs. Control diet + HAZ $p<0.05$ for both 100 and 500 μg hazelnut stimulation.

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CHAPTER EIGHT

8.0 Working model and future studies

From this work, the working model so far is hazelnut applied to the skin of mice can be taken up and presented via MHC II by Langerhans' cells to CD4+ Th2-cells. This causes the CD4+ Th2-cells to produce IL-4, the class switch factor for IgE synthesis, causing B-cells to make IgE specific to hazelnut. This IgE specific to hazelnut can then bind to mast cells and basophils. Upon oral allergen exposure, the IgE on these mast cells and basophils is cross-linked causing them to degranulate, releasing mediators such as histamine. These mediators cause the signs and symptoms of systemic anaphylaxis.

With a well-characterized model of tree-nut allergy, there are further studies that can now be proposed. 1) Determine the molecular mechanisms underlying tree-nut allergy, using gene knockout mice (e.g. stat KO) could assess the role of STAT6 in Th2 development as well as systemic anaphylaxis. Also different techniques could be used, for example RNAi (RNA interference) could be used to determine the exact signals needed to trigger an allergic response. Using RNAi the role of GATA3 could be assessed to determine if this model is GATA3 dependent. 2) With this model more studies could be done assessing therapies of food allergy, either in a dietary role (n-3 fatty acids, pro-biotics) or even a pharmaceutical avenue, possibly uncovering different approaches that could be used to slow down or reverse the increasing trend of food allergy. After the n-3 (DHA and EPA) study was done, we further characterized the systemic anaphylaxis and threshold dose to cause an anaphylactic response, now we could determine if DHA and EPA enrichment could affect clinical responses. 3) The effect of different food

processing techniques on tree-nuts could be assessed to see if tree-nuts could be processed in a certain way to make them less allergenic, therefore trace amounts could possibly be tolerable to allergenic patients. 4) Using a similar approach that we used for hazelnut, the allergenicity of various genetically modified foods could be assessed. Using a number of strains of mice and an array of doses, the relative allergenicity could be determined. With this model both phases of allergy can be studied, the sensitization phase, as well as the effector phase. If something can be done to decrease either phase, there would be a win in the fight against food allergy.