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LONG-TERM CHARACTERISTICS OF HAZELNUT ALLERGY AND MILK ALLERGY IN AN ADJUVANT-FREE MOUSE MODEL

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M.S. degree in Food Science and Human Nutrition

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By

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ABSTRACT

LONG-TERM CHARACTERISTICS OF HAZELNUT ALLERGY AND MILK ALLERGY IN AN ADJUVANT-FREE MOUSE MODEL

By

Babu Gonipeta

An adverse, immune mediated clinical reaction to food protein in sensitized subjects is known as food allergy. There are two types of food allergies: those that are usually out grown (known as transient food allergy); and those that are rarely out grown (known as persistent food allergy). The immune mechanism behind transient and persistent food allergies is incompletely understood at present. To address this problem, the present study was conducted using an adjuvant-free mouse model of food allergy. In the first part, long-term characteristics of hazelnut allergy were studied. We found that hazelnut allergy once established persists for long periods (up to 8 months), due to long-lasting, memory IL-4 and IgE responses. In the second part of the study, we established an adjuvant-free mouse model of milk allergy. In the third part of the study, long-term characteristics of cow's milk allergy were studied. We found that, upon allergen withdrawal, cow's milk allergy readouts of memory IgE and IL-4 responses were short lived. Surprisingly, whey protein induced hypothermia in both healthy and allergic old mice, thus complicating analysis of milk induced systemic anaphylaxis in aged mice. These results have advanced the knowledge of immune mechanism behind persistent and transient type of food allergies in this mouse model.

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APC	antigen presenting cells
ALA	alpha lactalbumin
BLG	beta lactoglobulin
BSA	bovine serum albumin
CD	clusters of differentiation
CMA	cow's milk allergy
CMP	cow's milk protein
ELISA	enzyme linked immunosorbent assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
ng	nano grams
OD	optical density
PBS	phosphate buffered saline
pg	pico grams
SD	standard deviation
SE,	standard error
SIgE	specific IgE
Th	T helper lymphocyte

CHAPTER ONE

Introduction

Abnormal immune system-mediated clinical response to harmless environmental substance such as protein is known as allergy or immediate hypersensitivity. When this happens with food proteins it is termed as food allergy. Thus, food allergy can be defined as an adverse immune system-mediated reaction to food proteins in sensitized individuals (1).

Although food allergies are immune system mediated adverse reactions, not all adverse reactions to foods are food allergies. There are some immune system independent adverse reactions to foods, such as food intolerance, this may be due to the food itself (e.g. scrombroid fish poisoning) or due to metabolic disorders in the host (e.g. lactase deficiency) or due to some pharmacological properties of certain foods (e.g. tyramine in aged cheese) (2).

Immune system mediated hypersensitivity reactions have been classified by Coombs and Gell into four types: Type-I, II, III and IV **(Fig. 1-2).** Most food allergies are classified as immediate or Type-I hypersensitivity reactions. Because, they occur immediately, usually within minutes, after exposure to the food in sensitized subjects (3). Type-I hypersensitive reaction is mediated by IgE antibody

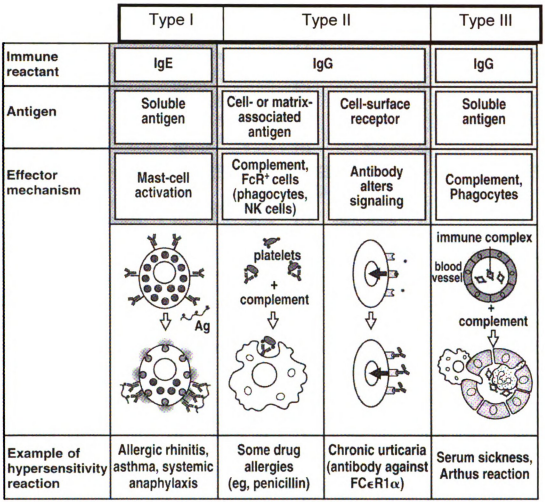

Fig. 1: Type I, II and III hypersensitivity reactions. Types I, II and III are antibody-mediated. Type-I responses are mediated by IgE, which induces mast-cell activation, whereas type-II and type-III are mediated by IgG. Type-II responses are directed against cell-surface or matrix antigens, whereas type-III responses are directed against soluble antigens, and the tissue damage involved is caused by responses triggered by immune complexes (Source: Ref. No (4)).

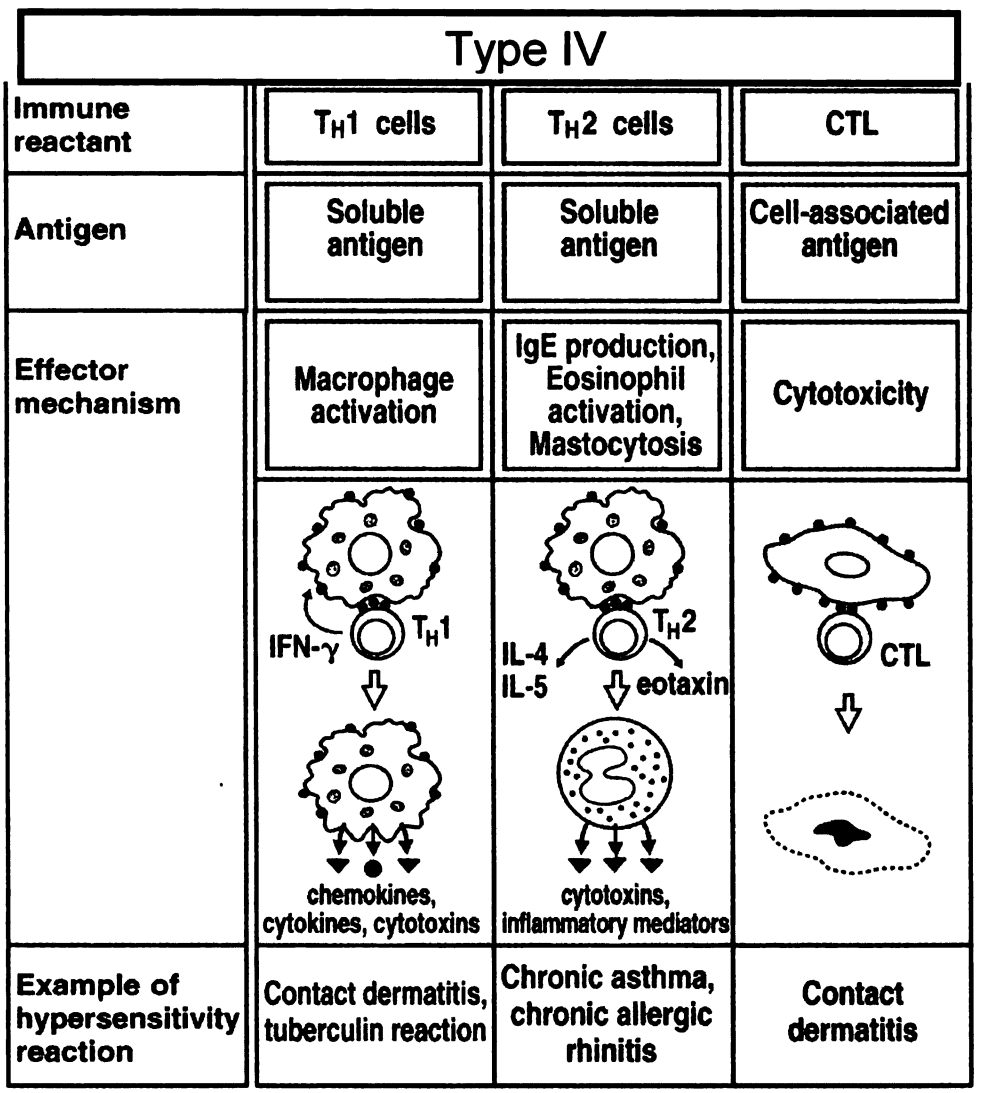

Fig. 2: Type IV hypersensitivity reaction. This is a T-cell mediated and can be subdivided into three groups. In the first group, tissue damage is caused by the activation of macrophages by TH1 cells, which results in an inflammatory response. In the second, damage is caused by the activation of eosinophilic inflammatory responses by TH2 cells; in the third, damage is caused directly by T cells (Source: Ref. No (4)).

against the soluble antigen. Airway allergies and most food allergies belong to this group.

In Type-II hypersensitive reactions, IgG and IgM play a vital role. Here antibodies bind to insoluble antigens and activate the complement system, which in turn initiates the release of mediators that are responsible for the disease. Examples include drug allergies, and autoimmune hemolytic anemia.

In Type-III hypersensitive reactions, IgG and IgM antibodies bind to circulating antigens and form large immune complexes. Failure to clear these complexes from the body leads to the disease conditions such as systemic lupus erythematosus (SLE).

Type-IV reactions are delayed hypersensitive reactions mediated by $CD4^+$ T-cells and require intact antigen presenting cells like dendritic cells and macrophages. The Type-IV mechanism is considered to be a major cause of gluten enteropathy. In the case of milk allergy, it is proposed that in addition to a Type-I mechanism, in some cases, a Type-IV mechanism may also be involved (5).

Significance of food allergy

Food allergy is a significant public health problem because of the number of people affected and the severity of the reactions. It is estimated to affect about 6% of young children and 3%-4% of adults in westernized countries, and the prevalence appears to be increasing (6). It is also the leading cause of anaphylactic events treated in hospital

emergency departments (7). Recent epidemiological data suggests that around 30,000 emergency room visits and 150-200 deaths per year in America are due to food-induced anaphylactic reactions (7-9).

Food allergy is also a significant challenge to the food and restaurant industries, as it is the leading cause for class-1 food recalls and food related lawsuits against restaurants (10, 11). Similarly, the agriculture and biotech industries face the challenge of potential allergenicity of foods in developing novel food crops by genetic engineering. For example, the recent episode of contamination of the human food chain by genetically engineered corn (Star link®) led to a food recall at an estimated cost of ~ 100 million dollars (12-15).

Avoiding exposure to allergenic food is the only means of preventing a food allergic reaction. The need to avoid an allergenic food requires care when buying food and preparing meals. Extreme precautions may be taken for fear of a potentially severe reaction caused by the accidental intake of an allergenic food. As a consequence, food allergy can significantly reduce the quality of life of sufferers and their families and can lead to nutritional deficits. Indeed, it is suggested that some food allergic individuals experience a lower quality of life than patients with other chronic conditions such as Type- 1 diabetes mellitus (16).

The 'red-flag' allergenic foods

Any food has the potential to be allergenic in sensitized subjects. A large number of foods (in excess of 160) have been reported to provoke allergenic reactions in sensitive individuals (17). However, the number of allergenic foods of clinical importance is much lower and those relevant to public health are often limited to fewer than a dozen food groups (18). According to the US FDA, the following foods are reported to cause allergy in 90% of the cases: Egg, milk, wheat, soy, peanuts, tree nuts, fish and shellfish; these foods are often called 'the red-flag foods' (19-21). According to the Canadian Food Safety Authority the following foods are considered major allergenic foods: egg, milk, wheat, soy, peanuts, tree nuts, fish, shellfish, sesame seeds and sulphites (22). According to the European Union, the following foods are considered major allergenic foods for regulatory purpose: egg, cow's milk, fish, peanuts, tree nuts, soy, celery, cereals containing gluten, crustacean (crab, crayfish, lobster and shrimps), sesame, mustard, and sulphites (23).

Mechanism of food allergy

Though the basic mechanism of food allergy is not completely clear at present, it can be explained in two phases namely the sensitization phase and the effector phase **(Fig. 3)** (24). In the sensitization phase when the body encounters a particular allergen for the first time, the antigen presenting cells present it to B-lymphocytes. This leads to production of

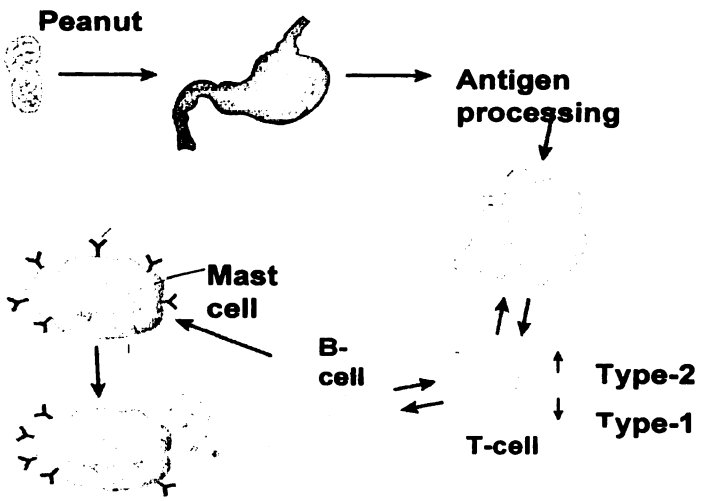

Fig. 3: Mechanism of peanut allergy development in humans.

Foods enter the gastrointestinal tract and undergo protein digestion and then antigen (Ag) processing. Some Ag enters the blood stream to be distributed at distal sites throughout the body. As the antigen-presenting cell presents Ag to the T cell, specific cytokines are produced. In the peanut-allergic individual, T cells secrete increased amounts of IL-4, IL-5, and IL-13, among other mediators, and reduced amounts of IFN-γ when compared to secretion in an individual who is not allergic to peanuts. The T cell in turn regulates the eventual peanut-specific IgE production by B cells. Peanut-specific IgE is attached to mast cells in the gastrointestinal tract, skin, and respiratory tract mucosa. Subsequently, on ingestion of peanuts, in the peanut-allergic individual, the protein is digested and the Ag binds peanut-specific IgE on the mast cell, causing activation of the mast cell with mediator release at the mucosal site. Clinical symptoms ensue (Source: Ref. No (25)).

allergen specific IgE antibodies with the help of T-lymphocytes. These IgE antibodies enter circulation and bind to Fc receptors on mast cells. When this sensitized cell is exposed to the same allergen for a second time, the effector phase sets in. When the IgE antibodies present on mast cells are cross linked with the allergen, degranulation of mast cells results leading to release of mediators such as histamine that are responsible for the symptoms of the food allergy. Common symptoms of food allergy include skin reactions such as hives, gut reactions, respiratory distress or cardiovascular reactions. Sometimes all these symptoms occur simultaneously resulting in a fatal situation known as systemic anaphylaxis or anaphylactic shock (26, 27).

Types of food allergies

Based on clinical course and natural history, food allergies are broadly classified into two types: transient and persistent (28). Food allergies that usually resolve include those caused by milk, egg, soy, and wheat. These allergies are typically present in infancy and usually resolve by the age of 3-5 years (28). Food allergies that frequently persist include those caused by peanut, tree nuts, fish and shell fish (28).

Cow's milk allergy (CMA) is one of the most common food allergies in early childhood with an incidence of 2.5% among infants in US. A majority of infants with CMA outgrow their allergy by three years of age, whereas a small percentage (15%) of this population has persistent allergy to cow's milk (29). In the case of egg allergy, approximately 50% of egg allergic-children outgrow the allergy by the age of 3 years (30). The median age of resolution of wheat allergy is approximately 6.5 years (31).

Peanut allergy is estimated to affect about 1% of the population in the USA (32). The expected natural course of peanut allergy is life-long persistence among the majority (80%) of patients, but some studies report about 20% of children with peanut allergy outgrow their allergy (33). It has been reported that only 9% of children with tree nut allergies such as cashew nut, walnut and pecan are likely to outgrow their allergy (34).

Mechanisms behind transient and persistent food allergies

The question remains why some food allergies are outgrown, while others are not. Elucidation of the mechanisms behind the persistent and transient food allergies is important for providing insights for therapeutic and preventive strategies for these food allergies. Several studies have looked at various factors to predict the ability to outgrow food allergies. The results from the literature are summarized here.

Cow's milk allergy (CMA): factors associated with persistence versus transient milk allergy

Schade et al (2001) reported that the down regulation of the Th2 cytokine (IL-4) response and up-regulation of Th1 cytokine (IFN-γ) response is associated with the development of spontaneous clinical tolerance to cow's milk in patients with CMA. The results of this study suggests that Th2 and Th1 cytokine balance may play a vital role in induction of tolerance to cow's milk (35).

Tiemessen et al (2004) reported that T cells derived from donors with persistent CMA were associated with production of IL-4 and IL-13, where as T cells from control subjects were characterized by production of IL-10 and IFN-γ. These data suggested that activated allergen–specific T cells might contribute to an active form of immunosuppression in vivo through the production of IL-10 (36).

The presence of specific immune components and genetic predisposition may play a role in persistence of CMA. Karlsson et al (2004) reported that children who outgrew CMA had higher frequencies of circulating T regulatory cells ($CD4^+$ $CD25^+$) compared with children that had persistent CMA (37). There is also evidence in the literature which suggests that genetic predisposition may have a very important role in persistence of CMA (38).

Other studies have shown that patients who are allergic to casein proteins most often have persistent CMA (29). Chatchatee et al (2001) reported that patients with persistent CMA have IgE directed to different epitopes than patients with transient CMA (39).

Factors associated with ability to outgrow egg allergy.

Noma et al (1996) reported that the trigger for ability to outgrow hen egg allergy may be related to induction of Th1 type cells to produce IFN-γ resulting in suppression of Th2 type response like IL-4 production (40). Shek et al (2004) found that the rate of decrease in food sIgE levels over time was predictive for the likelihood of developing tolerance in

both milk and egg allergy (41). Savage et al (2007) reported that most patients with egg allergy are likely to develop tolerance by late childhood, with the exception of patients with an egg specific IgE levels greater than 50 kU/L (42).

Mechanisms of ability to outgrow peanut allergy

The expected natural course of peanut allergy is life-long persistence among the majority of patients. One study showed that about 20% of children with peanut allergy outgrow it (33). Turcanu et al (2003) reported that children who outgrew peanut allergy exhibited a shift in peripheral blood lymphocyte profile from Th2 (IL-4, IL-5, IL-13) to a Th1 profile (IFN-γ , TNF- α) (43).

Table 1: Proposed mechanisms behind persistent and transient food allergies

Type of food allergy	Natural history	Associated with	Reference
Cow's milk allergy	Persistent	• ↑ Th2 (IL-4)	(36)
		• IgE to casein	(23)
			(38)
Cow's milk allergy	Transient	• Genetic • ↑ Th1 (IFN-γ) > Th2	(35)
		• IgE to non-casein protein	(23)
		• ↑ T- reg cells ($CD4^+CD25^+$)	(31)
Egg allergy	Transient	• ↑ Th1 (IFN-γ) > Th2	(34)
Peanut allergy	Transient	• ↑ Th1 (IFN-γ) > Th2	(37)
Tree nut allergy	Transient	• Low sIgE levels	(28)
Wheat allergy	Transient	• Low sIgE levels	(25)

Tree nut allergy: mechanism of persistence

Very little is known about factors that explain why tree nut allergies in general are rarely outgrown. Fleischer et al 2005 reported that tree nut specific IgE levels are an indicator that can be used to assess the likelihood of developing oral tolerance (34). Thus, they found that patients with tree nut allergies (cashew nut, walnut and pecan) will likely outgrow their allergy if their tree nut specific IgE levels are 5 kU (A)/L or less. Notably, there is no information available in the literature on the mechanisms of persistence of hazelnut allergy.

Potential role of differential immune memory to explain transient vs. persistent food allergy

As part of this study, we have been pursing the idea that the nature of immune memory might explain why some food allergies are outgrown but not others. For example, it is possible that short lived T cell and/or B cell memory might lead to ability to outgrow food allergy more rapidly. On the other hand a food that triggers a long-lasting T cell and/or B cell memory response might lead to a persistent food allergy phenotype. However, properly designed long-term studies have not been done before. In the following section, the literature on the general aspects of the mechanism of T cell and B cell memory is reviewed briefly.

The memory immune response is a characteristic property of the adaptive immune system, which promotes an enhanced, rapid and long-lived immune response. Antigen

specific T and B lymphocytes of the adaptive immune system mediate the memory immune response.

Memory T-cells

Memory T-cells are generated following an initial priming event in which naïve T-cells are activated by antigen presenting cells (APC). Activated naïve T-cells proliferate and differentiate into several types of effector T-cells. Most of these effector T-cells die after a brief life span and a subset of primed antigen-specific T-cells develop into memory T-cells by mechanisms that are not yet completely defined **(Fig.4)** (44).

Memory T-cells and naïve T-cells are distinguished based on phenotypic, functional and homing properties (45). Phenotypically both naïve and memory T-cells have common properties like small size and low level expression of the high affinity IL-2Rα (CD25). However memory T-cells differ from naïve T-cells by expressing elevated levels of CD44 and CD11a (46, 47).

Functionally, memory T-cells and naïve T-cells exhibit similarities in producing effector molecules like IFN-γ or IL-4 and IL-5, but they differ in the stimulation time required to produce these effector molecules. Memory T-cells produce cytokines within hours of stimulation where as naïve T-cells require days of sustained activation to differentiate into effector cytokine producers (48). Other functional attributes of memory-T cells include rapid recall response and reduced activation threshold. Memory T-cells can respond to diverse antigen presenting cell (APC) types such as resting B-cells,

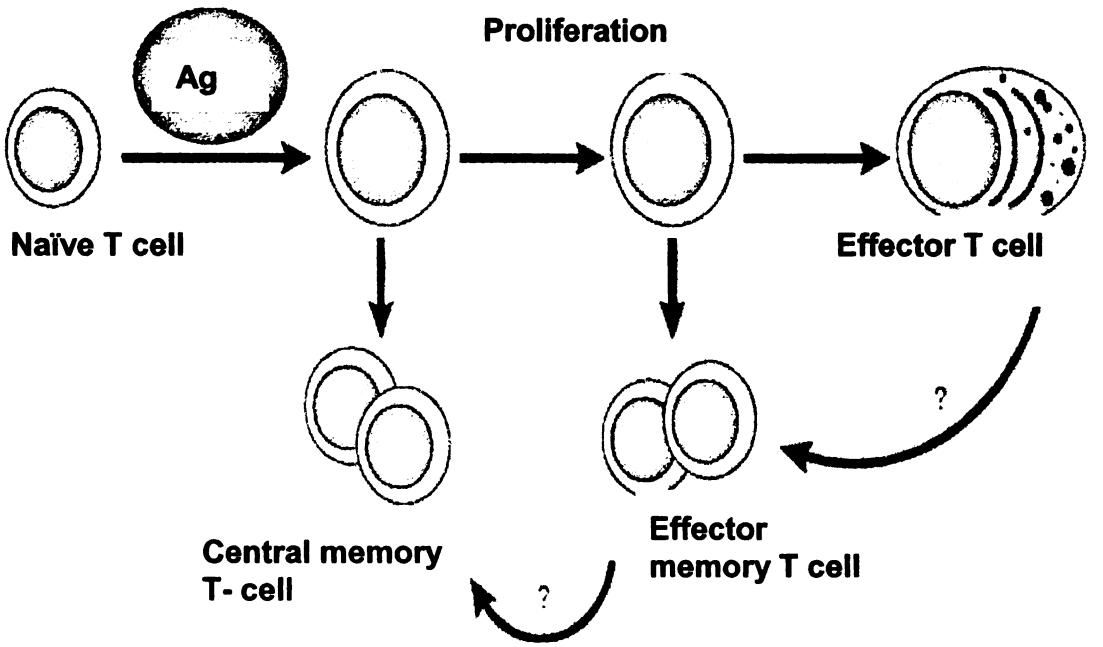

Fig. 4: Lineage pathway for the generation of effector and memory T-cells. After activation, cells differentiate into effector T- cells. Memory T-cells are thought to be generated by divergence from this pathway or directly from effector cells. The model propose two subsets of memory cells: 1) quiescent, central memory cells that recirculate from bone marrow to secondary lymphoid organs, and 2) effector memory cells that migrate through tissues and deliver a very rapid response on reactivation with antigen (Source: Ref. No (49)).

macrophages, endothelial cells as well dendritic cells which are primary APC for naïve T-cell activation (50, 51). Memory T-cells also differ from naïve T-cells in expression of homing receptors which reflect their diverse abilities to migrate to lymphoid and non-lymphoid tissue (52).

Memory B-cells and plasma cells:

Naïve B-cells are activated and proliferate at the margins of secondary lymphoid organs such as spleen and lymph nodes when they encounter with antigen. B-cell activation is a T-cell dependent activation process (Fig.5). An activated B-cell can then continue down one of the two pathways: 1) it can differentiate into antibody secreting short- lived plasma cells in the periphery of secondary lymphoid organ or 2) migrate into the center of secondary lymphoid organs and initiate a germinal center reaction resulting in the generation of high affinity memory B-cells (53, 54). Precursors of long lived plasma cells are most likely generated in the germinal center reaction and then migrate to the bone marrow, which are key to maintaining long–term humoral immunity (55).

In summary the current hypotheses to explain the longevity of antibody response in the absence of re-exposure to antigen are: 1) long lived plasma cells in the bone marrow that secrete specific antibody levels for extended periods (56) and 2) plasma cells are formed continuously from memory B-cells in an antigen independent manner due to polyclonal activation (57).

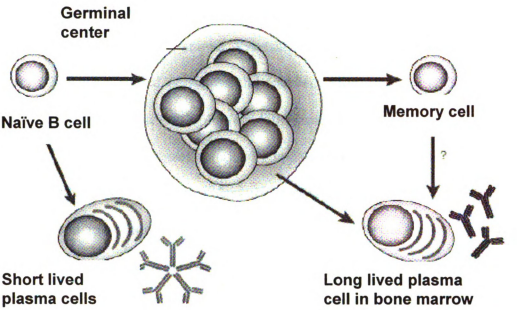

Fig. 5: Generation of memory B-cells and effector B-cells. Development of memory B-cells and effector B-cells (Plasma cells) occurs in two phases. Short lived plasma cells that make mostly IgM (but some IgG) are generated during the primary response and occupy sites, such as the splenic red pulp or lymph node medulla. B-cells are also seeded to the follicle to form germinal centers in the early phase. The second phase involves the formation of the memory B-cell pool and seeding of long- lived plasma cells to the bone marrow (making predominantly switched isotype antibodies). Plasma cells are terminally differentiated and do not give rise to memory cells. Pathways designated by arrows are driven by antigen and T-cell help (Source: Ref. No (49)).

Rationale for the present study:

Even though much is becoming known about the mechanisms of food allergies in general, the mechanism behind persistent and transient food allergies is incompletely understood at present. It is noteworthy that the long-term characteristics of T-cell and B-cell memory in food allergy are not completely understood. It is also unclear whether differential immune memory could explain the persistent or transient nature of food allergies. Improved knowledge on such mechanisms might provide clues to develop novel methods to prevent and treat food allergies.

We sought to address this area of research using an adjuvant free mouse model of hazelnut allergy that our laboratory had developed earlier (58). Furthermore, we also developed a novel adjuvant-free CMA mouse model and studied long term immune memory in this model.

Hypotheses and objectives of the present study

The present study was conducted in three parts: in Part-I, characteristics of long-term hazelnut allergy was studied using an adjuvant free mouse model that our laboratory had developed earlier (58). In Part-II, an adjuvant-free mouse model of CMA was developed. In Part-III, characteristics of long-term CMA was studied.

Part-I

Statement of the Problem

Hazelnut allergy is thought to be a persistent food allergy in humans; however we did not know if hazelnut allergy is persistent in this mouse model. Furthermore, characteristics of long-term immune memory in this model were also unknown. Addressing this problem helps in understanding mechanism behind persistence of hazelnut allergy and also validates our mouse model for human disease.

Hypothesis

Hazelnut allergy biomarkers once established, will persist chronically, even after allergen withdrawal in our model.

Objectives

This hypothesis was tested with the following objectives: 1) to determine the hazelnut allergy biomarkers in hazelnut allergic mice after 3 months of allergen withdrawal; 2) to determine the hazelnut allergy biomarkers in hazelnut allergic mice after 5 months of allergen withdrawal; and 3) to determine the hazelnut allergy biomarkers in hazelnut allergic mice after 8 months of allergen withdrawal.

Part-II

Statement of the Problem

An adjuvant- free mouse model was not available for CMA when this work was started. Therefore, we tested the following hypothesis.

Hypothesis

Transdermal exposure to cow's milk whey protein will trigger allergic immune response and sensitize mice for clinical features of CMA in the absence of adjuvant in BALB/c mice.

Objectives

This hypothesis was tested with the following objectives: 1) to study IgE response in BALB/c mice following transdermal exposure to milk whey proteins; 2) to study clinical reaction of BALB/c mice to oral milk whey protein exposure; and 3) to study spleen cell IL-4 response in allergic vs. control mice.

Part-III

Statement of the Problem

CMA is a transient type of food allergy in humans and it is usually outgrown by the age of 3-5 years. However, it was unclear whether CMA would be short lived or long-lasting in our mouse model. The characteristic of long-term immune memory in this model were also unknown. Therefore, we tested the following hypothesis.

Hypothesis

CMA biomarkers will be short lived in our adjuvant-free mouse model.

Objectives

This hypothesis was tested with the following objectives: 1) to determine the CMA readouts in milk allergic mice after 3 months of allergen withdrawal; 2) to determine the CMA readouts in milk allergic mice after 5 months of allergen withdrawal; and 3) to determine the CMA readouts in milk allergic mice after 8 months of allergen withdrawal.

Organization of the thesis

Studies conducted in Part-I, Part-II and Part-III is presented in a manuscript style in Chapters 2, 3 and 4 respectively. Chapter 2 deals with the characteristics of long-term hazelnut allergy in an adjuvant-free mouse model. This work is under revision for publication in, *International Archives of Allergy and Immunology*. Chapter 3 deals with the development of an adjuvant-free mouse model for CMA. This work has been accepted for publication in the *Journal of Dairy Science*. Chapter 4 deals with the characteristics of long-term cow's milk allergy in an adjuvant-free mouse model. This is followed by a summary section and suggested future studies.

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CHAPTER TWO

(This work has been accepted for publication in *International Archives of Allergy & Immunology*)

LONG-TERM CHARACTERISTICS OF HAZELNUT ALLERGY IN AN ADJUVANT-FREE MOUSE MODEL

Abstract

Clinically it is recognized that tree nut allergies such as hazelnut allergy are not usually outgrown. Specific mechanisms underlying persistence of such food allergies are incompletely understood. Here we studied the natural history and the long-term immune and clinical characteristics of hazelnut allergy in an adjuvant-free mouse model. BALB/c mice were sensitized to hazelnut protein using a transdermal sensitization protocol that does not use adjuvant. After establishing sensitization, exposure to hazelnut was withdrawn for 3, 5 or 8 months. The fate of circulating IgE antibodies was monitored. Subsequently, mice were given booster exposures and examined for memory IgE antibody and spleen cell IL-4 responses. Clinical characteristics and hypothermia responses upon oral allergen challenge were studied. Upon allergen withdrawal, circulating hazelnut specific IgE antibody levels began to drop. Nevertheless, IgE responses once established remained at significantly high levels for up to 8 months (the last time point studied) despite withdrawal of allergen exposure. Memory IgE responses to booster exposures were robust after 3, 5 or 8 months of allergen withdrawal. Furthermore, significant clinical reactivity to oral hazelnut challenge, and hypothermia responses were demonstrable at each of these time points. Long-lasting spleen cell memory IL-4 responses to hazelnut were detectable in these mice explaining the mechanism of sustenance of IgE responses and clinical sensitization. Hazelnut allergy once established persists for long periods, despite withdrawal of allergen exposure, due to long-lasting, memory IL-4 and IgE responses.

INTRODUCTION

Food allergies that are mediated by IgE antibodies, afflict 6-8 % of children and ~2-4% of adults in westernized countries including Europe and the United States (1, 2). Any IgE mediated food allergy has the potential to trigger life-threatening systemic anaphylaxis in humans (2). However, peanut and tree-nut allergies are disproportionately linked to severe, potentially fatal anaphylactic reactions (3, 4). Furthermore, whereas milk and egg allergies are generally outgrown in most cases, peanut allergy has been reported to be rarely outgrown (~20 % cases) (5-8). It is also reported that approximately 9% of children with tree nut allergies such as cashew nut, walnut and pecan will outgrow their allergy (9). In contrast, hazelnut allergy, once initiated, is thought to persist for life in humans for reasons that are not completely understood (10, 11).

The mechanisms underlying why some IgE mediated food allergies are outgrown while others remain persistent is incompletely understood at present (12-14) . Thus, Turcanu et al.(2003) reported that children who outgrew peanut allergy exhibited shift in their peripheral blood lymphocyte profile from Th2 to a Th1 phenotype (15). In contrast, those who remained allergic maintained a Th2 dominated profile (15). In milk and egg allergy, there are similar reports implicating either Th1/Th2 imbalance or involvement of $CD4^+$ $CD25^+$ T regulatory cells in outgrowing clinical sensitivity (16-18). In contrast to these food allergies, the mechanism underlying persistence of tree nut allergies in general and hazelnut allergy in particular is largely unknown.

We have previously reported an adjuvant-free mouse model of hazelnut allergy (19). The major features of this model include: i) induction of dose dependent IgE antibody response to transdermal hazelnut protein exposure; (ii) clinical signs of systemic anaphylaxis and hypothermia response upon oral challenge with hazelnut; and (iii) significant type-2 cytokine responses to hazelnut. Although this model resembles many features of human disease, it is not known whether hazelnut allergy once established would persist in this model as it does in humans.

To address this question, we studied the characteristics of long-term hazelnut allergy in this mouse model. We found that hazelnut allergy once established remains persistent for up to 8 months even after allergen withdrawal. Furthermore, we also found that persistence of memory IL-4 and IgE responses are associated with long-lasting clinical reactivity to hazelnut.

MATERIALS AND METHODS

The following materials were purchased from sources as indicated in the parenthesis. Hazelnut protein extract (Greer Labs, Lenoir, NC, USA); protein content was measured by Lowry-Folin assay; LPS content of this material was tested and found to be <0.5 pg/mg of protein as measured by LAL assay (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA); Biotin conjugated Rat anti-mouse IgG1 and IgE antibodies; paired antibodies and recombinant standards for mouse IL-4 (BD PharMingen, San Diego, CA, USA); *p* nitro phenyl phosphate (Sigma, St Louis, MO, USA); Streptavidin

alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); Protein-G (GE Health care, NJ, USA).

Transdermal sensitization and long term studies

BALB/c female mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). Only adult animals (6-8 weeks age) were used in the study and they were on casein free JL Rat & Mouse/Auto 6F 5K52 lab diet (PMI Nutrition International, Brentwood, MO). All animal procedures used were in accordance with Michigan State University policies. Transdermal exposure experiments were performed using a modified method that we described before (20). Groups of mice (n=10, per group unless indicated otherwise) were exposed to saline or hazelnut protein (1 mg per mouse in saline per application); each mouse had the reagent applied to the skin of the back that had the hair clipped-off and covered with a non-latex non-occlusive bandage for 1 day. Mice were rested for 4 days. Then the cycle of exposure to saline or hazelnut protein was continued for six to eight weeks until IgE antibodies specific to hazelnut protein were detected.

After sensitization mice were divided into three groups and allergen withdrawn for 97 days (3 month study), 162 days (5 month study), and 252 days (8 month study). To study memory responses, the first group (3 month study) received 2 transdermal booster exposures with saline or hazelnut protein on day 97 and day 104; the second group (5 month study) received 2 booster exposures with saline or hazelnut protein on day 162 and day 169; and the third group (8 month study) received 2 transdermal booster exposures with saline or hazelnut protein on day 252 and day 259. Blood samples were collected for

every 15 days during the entire period by the saphenous vein and plasma was used in the antibody analysis.

Measurement of hazelnut protein specific antibodies

We have previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgE and IgG1 antibody analyses (21). The ELISA procedure used in this study was essentially as described (21).

Induction of systemic anaphylaxis, measurement of rectal temperature

Groups of hazelnut protein sensitized and saline exposed mice were orally challenged with hazelnut protein (15 mg/mouse) per mouse on days 115 (3 month study), 180 (5 month study) and 270 (8 month study) respectively, using mouse feeding needles (22 gauze, Popper and Sons Inc., NY). Rectal temperature was measured using a temperature probe (Physitemp Instruments, Inc., NJ, USA) before and at 30 minutes after oral challenge. Mice were sacrificed on the day of the oral challenge.

Spleen cell culture and cytokine analyses

Spleen cells were harvested and standard cell cultures were setup essentially as described (19). Briefly, spleen cells were cultured (7.5 million/ml) in the absence and presence of hazelnut protein (100 and 500 μg/ml). Cell culture supernatants were harvested for use in cytokine analyses using pre-optimized ultra sensitive assays (assay sensitivity: IL-4: 3.1 pg/ml).

RESULTS

Allergic (IgE) response to hazelnut once established remains persistent for up to 8 months despite allergen withdrawal.

We studied the fate of circulating hazelnut specific IgE antibodies in three groups of mice that had been sensitized to hazelnut. Thus, following allergen withdrawal, during the three, five and the eight month withdrawal period, as expected IgE levels started to fall during this period (Fig. 6, 7, 8). Nevertheless, significant IgE levels were noted in all three groups despite prolonged allergen withdrawal.

Memory IgE response to hazelnut persists for long periods of time despite withdrawal of allergen exposure

In order to test the hypothesis that hazelnut elicited IgE response has long lasting memory, we examined memory IgE responses in these mice upon booster transdermal exposure to hazelnut. We used two booster exposures because, two transdermal exposure to hazelnut protein, by itself, do not elicit significant allergic response or clinical reactivity in mice (data not shown). As evident, there was significant memory IgE response to booster exposure in these mice after three, five and eight months of allergen withdrawal (Fig. 9).

Hypothermia response to oral challenge with hazelnut in mice: long-term persistence of clinical reactivity

We examined the hypothermia response to oral challenge in these mice. As evident, mice that had been transdermally exposed to saline did not show any significant hypothermia.

In contrast, mice sensitized to hazelnut, despite allergen withdrawal for 3, 5 or 8 months (Fig. 10) exhibited consistent hypothermia response.

Memory IL-4 response to hazelnut persists long time despite withdrawal of allergen exposure

In order to examine the underlying mechanism of long lasting hazelnut allergy, we examined spleen cell IL-4 response to hazelnut. Hazelnut protein significantly activated a recall IL-4 response by spleen cells from hazelnut allergic mice despite withdrawal of allergen exposure for 3, 5, and 8 months (Fig. 11).

DISCUSSION

Here we report several important and novel findings from this study: (i) IgE response to hazelnut in BALB/c mice once established remains persistent at significant levels for at least 8 months despite allergen withdrawal; (ii) despite long-term withdrawal of allergen, mice once sensitized to hazelnut, exhibit robust memory IgE response to transdermal allergen exposure; (iii) memory IgG1 response to hazelnut also remains persistent suggesting that long lasting memory is not specific to IgE isotype *per se*; rather the immune response to hazelnut is characterized by a long-lasting memory response; (iv) hazelnut elicits a long-lasting memory spleen cell IL-4 response and that an IL-4 response once established is boosterable despite allergen withdrawal for up to 8 months; (v) the hypothermia response also remains persistent in this model of hazelnut allergy. These data demonstrate for the first time that clinical sensitization to hazelnut, once

established, persists for a long time in this mouse model due to long lasting memory IgE and IL-4 responses.

We used the BALB/c strain of mice to study characteristics of long term hazelnut allergy because this strain was used to establish the mouse model of hazelnut allergy that we have described before (19). Furthermore, since gene knockout mice are available in a BALB/c genetic background, this strain is also suitable for future studies to explore the mechanism of persistence of IL-4, IgE clinical reactivity and hypothermia response in this model.

The immune and clinical consequence of transdermal exposure to food proteins in humans is unknown at present. A few recent studies including ours demonstrate that transdermal exposure to allergenic food proteins such as hazelnut, ovalbumin, cashew nut and sesame seed can result in clinically significant sensitization (22-25). These data demonstrate, for the first time that just two transdermal exposures during the allergen withdrawal period can trigger a vigorous booster immune response with consequent clinical reactivity upon oral challenge.

Efforts to develop a mouse model of food allergy may be grouped into two types: (i) adjuvant-based approaches; and (ii) adjuvant-free approaches (25, 26). Here we demonstrate the utility of an adjuvant-free mouse model of hazelnut allergy to study the long-term characteristics of hazelnut allergy. Furthermore, our findings that hazelnut allergy is a persistent type of food allergy in this mouse model similar to the human

hazelnut disease, adds validity to using this mouse model for further basic and applied studies.

Previous studies focused on elucidating the mechanisms underlying persistence of food allergies. For example specific studies on cow's milk allergy have reported that high levels of IL-4 and inability to develop T regulatory cells ($CD4^+$ $CD25^+$) are the main reasons behind persistence of cow's milk allergy (13, 17). Shek et al (2004) reported that the rate of decrease in food specific IgE levels over time was predictive for the likelihood of developing tolerance in both milk and egg allergy (27). Another study reported that the trigger for outgrowing hen egg allergy and peanut allergy may be due to the induction of Th1 type cells to produce IFN-γ resulting in suppression of Th2 type response (15, 18). Thus many studies emphasize that long lasting (Th2 response) IL-4 and IgE levels are vital for persistence of food allergies.

Here we demonstrate for the first time long-term characteristics of hazelnut allergy using an adjuvant free mouse model. Based on these data, we propose that hazelnut elicits a long lasting memory IL-4 response that sustains a persistent IgE response thus explaining the mechanism underlying persistent clinical sensitivity to hazelnut despite allergen withdrawal for long periods of time.

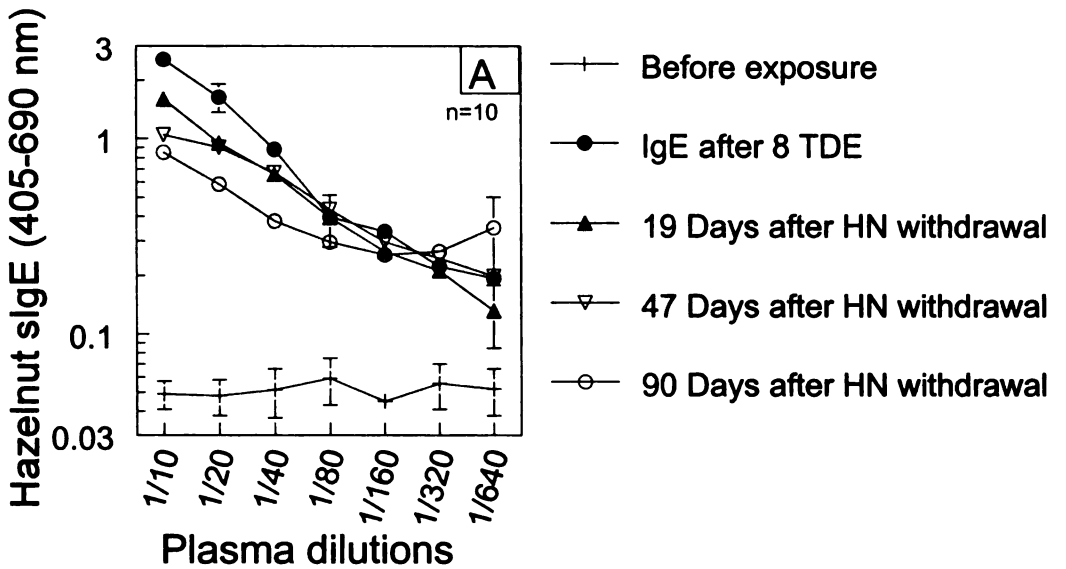

Figure 6: Circulating hazelnut specific IgE antibodies persist for long-periods despite allergen withdrawal for 3 months in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to hazelnut protein (0, 1 mg/mouse) as described in the methods. Specific IgE antibody levels were measured before exposure (pre) and at several time points after exposure and after allergen withdrawal.

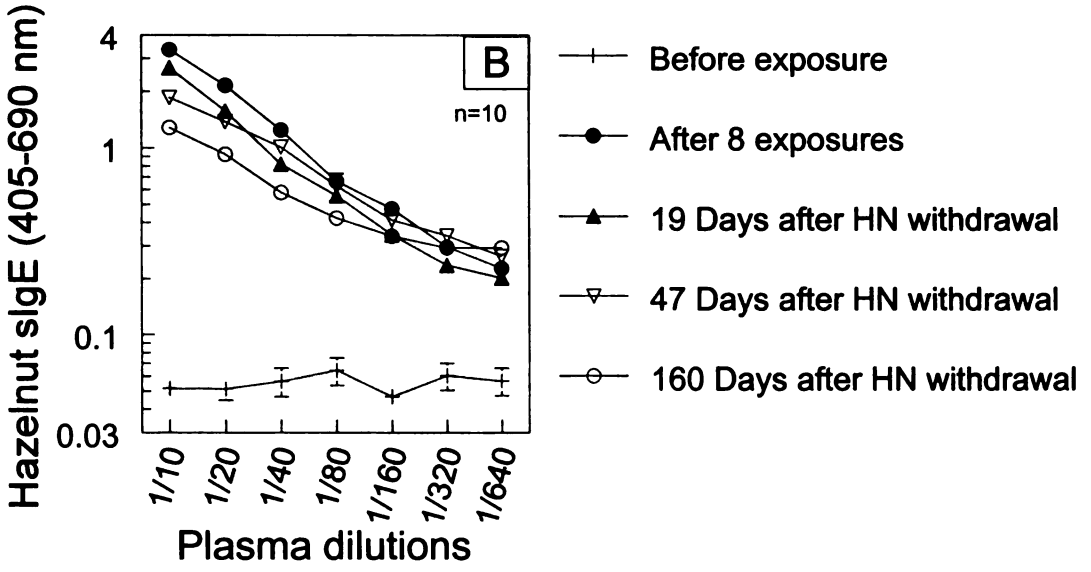

Figure 7: Circulating hazelnut specific IgE antibodies persist for long-periods despite allergen withdrawal for 5 months in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to hazelnut protein (0, 1 mg/mouse) as described in the methods. Specific IgE antibody levels were measured before exposure (pre) and at several time points after exposure and after allergen withdrawal.

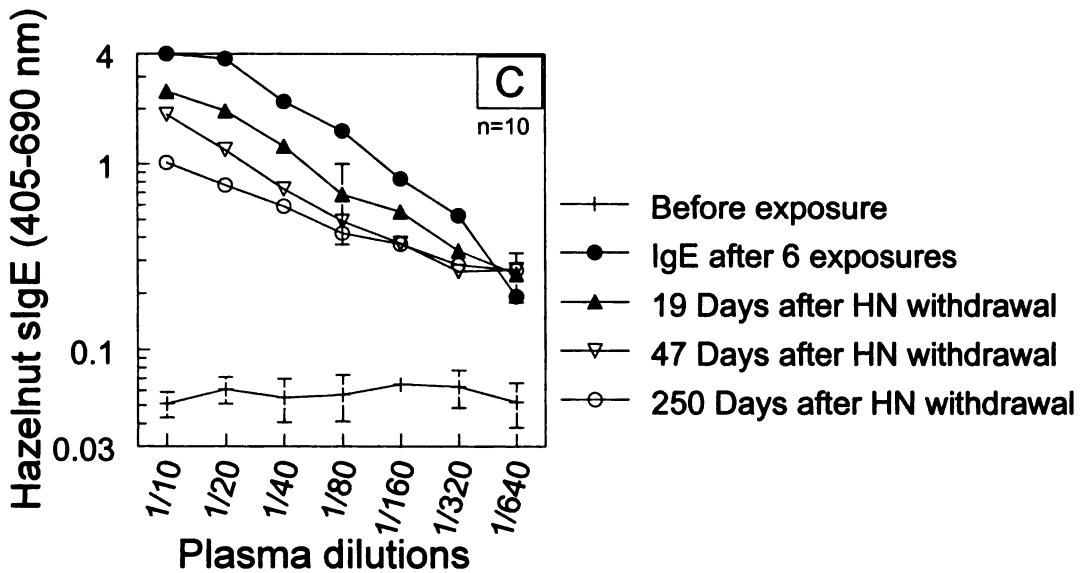

Figure 8: Circulating hazelnut specific IgE antibodies persist for long-periods despite allergen withdrawal for 8 months in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to hazelnut protein (0, 1 mg/mouse) as described in the methods. Specific IgE antibody levels were measured before exposure (pre) and at several time points after exposure and after allergen withdrawal.

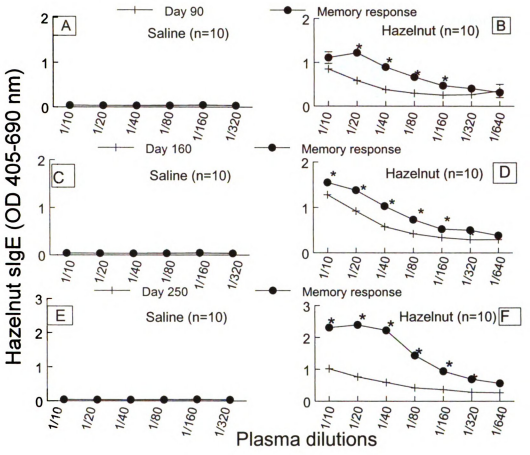

Figure 9: (A-F) Hazelnut elicits an IgE response that is characterized by long lasting memory in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to hazelnut protein (0, 1 mg/mouse) and then allergen was withdrawn as described in the protocols. Then mice were given two booster transdermal exposures and their memory IgE levels were measured. Data shown as mean +/- SE. Unpaired t-test results: Symbols with * are significantly different ($P<0.05$).

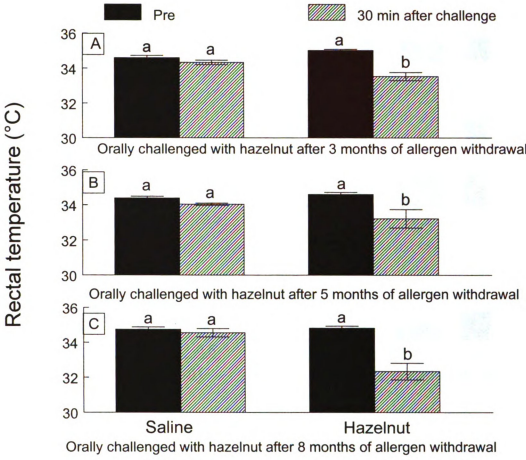

Figure 10: Long-lasting memory IgE response to hazelnut is associated with hypothermia response to hazelnut protein upon oral challenge

Groups of mice (n=10/group) were transdermally exposed to hazelnut protein (0, 1 mg/mouse) and then allergen was withdrawn as described in the methods. Later, mice were orally challenged with hazelnut protein (15 mg/mouse) and rectal temperature was monitored before and at 30 minutes after oral challenge. Data shown as mean +/- SE. ANOVA test results: Bars with different letters are significantly different ($P<0.05$).

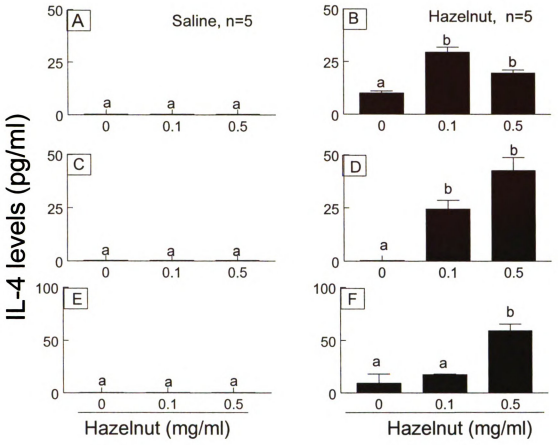

Figure 11: Hazelnut elicits long-lasting spleen cell memory IL-4 response.

Spleen cells were isolated from BALB/c mice from various groups and cultured with hazelnut protein (0.1, 0.5 mg/mL) HN or culture medium (CM) alone. Cell culture supernatants were harvested on day 3 and analyzed for cytokines using optimized ELISA. Data shown as average +/- SE of duplicate analyses. Significance was determined by ANOVA. Bars with different letters are significantly different ($P<0.05$).

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CHAPTER THREE

(This work has been accepted for publication in the *Journal of Dairy Science*)

AN ADJUVANT-FREE MOUSE MODEL TO EVALUATE THE ALLERGENICITY OF MILK WHEY PROTEIN

Abstract

Milk allergy is the most common type of food allergy in humans with potential for fatality. An adjuvant-free mouse model would be highly desirable as a pre-clinical research tool to develop novel hypoallergenic or non-allergenic milk products. Here we describe an adjuvant-free mouse model of milk allergy that uses transdermal sensitization followed by oral challenge with milk protein. Groups of BALB/c mice were exposed to milk whey protein via a transdermal route, without adjuvant. Systemic IgG1 and IgE antibody responses to transdermal exposure and systemic anaphylaxis and hypothermia responses to oral protein challenge were studied. Transdermal exposure resulted in a time and dose-dependent induction of significant IgE and IgG1 antibody responses. Furthermore, oral challenge of sensitized mice resulted in significant clinical symptoms of systemic anaphylaxis within 1 hour and significant hypothermia at 30 minutes post-challenge. In order to study the underlying mechanism, we examined allergen driven spleen cell Th2 cytokine (IL-4) response. There was a robust dose and time-dependent activation of memory IL-4 responses in allergic but not healthy control mice. These data demonstrate for the first time an adjuvant-free mouse model of milk allergy. It is expected that this model may be used not only to study the mechanisms of milk allergy, but also to evaluate novel milk products for allergenic potential and aid in the production of hypo/non-allergenic milk products.

Introduction

An immediate hypersensitivity response to food, commonly called food allergy, afflicts 6% of children and 3%-4% of adults in westernized countries including the United States (1). CMA is the most common food allergy with a prevalence of 2.5% among children and 0.3% among adults (1). There is a growing concern that food allergies are increasing at an alarming rate for reasons that are not well understood (1). Furthermore, since food allergies are potentially fatal, they are considered clinically significant and very dangerous immune-mediated disorders.

The mechanism underlying CMA is not completely understood at present. In general, milk allergies are classified as IgE-mediated and non-IgE mediated disorders (2). Whereas, non-IgE mediated CMA is generally considered not life-threatening, IgE mediated CMA is potentially fatal (3). The IgE mediated CMA involves production of IgE antibodies upon first exposure to milk protein (e.g., β-lactoglobulin, α-lactalbumin, caseins etc.) leading to sensitization of mast cells. Second and subsequent exposure to the same milk protein results in cross linking of mast cell bound IgE leading to activation and release of inflammatory mediators such as histamine. This results in clinical signs of disease such as hives, rashes, and in rare cases, potentially fatal systemic anaphylaxis (1).

There are many animal models described in the literature to study immune and allergic responses to milk proteins (4-6). However, most models use adjuvant to elicit allergic

response to milk proteins (4-6). While adjuvant-based models are very useful to study the immune response to milk protein in the context of an adjuvant as a co-factor, it has been suggested that use of adjuvant may interfere with evaluating the allergenic potential of novel proteins or chemicals because it enhances the risk of false positives (7). Consequently, adjuvant-free models might be more suitable for testing of novel proteins such as chemically or physically altered milk proteins to develop hypo/non-allergenic milk products. Therefore, we focused our efforts to develop an adjuvant-free mouse model of CMA in this study---a critical research need in the area of dairy science.

Materials and methods

The following materials were purchased from sources as indicated in parenthesis. Milk whey proteins extract (Greer Labs, Lenoir, NC, USA); protein content was measured by Lowry-Folin assay. Briefly, protein solution, in different dilutions, was first mixed with copper sulphate, and later Folin- Ciocal phenol reagent was added. The color reaction was read using absorbance at 750 nm. Bovine serum albumin was used to generate the standard curve (8). The LPS content was tested and found to be <0.5 pg/mg of protein as measured by the LAL assay. Briefly, samples were mixed with the LAL reagent and chromogenic substrate reagent. After incubation period (16 minutes) absorbance was measured at 405-410 nm (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA); Biotin conjugated rat anti-mouse IgG1 and IgE antibodies; paired antibodies and recombinant standards for mouse IL-4

(BD PharMingen, San Diego, CA, USA); *p* nitro phenyl phosphate (Sigma, St Louis, MO, USA); streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove,

PA); protein-G (GE health care, NJ, USA); purified BLG and ALA (Sigma Chemical); adult BALB/c female mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). The animal procedures used were approved by the Institutional Animal Care and Use Committee (Michigan State University).

Transdermal sensitization and bleeding

Adult female animals (6-8 weeks age) were used in the study and they were fed a casein free JL Rat & Mouse/Auto 6F 5K52 lab diet (PMI Nutrition International, Brentwood, MO). Transdermal exposure experiments were performed using a modified method that we described before (9). Groups of mice (n=5-10 per group) were exposed to saline or milk whey protein (1 mg and 2.5 mg per mouse per application); reagent was applied to the skin of the back that had the hair clipped-off and covered with a non-latex non-occlusive bandage for 1 day. Mice were rested for 4 days. Then the cycle of exposure to saline or milk whey protein was continued for six weeks. Blood samples were collected by the saphenous vein and plasma was used in the antibody analysis.

Measurement of milk whey protein specific IgE and IgG1 antibody levels

We have previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgG1 and IgE antibody analyses (10). The ELISA procedure used in this study was essentially as described by us (10).

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

Groups of milk whey protein sensitized and saline exposed mice were orally challenged with milk whey protein (15 mg/mouse) on day 13 following 6^{th} exposure using mouse feeding needles (22 gauze, Popper and Sons Inc., NY). Mice were then observed for signs of systemic anaphylaxis during the next 60 minutes. Clinical scoring (on a scale of zero to 5) was performed by 2 individuals according to the method described previously (11). 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 (Physitemp Instruments, Inc., NJ, USA) before and at 30 minutes after oral challenge.

Spleen cell culture and cytokine analyses

Spleen cells were harvested and standard cell cultures were setup essentially as described (12, 13). Briefly, spleen cells were cultured (7.5 million/ml) in the absence or presence of milk whey protein (100 and 500 μg/ml). Cell culture supernatants were harvested for use in cytokine analyses using a pre-optimized ultra sensitive assay (assay sensitivity: IL-4: 3.1 pg/ml).

Statistical Analysis

The Wilcoxon nonparametric test was used to compare treatment versus control for clinical scores. IgE antibody titer data was log-transformed and subsequently analyzed using a one sample t-test. ANOVA was used to analyze rectal temperature, IL-4 and purified milk protein data. SAS software was used for all statistical analyses (SAS Institute Inc, Cary, NC, USA). The statistical significance level was set to 0.05.

Results

Transdermal exposure to milk whey protein elicits a dose-dependent IgE antibody response

We performed dose-response and time-course experiments and analyzed antibody responses in mice following transdermal exposure to milk protein. Significant milk whey protein specific IgE antibody responses were observed at a dose of 1 mg/mouse after 6^{th} exposure (**Fig. 12A**). No IgE antibodies were detectable in the samples collected before allergen exposure or at any time point in saline control mice. A dose of 2.5 mg/mouse also elicited a significant IgE antibody response by 4^{th} exposure (IgE, titer 1066.6 ± 213.3, n=10 mice). IgE induction was confirmed by analyzing the plasma samples after depleting IgG1 and IgG2a with protein-G treatment (data not shown). Furthermore, we used purified BLG, ALA and casein to coat plates for ELISA and found that IgE was directed against BLG and ALA (**Fig. 12B**). In addition to IgE, significant IgG1 response was detected in milk whey protein exposed mice but not in saline control mice (**Fig. 13**).

Transdermal exposure to milk whey protein is sufficient to sensitize BALB/c mice for clinical signs of systemic anaphylaxis in response to oral challenge with milk protein

After confirming an IgE response, mice were orally challenged with milk whey protein and observed for clinical reactions. As evident, only transdermal sensitized mice but not saline exposed mice exhibited immediate and significant clinical symptoms of systemic anaphylaxis (**Fig. 14A**).

Clinical symptoms of systemic anaphylaxis are associated with significant hypothermia

We tested rectal temperature of mice before and 30 minutes after oral challenge with milk whey protein. Following oral challenge, only mice that had been transdermally sensitized to cow's milk whey protein showed a significant drop in rectal temperature (**Fig. 14B**).

Milk allergic mice but not healthy control mice exhibit a dose-dependent milk whey protein driven memory IL-4 response

We studied dose-response and time-course of IL-4 response in healthy and milk allergic mice using spleen cell culture. A significant IL-4 response was observed on day 3 of culture at both doses of milk whey protein in milk allergic mice but not in control healthy mice (**Table 2**).

Discussion

There are three important and novel findings from this study: (i) transdermal exposure of BALB/c mice to milk whey protein (in the absence of adjuvant) results in a significant systemic allergic (IgE) response; (ii) exposure to milk whey protein via the skin is also sufficient to clinically sensitize mice for immediate hypersensitivity reactions such as systemic anaphylaxis and hypothermia in response to oral challenge with the milk whey protein; and (iii) the mechanism underlying allergic response to transdermal milk whey protein exposure involves activation of the prototypic Type-2 cytokine, IL-4.

We chose BALB/c mice in this study to examine allergic responses to milk whey protein because: (i) this strain has been used in allergy studies previously by many in the field including us; however, whether they develop allergic response to transdermal exposure to milk protein in the absence of adjuvant, was unknown (12-14); and (ii) since gene knockout mice are available in BALB/c genetic background, this strain is desirable for conducting mechanistic studies on milk allergy.

It is very common for humans especially children to get exposed to milk proteins via skin. However, the immune and clinical consequences of such transdermal exposure are not completely clear at this time. Emerging evidence indicates that transdermal exposure to allergenic food proteins can have clinical consequence, at least, in mice. Thus, transdermal exposure to other allergenic food proteins such as hazelnut, cashew nut and sesame seed can result in both immune activation and clinical sensitization for immediate hypersensitivity reaction including systemic anaphylaxis (12-14). Others have reported a

delayed hypersensitivity response to peanut *via* skin exposure in the ear using a tape stripping method (15). They reported that mice also suffered from signs of anaphylaxis (15). Thus, in addition to the above food proteins, here we demonstrate that transdermal exposure to milk whey protein also can activate the immune system response (especially IL-4 and IgE) leading to clinical sensitization for systemic anaphylaxis in mice.

There are several CMA models reported in the literature with many using adjuvant to demonstrate a robust allergic or immune response in mice. Thus, C3H/Hej mice were shown to develop clinical CMA disease (such as atopic dermatitis and systemic anaphylaxis) following oral exposure to milk protein along with cholera toxin adjuvant (5, 6, 16) other models have used an IgE response but not a clinical disease readout (4). Here we demonstrate for the first time that it is possible to use an adjuvant-free transdermal approach to develop a mouse model of CMA that includes not only immune response readouts (IgE and IL-4) but also a clinical readout of systemic anaphylaxis and a physiological readout of hypothermia.

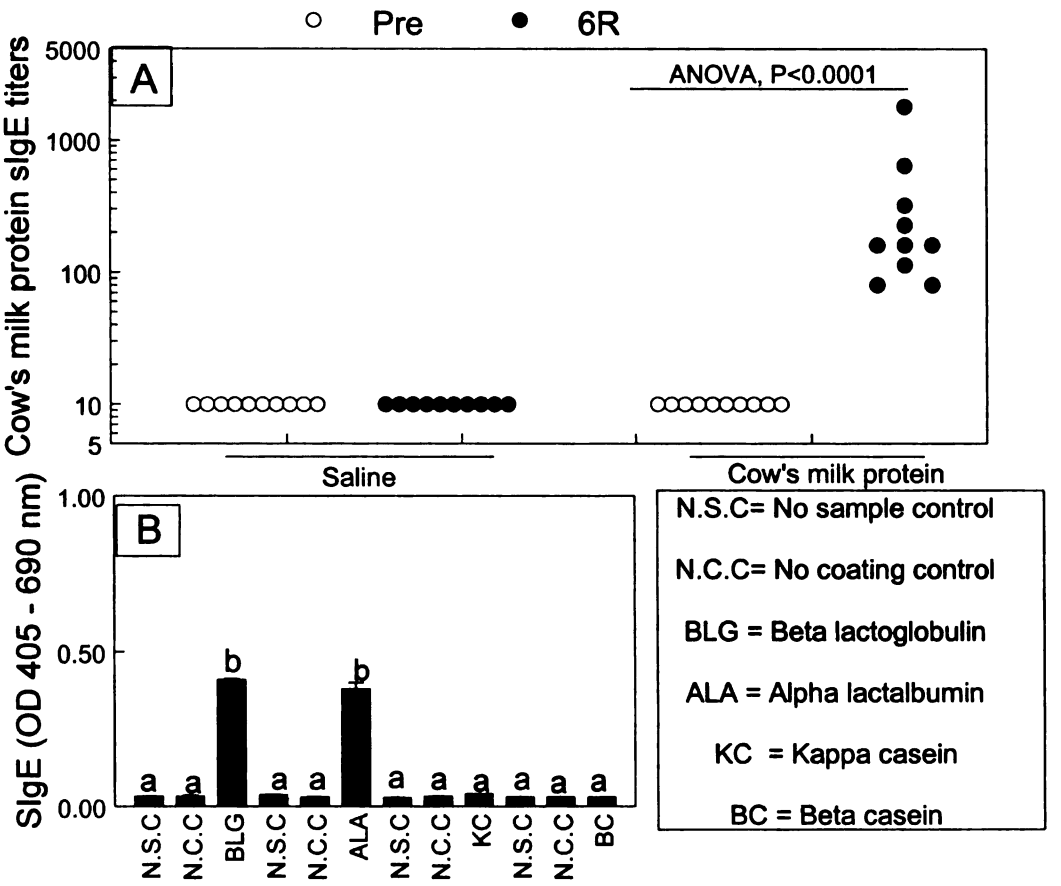
One previous study using BALB/c mice and cholera toxin adjuvant reported that BABL/c mice are '*genetically resistant*' to CMA (17). In contrast, others reported that BALB/c mice can develop an allergic response to milk following oral exposure to milk protein along with cholera toxin adjuvant (6). Using a different approach—that is transdermal exposure, we demonstrate that BABL/c mice are indeed susceptible to CMA even in the absence of adjuvant as a co-factor.

Development of novel milk products containing hypo/non-allergenic milk proteins is a major area of interest in the field of dairy science and the functional foods (18, 19). Testing such novel products in adjuvant-based CMA models poses problems of interpretation. For example, although a chemically or physically altered whey protein might be intrinsically hypo-allergenic, when used in adjuvant-based models, it may test positive for allergenicity due to adjuvant effect. With the adjuvant-free model that we describe here, we now provide an improved opportunity to study the *intrinsic* allergenicity of chemically or physically altered milk proteins in the absence of adjuvant effect.

It is largely unclear whether exposure to allergenic foods such as milk whey proteins via skin might lead to food allergy in humans. There is extensive discussion on this topic in the most-recent literature (20, 21). One epidemiological study suggested that peanut allergy in children following skin exposure to peanut (20). We are not aware of such a study on CMA in humans as yet but we directly demonstrate this possibility in BALB/c mice. Consequently, we suggest that future investigations on human CMA consider transdermal exposure to milk protein as a possibility in the pathogenesis of CMA in humans.

In conclusion: (i) we demonstrate for the first time a novel mouse model that utilizes transdermal sensitization followed by oral challenge that does not use adjuvant; (ii) this model might be useful to study mechanisms of CMA; and (iii) this model is also expected to serve as a research tool to evaluate novel milk products for allergenic potential and aid

in the production of hypo/non-allergenic milk products and development of new preventive and therapeutic methods for CMA.



Figures 12: BALB/c mice exhibit systemic allergic response to transdermal exposure with cow's milk whey protein. Groups of mice (n=10 per group) were exposed to milk whey protein (1 mg/mouse; Greer Lab.) via transdermal route 6 times over a period of six weeks. Plasma samples were collected before transdermal exposure (Pre), and after 6 exposures (6R). (A.) Cow's milk protein-specific IgE (sIgE) titers were measured using an optimized indirect ELISA. * ANOVA, 6R vs. pre: $p<0.0001$ (B.) Binding of IgE from 6R sample to different purified milk proteins (Sigma). ANOVA: Bars labeled with different letters are significantly different.

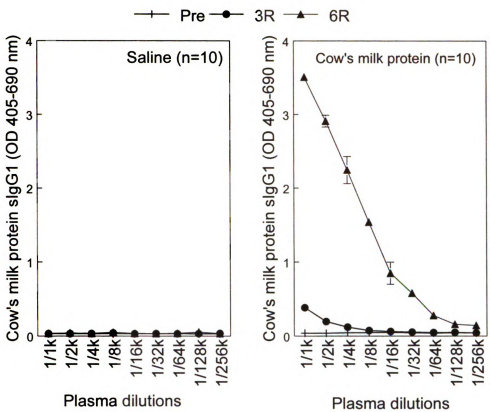
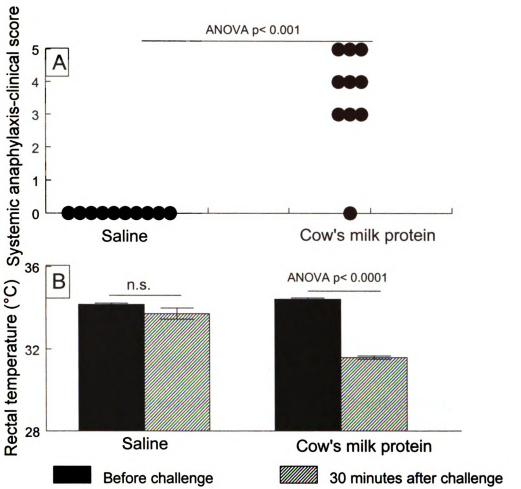


Figure 13: BALB/C mice exhibit robust IgG1 response to milk whey protein upon transdermal exposure. Groups of mice (n=10 per group) were exposed to milk whey protein (1 mg/mouse) via a transdermal route 6 times over a period of six weeks. Plasma samples were collected before transdermal exposure (Pre), after 3 exposures (3R) and after 6 exposures (6R). Cow's milk protein-specific IgG1 (sIgG1) levels were measured using an optimized indirect ELISA (OD 450-690 nm).



Figures 14: Systemic anaphylaxis and hypothermia in BALB/c mice following oral milk whey protein challenge. BALB/c mice (n=10 per group) were exposed to saline or milk whey protein (1 mg/mouse) via transdermal exposure 6 times. After confirming IgE responses, mice were orally challenged with milk whey protein (15 mg/mouse). (**A.**) Mice were examined for clinical signs of systemic anaphylaxis during the 1 hour post challenge period as described in the text. (**B.**) Rectal temperatures were recorded before and 30 minutes after oral challenge. Data shown are average +/- SE. Differences were compared using ANOVA; ns= not significant.

Table 2: Milk allergic BALB/c mice but not healthy control mice exhibit robust memory IL-4 response to cow's milk protein

Cow's milk protein used in cell culture (mg/ml)	IL-4 levels (pg/ml) in cell culture supernatant (Day-3)	
	Saline control mice (n=5)	Milk allergic mice (n=5)
0	0.50 [c]	28.99 ± 0.43 [b]
0.1	3.90 ± 1.64 [c]	251.38 ± 8.6 [a]
0.5	0.28 [c]	261.86 ± 11.3 [a]

- Data shown are average +/- SE of triplicate analysis.
- ANOVA: numbers labeled with different letters are significantly different ($P<0.05$).

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CHAPTER FOUR

LONG-TERM CHARACTERISTICS OF COW'S MILK ALLERGY IN AN ADJUVANT-FREE MOUSE MODEL

Abstract

Specific changes in immune memory underlying the ability to outgrow CMA are incompletely understood. Here we studied the long-term immune memory and clinical characteristics of CMA in an adjuvant-free mouse model. BALB/c mice were sensitized to cow's milk whey protein using a transdermal sensitization protocol that we reported earlier. Following sensitization, exposure to milk whey protein was withdrawn for 3, 5 or 8 months. Fate of circulating IgE and IgG2a antibodies was monitored. Subsequently, mice were given booster exposures and examined for memory IgE, IgG2a, and spleen cell IL-4 and IFN-γ responses. Hypothermia response, as a quantifiable readout of systemic anaphylaxis, upon oral milk allergen challenge was studied. Following allergen withdrawal, IgE antibody levels began to drop. Whereas, significant memory IgE response to booster exposures was evident after 3 months of allergen withdrawal, a weak or no memory IgE response was noted after 5 or 8 months of allergen withdrawal. Spleen cell memory IL-4 responses to cow's milk whey protein was absent after 3, 5 or 8 months of allergen withdrawal. Milk sensitized mice, upon 8 months of allergen withdrawal, exhibited elevated IFN-γ and IgG2a responses suggesting a shift to a TH1 immune profile. Surprisingly, both allergic and healthy old mice exhibited a hypothermia response upon oral allergen challenge, and complicated the analysis of hypothermia readout in old mice. In summary, upon withdrawal of allergen exposure, milk whey protein specific memory IgE, and memory IL-4 do not persist for long periods, and there is a shift towards elevation of TH1 responses as mice age.

Introduction

Cow's milk allergy (CMA) is the most common food allergy in early childhood with an incidence of 2.5% among infants and 0.3% in adults in the USA (1). Allergy to cow's milk is the most important food allergy in infancy. A majority of infants with CMA (~85%) outgrow their allergy by three years of age (2). The question remains why some children outgrow their CMA and others do not (2). Elucidation of the mechanisms behind the ability to outgrow CMA is important for providing insights to improve therapeutic and preventive strategies for CMA.

Several studies suggest that the ability to outgrow CMA is associated with multiple immune changes. Thus, changes in milk-specific IgE levels, changes in IL-4, IFN-γ cytokine profile and altered T regulatory cells ($CD4^+$ $CD25^{+)}$ have been suggested to have prognostic value in predicting clinical tolerance to CMA in children. However, characteristics of long-term T and B-cell memory responses and their relation to the development of clinical tolerance to CMA remains poorly understood in humans or in mouse models of CMA (3-5).

Here, we studied the persistence of CMA in an adjuvant-free mouse model that we developed earlier (Chapter 3). We found that whereas, milk specific memory IgE response and memory IL-4 response declined upon allergen withdrawal, memory IgG2a and IFN-γ (both markers of TH1 responses) responses were elevated as mice aged. Surprisingly, both allergic and healthy old mice exhibited hypothermia upon oral milk

whey protein administration, and thus complicated the analysis of this readout in old mice.

Materials and methods

The following materials were used: milk whey protein extract (Greer Labs, Lenoir, NC, USA); protein content was measured by Lowry-Folin assay; LPS content of this material was tested and found to be <0.5 pg/mg of protein as measured by LAL assay (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA); biotin conjugated rat anti-mouse IgG1, IgG2a and 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); protein-G (GE health care, NJ, USA).

Transdermal sensitization and long term studies

BALB/c female mice were purchased from The Jackson Lab (Bar Harbor, Maine, USA). Only adult animals (6-8 weeks age) were used in the study and they were fed a casein free JL Rat & Mouse/Auto 6F 5K52 lab diet (PMI Nutrition International, Brentwood, MO). All animal procedures used were in accordance with Michigan State University policies. Transdermal exposure experiments were performed using a modified method that we described before (6). Groups of mice (n=10, per unless indicated otherwise) were exposed to saline or cow's milk whey protein (1 mg per mouse per application); each mouse had the reagent applied to the skin of the back that had the hair clipped-off and

covered with a non-latex non-occlusive bandage for 1 day. Mice were rested for 4 days. Then the cycle of exposure to saline or milk whey protein was continued for six to eight weeks until IgE antibodies specific to milk protein were detected.

After sensitization, mice were divided into three groups and allergen withdrawn for 97 days (3 month study), 162 days (5 month study), and 252 days (8 month study). To study memory responses, the first group (3 month study) received 2 transdermal booster exposures with saline or milk whey protein on day 97 and day 104; the second group (5 month study) received 2 booster exposures with saline or milk whey protein on day 162 and day 169; and the third group (8 month study) received 2 transdermal booster exposures with saline or milk whey protein on day 252 and day 259. Blood samples were collected every 15 days during the entire period by the saphenous vein and plasma was used in the antibody analysis.

Measurement of milk protein specific IgE, IgG1and IgG2a antibody levels

We previously described optimization of enzyme linked immunosorbent assay (ELISA) for food specific IgE, IgG1 and IgG2a antibody analyses (7). The ELISA procedure used in this study was essentially as described by us (7).

Measurement of rectal temperature

Groups of milk whey protein sensitized and saline exposed mice were orally challenged with milk protein (15 mg/mouse) per mouse on days 115 (3 month study), 180 (5 month study) and 270 (8 month study), using mouse feeding needles (22 gauze, Popper and Sons

Inc., NY). Rectal temperature was measured using a temperature probe (Physitemp Instruments, Inc., NJ, USA) before and at 30 minutes after oral challenge. Mice were sacrificed on the day of the oral challenge.

Spleen cell culture and cytokine analyses

Spleen cells were collected and used for cell culture as described (8, 9). Briefly, spleen cells were cultured (7.5 million/ml) in the absence or presence of milk whey protein (100 and 500 μg/ml). Cell culture in the presence of medium alone was used as a negative control and phorbol myristate 13-acetate (1 μg/ml) and ionomycin (1 μg/ml) were positive controls. Cell culture supernatants were harvested for use in cytokine analyses using pre-optimized ultra sensitive assays (Assay sensitivity: IL-4: 3.1 pg/ml, IFN-γ: 0.39 pg/ml).

Results

Allergic (IgE) response to cow's milk whey protein does not persist upon allergen withdrawal

We studied the fate of circulating milk whey protein-specific IgE antibodies in three groups of mice that had been sensitized to milk whey protein. As evident, IgE levels started to fall during three, five and eight months of allergen withdrawal (Fig. 15, 16, 17).

Memory IgE response to cow's milk whey protein persists for less than five months upon withdrawal of allergen exposure

We examined memory IgE response in mice upon booster transdermal exposure to milk whey protein. As evident, there was significant memory IgE response to booster exposure

only after three months of allergen withdrawal but not after five and eight months of allergen withdrawal (Fig. 18).

Oral administration of cow's milk whey protein to old mice induces hypothermia in both healthy and milk allergic mice

We examined the hypothermia response to oral challenge in mice. As evident, oral administration of cow's milk whey protein to old mice exhibited consistent hypothermia in both healthy (saline exposed) and allergic mice (exposed to cow's milk whey protein) (Fig. 19). In contrast, young adult mice did not exhibit hypothermia unless they were allergic to cow's milk (See Chapter 3).

Memory IL-4 response to cow's milk whey protein is short lived

We examined spleen cell IL-4 response to cow's milk protein. There was no significant memory IL-4 response to cow's milk protein after 3, 5 and 8 months of allergen withdrawal (Fig. 20).

Cow's milk allergic mice exhibited elevated TH1 immune response upon allergen withdrawal as mice aged

As mice aged, cow's milk sensitized mice exhibited an elevated IgG2a response after 8 months of allergen withdrawal (Fig. 22). Furthermore, cow's milk whey protein also elicited a significant spleen cell IFN-γ response (Fig. 21) in old milk sensitized mice but not in saline control mice.

Discussion

Here we report the following important and novel findings: (i) cow's milk whey protein induced memory B-cell response as measured by IgE levels persists for less than 5 months when allergen was withdrawn; (ii) as mice aged, memory B cell response as measured by IgG2a levels increased upon allergen withdrawal (iii) cow's milk whey protein induced memory T helper (TH) 2-cell response as measured by IL-4 persists for less than 3 months when allergen was withdrawn; (iv) memory TH1 response as measured by IFN-γ increased upon allergen withdrawal in aged mice; and (v) surprisingly, oral administration of cow's milk whey protein to old mice induced hypothermia in both healthy and milk allergic mice; consequently, this complicated analysis of the hypothermia response in old mice.

We used BALB/c mice to study characteristics of long term CMA because this strain was used to establish the mouse model of CMA that we described in an earlier chapter. Furthermore, since gene knockout mice are available in a BALB/c genetic background, this strain is also suitable for future studies to explore the mechanism by which short-lived IL-4, IgE and shift to TH1 profile in this model.

Several studies focused on identification of markers to predict which children might outgrow CMA (3-5). These studies showed that children with long-lasting CMA present higher levels of IgE to cow's milk than those who outgrow it (3). Another human study reported that the rate of decrease in food specific IgE levels over time was predictive for the likelihood of developing tolerance in both milk and egg allergy (10). It is noteworthy

that findings from our adjuvant-free mouse model study also demonstrated this characteristic—i.e., the circulating IgE antibodies did not persist for long- periods in our model.

Previous studies suggested that the ability to outgrow CMA in humans is associated with a shift from a TH2 to a TH1 cytokine response. Thus, Schade et al (2000) reported that the down regulation of the TH2 response (as measured by IL-4 responses) and up-regulation of TH1 response (as measured by IFN-γ responses) is associated with development of spontaneous clinical tolerance to cow's milk in patients with CMA (4). We observed that in our mouse model, whereas a memory IL-4 response was short lived (for less than three months), there was a significant elevation in type-1 cytokine (IFN-γ) response as the age of sensitized mice advanced. Furthermore, IgG2a levels (another indicator of TH1 response in mice), also increased as mice aged. Thus, this feature of our model is also similar to the human phenotype.

We were surprised to note the hypothermia response in both healthy and milk sensitized old mice upon oral administration of cow's milk whey protein. In view of this complication, it was not possible to conclude whether an allergen-induced hypothermia response was persistent or transient in this model.

We do not know at this time why only aged mice (with or without sensitization) but not young mice (without sensitization) exhibit hypothermia upon oral milk whey protein administration. A previous study reported that intraperitoneal administration of

hydrolyzed whey protein in spontaneously hypertensive rats leads to hypotension (11). Therefore, it is possible that the hypothermia response observed in our study may be secondary to a drop in blood pressure caused by whey protein administration. Further studies are required to test this possibility and to find out whether oral administration of cow's milk whey protein can alter circulating molecular regulators of blood pressure and body temperature (e.g., angiotensin-I converting enzyme and angiotensin I and II levels).

Previous studies of milk allergy in mouse model including ours showed that exposure to cow's milk protein can result in clinically significant sensitization (12-15). However, none of these studies evaluated long term immune and clinical consequences of cow's milk whey protein sensitization. Also no previous studies examined whether CMA in mouse models is transient or persistent (12-15). Thus, our data demonstrate the characteristics of long term of CMA in an adjuvant free mouse model for the first time.

We observed that memory IgE and IL-4 responses to cow's milk whey protein are short lived and up regulate a TH1 (IFN-γ and IgG2a) response upon allergen withdrawal as mice aged. These data suggest that these features are similar to humans who outgrow CMA (4) (10). This adds validity to the use this mouse model for further basic and applied studies on CMA. However, the non-specific hypothermia response noted in aged mice is a major disadvantage of this model. Because, due to this peculiar feature it is not possible to study the milk allergen specific hypothermia responses (that is a quantifiable readout of systemic anaphylaxis) in old mice.

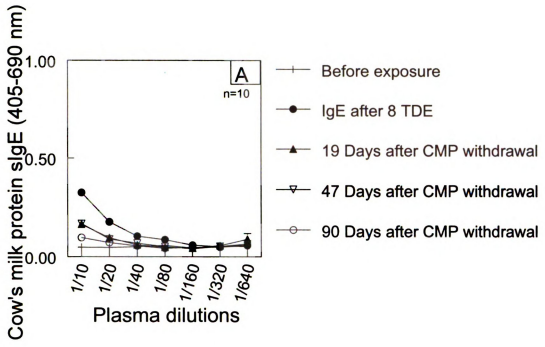

Figure 15: Circulating cow's milk specific IgE antibodies do not persist for long-periods upon allergen withdrawal for 3 months in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to cow's milk whey protein (0, 1 mg/mouse) as described in the methods. Specific IgE antibody levels were measured before exposure (pre) and at several time points after exposure and after allergen withdrawal.

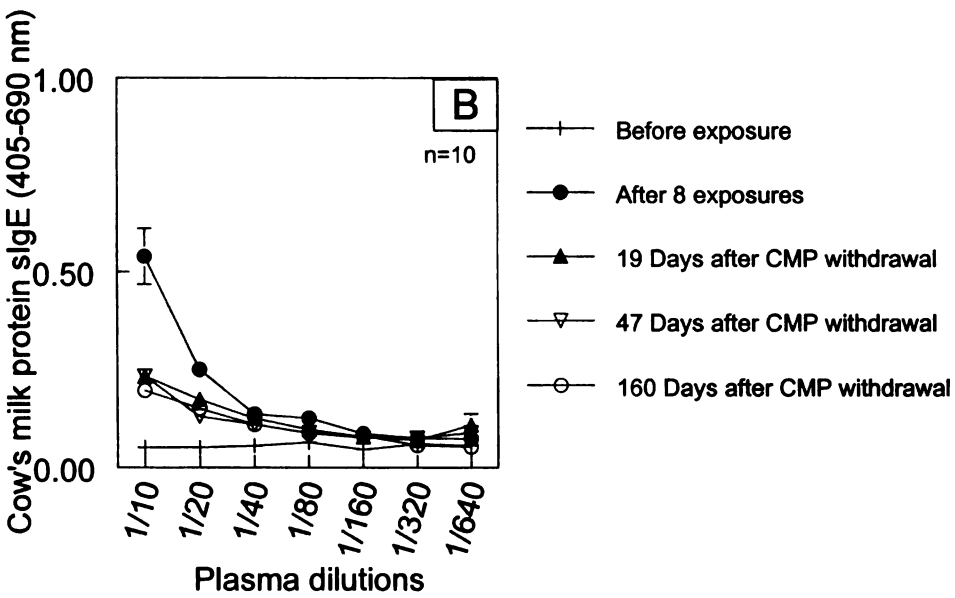

Figure 16: Circulating cow's milk specific IgE antibodies do not persist for long-periods upon allergen withdrawal for 5 months in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to cow's milk whey protein (0, 1 mg/mouse) as described in the methods. Specific IgE antibody levels were measured before exposure (pre) and at several time points after exposure and after allergen withdrawal

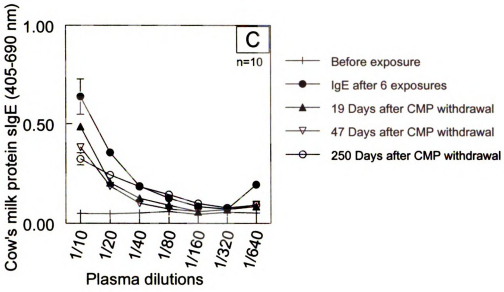

Figure 17: Circulating cow's milk specific IgE antibodies do not persist for long-periods upon allergen withdrawal for 8 months in BALB/c mice. Groups of mice (n=10/group) were transdermally exposed to cow's milk whey protein (0, 1 mg/mouse) as described in the methods. Specific IgE antibody levels were measured before exposure (pre) and at several time points after exposure and after allergen withdrawal

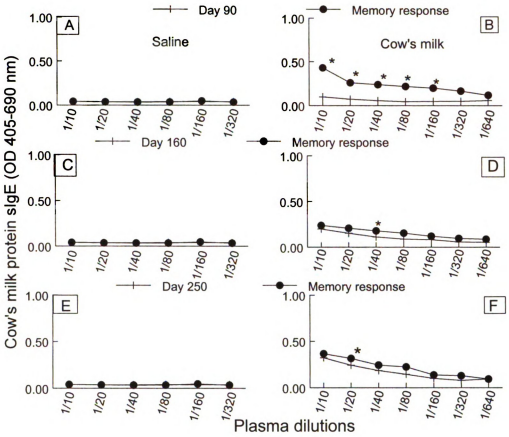

Figure 18: (A-F) Memory IgE response to cow's milk whey protein persists for less than five months. Groups of mice (n=10/group) were transdermally exposed to cow's milk whey protein (0, 1 mg/mouse) and then allergen was withdrawn as described in the methods. Then mice were given two booster transdermal exposures and their memory IgE levels were measured. Data shown is mean +/- SE. Unpaired t-test results: Symbols with * are significantly different ($P<0.05$).

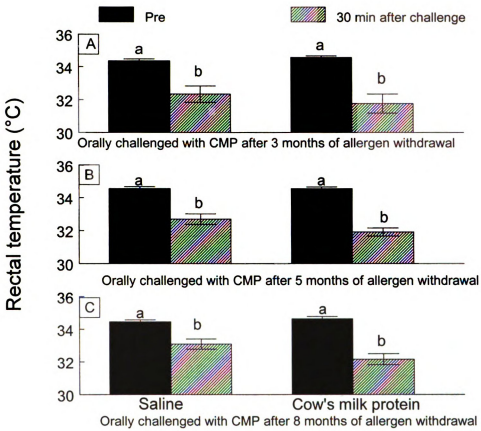

Figure 19: Oral administration of cow's milk whey protein to old mice induces hypothermia in both healthy and milk allergic mice. Groups of mice (n=10/group) were transdermally exposed to cow's milk whey protein (0, 1 mg/mouse) and then allergen was withdrawn as described in the methods. Later, mice were orally challenged with cow's milk whey protein (15 mg/mouse) and rectal temperature was examined before and at 30 minutes after oral challenge is shown Fig. 16 (A, B, C). Data shown is mean +/- SE. ANOVA test results: Bars with different letters are significantly different ($P<0.05$).

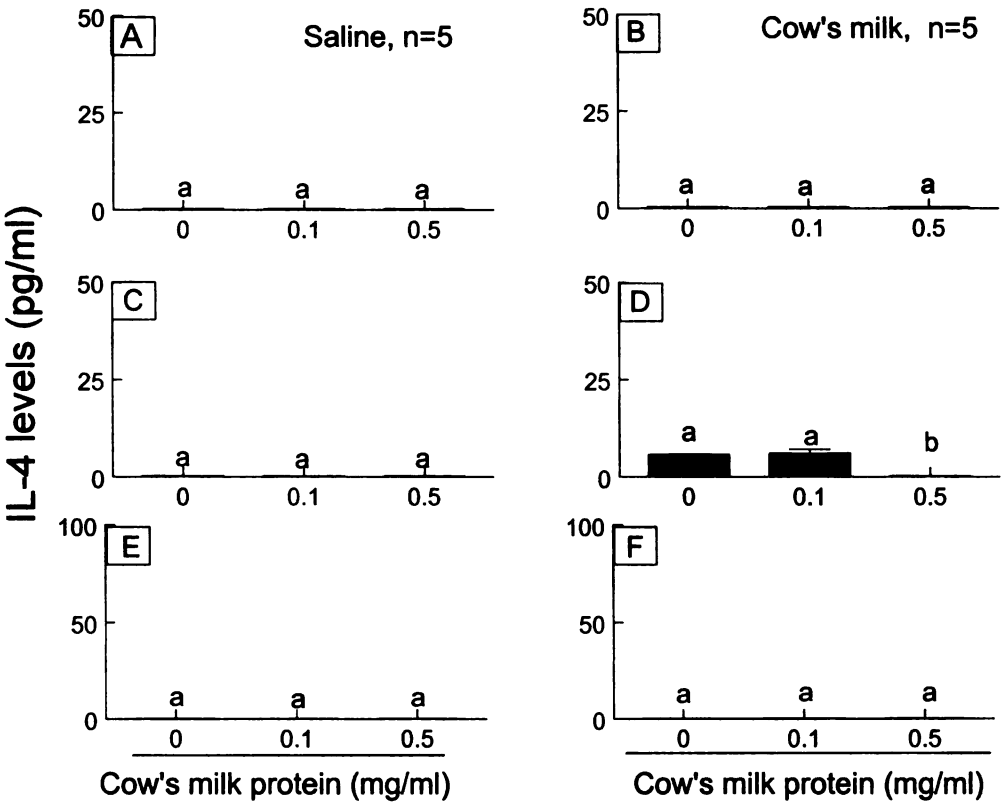

Figure 20: (A-F) Memory IL-4 response to Cow's milk whey protein is short lived. Spleen cells were isolated from BALB/c mice from various groups and cultured with cow's milk whey protein (0.1, 0.5 mg/mL; CMP) or culture medium (CM) alone. Cell culture supernatants were harvested on day 3 and analyzed for cytokines using optimized ELISA. Data shown is average +/- SE of duplicate analyses. Significance was determined by ANOVA. Bars with different letters are significantly different ($P<0.05$).

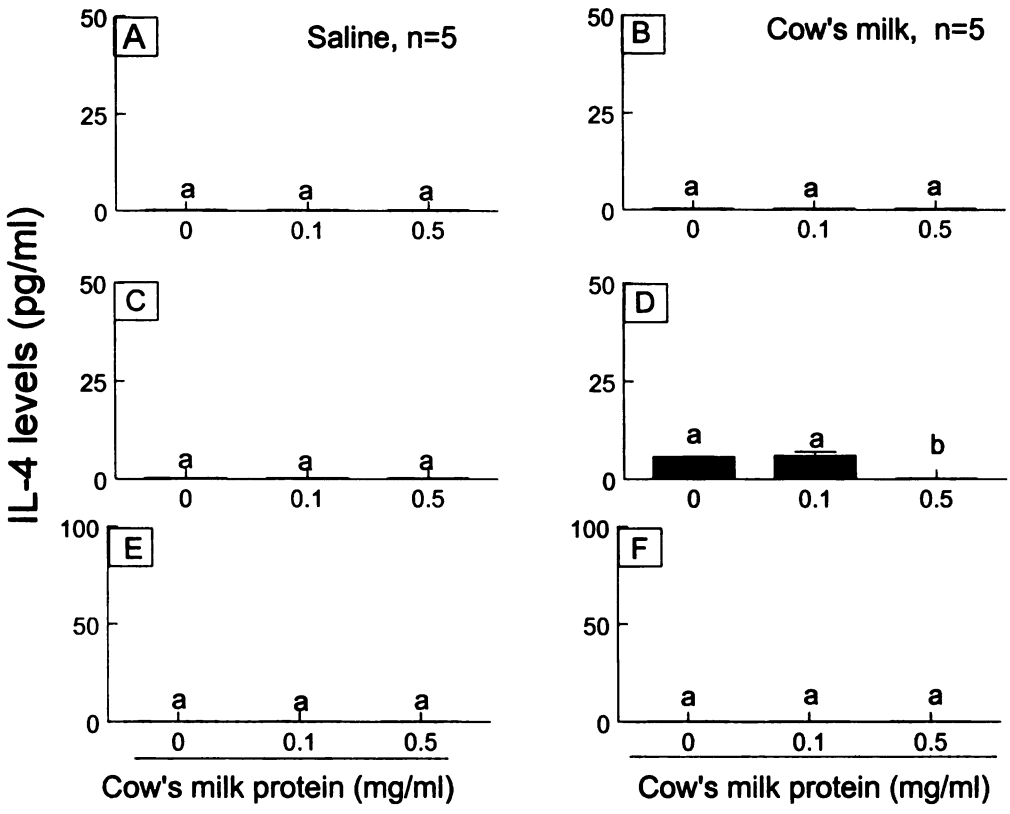

Figure 21: Cow's milk whey protein elicits IFN-γ response in milk-sensitized mice upon allergen withdrawal as age advances. Spleen cells were isolated from BALB/c mice from various groups and cultured with cow's milk whey protein (0.1, 0.5 mg/mL; CMP) or culture medium (CM) alone. Cell culture supernatants were harvested on day 3 and analyzed for cytokines using optimized ELISA. Data shown is average +/- SE of duplicate analyses. Significance was determined by ANOVA test. Bars with different letters are significantly different ($P<0.05$).

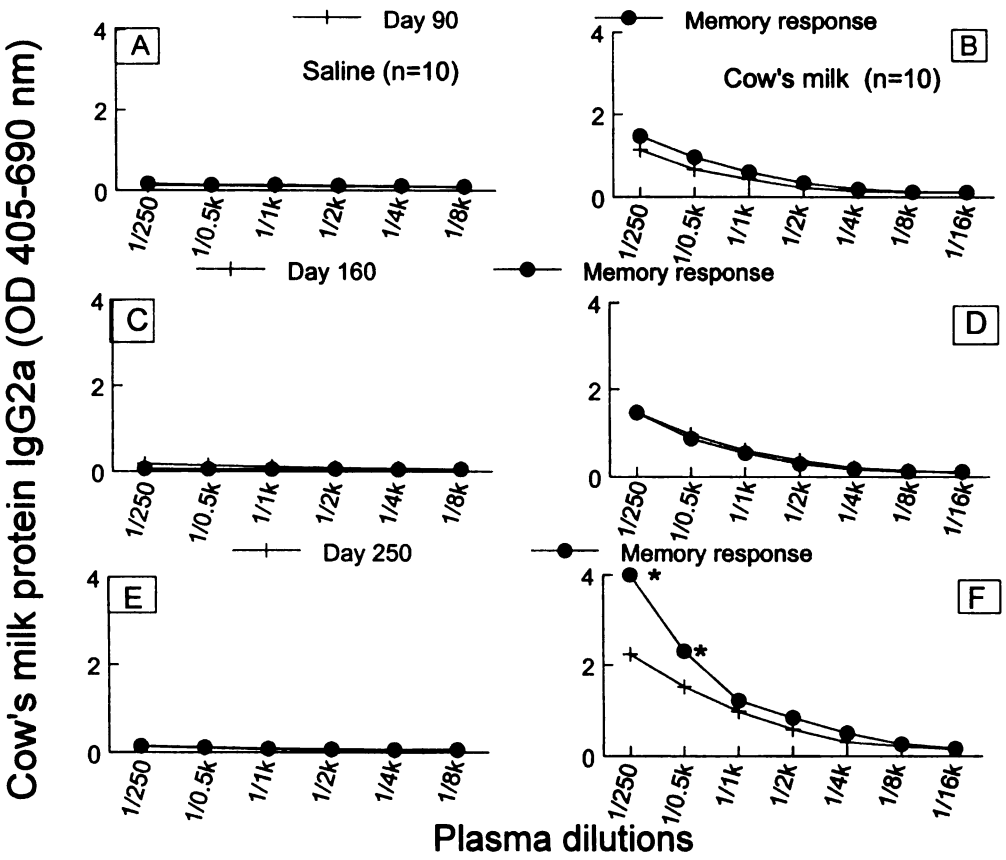

Figure 22: (A-F) Cow's milk whey protein sensitized mice exhibit elevated IgG2a response upon allergen withdrawal and as age advances. Groups of mice (n=10/group) were transdermally exposed to cow's milk whey protein (0, 1 mg/mouse) and then allergen was withdrawn as described in the protocols. Then mice were given two booster transdermal exposures and their memory IgG2a levels were measured. Data shown is mean +/- SE. Unpaired t-test results: Symbols with * are significantly different ($P<0.05$).

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SUMMARY AND FUTURE DIRECTION

In Part-I, long-term characteristics of hazelnut allergy were studied in an adjuvant-free mouse model. We found that hazelnut protein induced memory B-cell (IgE) response and memory T cell (IL-4) response persisted up to 8 months after allergen was withdrawn. These data support the following model to explain why hazelnut induces a persistent type of food allergy: upon entering the body, hazelnut activates the immune system leading to the establishment of a long-lasting memory B cell (IgE) response as well as memory T cell (IL-4) response. Consequently, clinical sensitization to hazelnut is not outgrown (**Fig. 23**).

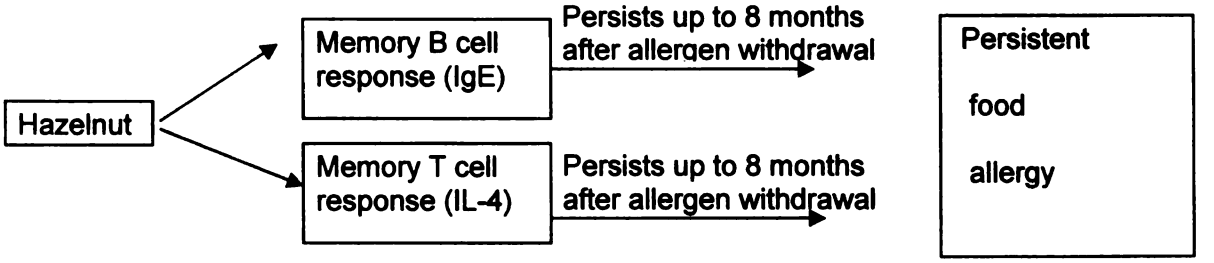

Fig. 23: Proposed model to explain why hazelnut allergy once established is not outgrown in our adjuvant-free mouse model.

In Part-II, we developed an adjuvant-free mouse model of CMA. We then used this model in Part-III, to study the characteristics of long-term CMA. We found that cow's milk whey protein induced short lived memory B-cell (IgE) and memory T-cell (IL-4) responses. An enhanced TH1 (IFN-γ and IgG2a) response occurred upon allergen withdrawal as mice aged. These findings are similar to the features in some humans who outgrow their CMA. One surprising finding was that oral administration of cow's milk whey protein in old BALB/c mice caused hypothermia in both healthy and allergic mice. This complicated analysis of the hypothermia response in old mice. Based on this data, the following model is proposed to explain why CMA might be short lived in this model: upon entering the body, milk whey protein activates the immune system leading to the establishment of a short-lived memory B cell (IgE) response as well as memory T cell (IL-4) response. However, due to non-specific hypothermia induced by milk whey protein in old mice, we could not study the clinical consequence of such a short-lived immune response, although it is very likely that the clinical sensitization might be outgrown as well **(Fig. 24).**

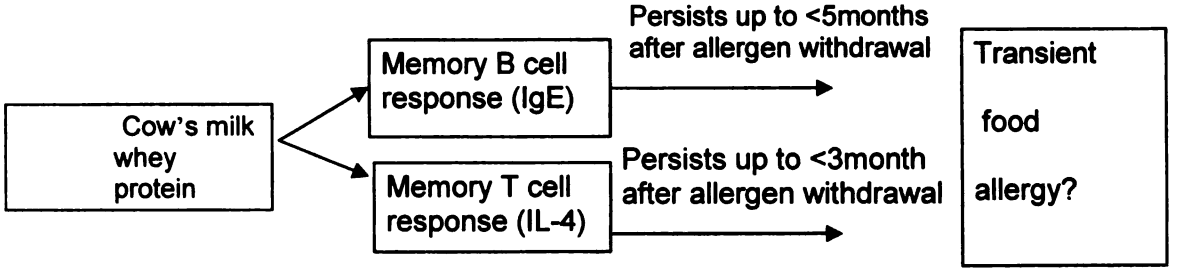

Fig. 24: Proposed model to explain why CMA might be short-lived in our adjuvant-free mouse model.

Future studies:

Hazelnut allergy model:

The following studies are suggested for the future: 1) determine why memory IgE and memory IL-4 responses are long lived in the case of hazelnut allergy. As part of this the factors (such as cytokines like IL-7, IL-15) could analyze longevity of the memory B-cells and T-cells. 2) Conduct phenotype analyses of memory B cell and memory T cells in this mouse model. For example one could track appearance and survival of CD45R0, CD45RA positive cells for T-cell, CD27 and CD19 for B-cell. 3) A recent study implicates a major role for IL-33 in systemic anaphylaxis in mice and humans (1); therefore, one could study the role of IL-33 in systemic anaphylaxis in this mouse model.

Milk allergy model:

With this model the following further studies can be conducted: 1) determine why memory IgE and memory IL-4 are short lived in the case of CMA. As part of this study one could study the factors that control longevity of the memory B-cells and T-cells. One could also study the detailed cytokine profile, which can unravel the mechanism of short lived memory response in the case of CMA. 2) One could study the molecular mechanism underlying hypothermia caused by oral administration of cow's milk whey protein in old mice by using angiotensin converting enzyme knock-out mice and also one could study whether angiotensin-II receptors are involved in this mechanism; one could also characterize which milk whey protein is specifically responsible for causing hypothermia; by using different pure proteins like beta lactoglobulin (BLG), alpha lactalbumin (ALA) etc. 3) Determine the role of IFN-γ in short lived memory responses

can be studied by using IFN-γ knock-out mice. 4) One could also use this adjuvant-free milk model to test the allergenicity of novel milk formulae that are developed for milk allergic infants. 5) Recently a study has shown that metformin can increase CD8-positive memory response (2). One can study this in our model to test whether metformin can also induce CD4-positive memory.

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