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Environmental and immunological factors associated with allergic disease in children

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“This is my interpretation”

-Mika

To Daniel, Lovisa & Emilia

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ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

- I. Böttcher MF, Björkstén B, **Gustafson S**, Voor T, Jenmalm MC. *Endotoxin levels in Estonian and Swedish house dust and atopy in infancy*. Clinical and Experimental Allergy 2003;33:295-300.
- II. **Tomičić S**, Norrman G, Fälth-Magnusson K, Jenmalm MC, Devenney I, Böttcher MF. *High levels of IgG₄ antibodies to foods during infancy are associated with tolerance to corresponding foods later in life*. Pediatric Allergy and Immunology 2008, in press.
- III. **Tomičić S**, Voor T, Jenmalm MC, Björkstén B, Böttcher MF. *Slower maturation of the secretory IgA system in Swedish than Estonian children – possibly caused by low microbial pressure and related to expression of allergy in sensitised individuals*. Submitted.
- IV. **Tomičić S**, Fälth-Magnusson K, Böttcher MF. *Dysregulated Th1 and Th2 responses in food-allergic children – a consequence of allergen avoidance?* Submitted.

ABSTRACT

Background: Allergic diseases are characterised by dysregulated immune responses. The first manifestation of the atopic phenotype is often food allergy, with symptoms like eczema. Food allergy in children is generally outgrown before 3 years of age, but a temporary food elimination diet is often advocated. The prevalence of allergic diseases has increased in affluent countries during the last decades, possibly as a consequence of a changed lifestyle leading to decreased microbial load.

Aim: To investigate humoral, mucosal and cell-mediated immunity in association to allergy and allergy development in young children and relate this to environmental factors.

Subjects: Two cohorts of children were investigated; 1) Children from countries with high (Sweden) and low (Estonia) prevalence of allergy that were followed prospectively from birth to 5 years of age. 2) Infants with eczema and suspected food allergy that were followed prospectively to 4 ½ years of age.

Methods: Endotoxin levels were analysed in house dust samples. Antibodies were measured in serum and saliva samples with ELISA. Food allergen induced cytokine responses were analysed in mononuclear cells.

Results: The microbial load, delineated as endotoxin levels, was higher in house dust from Estonia than Sweden and was, in Swedish children, inversely associated with sensitisation and clinical symptoms of allergy. The decreased microbial load in Sweden may have an impact on mucosal immune responses as different IgA antibody patterns were observed in Sweden and Estonian children with much lower secretory (S)IgA antibody levels and high proportion of non-SIgA, *i.e.* IgA antibodies lacking the secretory component, in the Swedish children. Moreover, low levels of SIgA were associated with clinical symptoms in sensitised children.

High IgG₄ antibody levels to food allergens during infancy were associated with faster tolerance development in food allergic children. Cytokine responses by mononuclear cells after allergen stimulation was up-regulated with age in children with prolonged food allergy, but not in children who develop tolerance before 4 ½ years of age, possibly because of the prolonged elimination diet in the former group.

Summary: Reduced microbial exposure in affluent countries may affect the mucosal immune responses during infancy, possibly resulting in an increased risk of developing allergic disease. High levels of IgG₄ antibodies during infancy are associated with faster achievement of tolerance in food allergic children. Allergen elimination during infancy may influence the regulatory mechanisms maintaining balanced immune responses to innocuous food antigens.

SAMMANFATTNING

Bakgrund: Födoämnesallergi, företrädesvis med eksem som symptom, är ofta det första tecknet på att ett barn är allergibenäget. Allergi mot födoämnen växer ofta bort före 3 års ålder, men under pågående sjukdom är det vanligt att orsakande födoämne utesluts ur barnets kost, en åtgärd som inte är extensivt vetenskapligt studerad. Förekomsten av allergier har under de senaste decennierna ökat i västvärlden, möjligen för att en förändrad livsstil lett till ett minskat mikrobiellt tryck.

Syfte: Att undersöka det humoral-, mukosala- och cellmedierade immunsvaret i relation till allergi och allergiutveckling hos små barn och relatera detta till faktorer i den yttre miljön, t.ex. mikrobiellt tryck och allergenfri kost.

Studiepopulationer: Två grupper har undersökts; 1) Barn från länder med hög (Sverige), respektive låg (Estland) förekomst av allergi har följts från födseln upp till 5 års ålder. 2) Barn med eksem och misstänkt födoämnesallergi har följts från diagnos, ställd före 2 års ålder, upp till 4 ½ års ålder.

Metoder: Mikrobiellt tryck, i form av endotoxinnivåer, analyserades i dammprover från svenska och estniska hushåll. Antikroppar i serum och saliv analyserades med ELISA och allergeninducerad cytokinproduktion i mononukleära celler utvärderades.

Resultat: Endotoxinnivåer i damm var högre i estniska jämfört med svenska hushåll och nivåerna var omvänt relaterade till sensibilisering och allergiska symptom hos de svenska barnen. Möjligen påverkar det låga mikrobiella trycket i Sverige det mukosala immunsvaret hos barnen, eftersom det fanns en stor skillnad i antikroppsmönster mellan de båda länderna. I Sverige var nivåerna av sekretoriskt (S)IgA i saliv mycket låga och många IgA antikroppar saknade den sekretoriska komponenten. Dessutom hade sensibiliserade barn med låga nivåer av SIgA oftare symptom än sensibiliserade barn med höga SIgA nivåer.

Höga IgG₄ nivåer under tidig barndom var associerat med en benägenhet att utveckla tolerans mot födoämnen hos allergiska barn. Cytokinsvar i mononukleära celler efter stimulering med födoämnesallergen, var högre hos allergiska jämfört med toleranta barn, vilket kan bero på att de allergiska barnen gått på eliminationsdiät under en längre period.

Summering: Den ökade allergiförekomsten i västvärlden kan delvis bero på att ett minskat mikrobiellt tryck förändrar de mukosala immunsvaren hos spädbarn. Höga nivåer av IgG₄ antikroppar tidigt i livet är associerat med toleransutveckling hos födoämnesallergiska barn. Det är möjligt att allergenelimination kan orsaka en försämrad reglering av cytokinsvar hos små barn.

ABBREVIATIONS

APC	Antigen Presenting Cell
AU	Arbitrary Units
BLG	β -lactoglobulin
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CMA	Cow's Milk Allergy
CMP	Cow's Milk Protein
CTLA	Cytotoxic T Lymphocyte-associated Antigen
CV	Coefficient of Variation
DBPCFC	Double-Blind Placebo-Controlled Food Challenge
DC	Dendritic Cell
ELISA	Enzyme-Linked ImmunoSorbent Assay
HSA	Human Serum Albumin
HSP	Heat Shock Protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAL	Limulus Amebocyte Lysat
LPS	Lipopolysaccharide
MAMPs	Microbe-Associated Molecular Patterns
OD	Optical Density
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cells
pIgR	Polymeric Immunoglobulin Receptor

Abbreviations

PP	Peyer's Patches
SC	Secretory Component
SCORAD	Severity Scoring of Atopic Dermatitis
SIgA	Secretory IgA
SIT	Sublingual ImmunoTherapy
SPT	Skin Prick Test
TIgA	Total IgA
TGF	Transforming Growth Factor
Th	T helper
TLR	Toll-Like Receptor

INTRODUCTION

Allergic diseases depend on dysregulated immune responses to normally innocuous substances. The origin and cause of the disease is multifactorial and the interaction between genetic disposition, allergen exposure and non-specific adjuvant factors is of importance. The prevalence of allergic diseases in developed countries has increased during the last decades and several contributing factors to this have been proposed, including changes in lifestyle and dietary habits, reduction in infections and environmental pollution. During infancy and childhood the most common manifestation of the disease is eczema and food allergy, up to date most often treated with allergen exclusion and skin care.

To institute relevant preventive and treatment strategies in children, it is important to understand how external environmental factors interact with the immature and developing immune system.

REVIEW OF THE LITERATURE

General aspects of the allergic disease

Allergic diseases are caused by a hypersensitivity reaction initiated by specific immunological mechanisms that cause tissue damage ¹. There are 4 types of hypersensitivity reactions, and they are antibody-mediated or cell-mediated. Immunoglobulin (Ig) E mediates type I responses and induces mast cell activation. Both type II and III are mediated by IgG antibodies, but 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. Finally, type IV hypersensitivity reactions are T cell mediated ².

Atopy is defined as a personal and/or familial tendency, usually in childhood or adolescence, to become sensitised and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. Therefore, atopy is a clinical definition of an IgE-antibody high-responder and the term atopic should only be used when the presence of IgE antibodies have been verified ¹. The terminology used to characterise hypersensitivity reactions is presented in figure 1. In this thesis the term allergy refers to IgE-mediated allergy.

Typical allergic symptoms are asthma, rhinitis, conjunctivitis, eczema and gastrointestinal symptoms ¹. The development of allergic diseases depends on several factors, *e.g.* genotype, when the first encounter with the allergen occurs, dose of allergen exposure, and the presence of non-specific adjuvant factors ³. The clinical allergic symptoms tend to vary with age, a phenomenon called the “atopic march”. The first manifestation of atopy is often atopic eczema and food allergy, usually appearing during the first 1 to 2 years of life ⁴. As the atopic march continues, allergies to foods are

typically outgrown and replaced in pre-school age by asthma and rhinoconjunctivitis to inhalant allergens, *e.g.* cat and birch ⁴. About 80% of children with atopic eczema early in life develop asthma and/or rhinitis later ⁵.

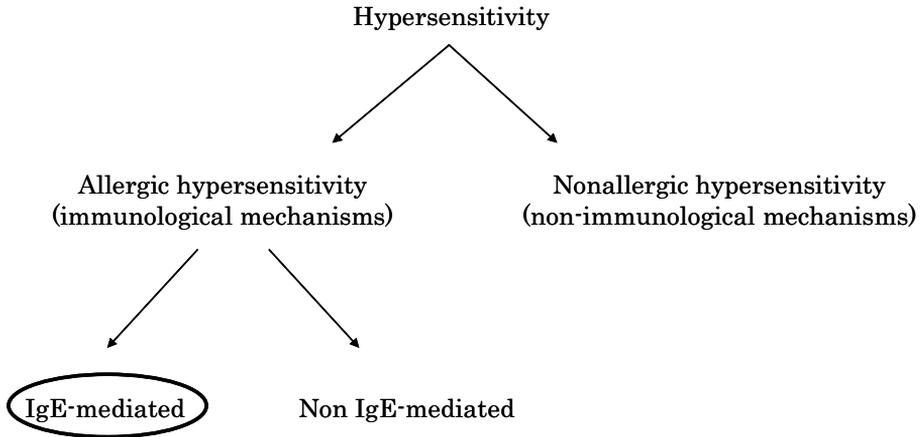


Figure 1. Terminology of hypersensitivity reactions.

The IgE-mediated allergic response can be divided into sensitisation, immediate hypersensitivity reactions and late phase reactions. When a non-pathogenic exogenous allergen penetrates into an organism, antigen presenting cells (APC), *e.g.* dendritic cells (DC), immediately take up and process the allergen in the endosome. The APC then present the allergen for T-cells that, either differentiate to T helper (Th)1 or Th2 cells (fig. 2). The process of sensitisation begins after generation of activated allergen-specific cluster of differentiation (CD)4+Th2 cells. They produce interleukin (IL)-4, causing B-lymphocytes to switch from production of IgM to IgE antibodies ⁶. The key cytokine in development of sensitisation and further allergic disease is therefore IL-4 ⁷. The majority of the IgE antibodies then attach to high affinity IgE receptors (FcεRI) on mast cells in tissue, but also on basophils in blood and activated eosinophils ⁶.

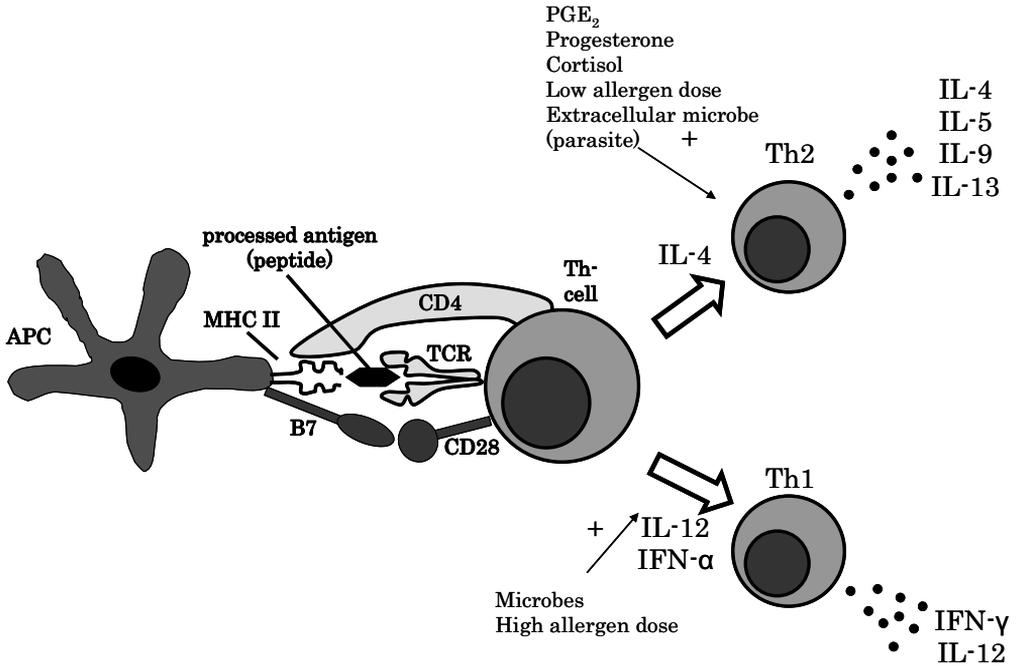


Figure 2. Schematic overview of antigen presentation and potential factors affecting the differentiation of Th1 and Th2 cells.

On re-exposure, the allergens immediately cross-link IgE antibodies on FcεRI expressing cells. The aggregation leads to activation of the cell, and in the case of mast cells, release of *e.g.* histamine, prostaglandins and cytokines, which generate clinical symptoms. This is called immediate hypersensitivity reactions ⁶. However, not all sensitised children develop symptoms and the reason for that is still an unsolved conundrum.

The last stage can be chronic and is called the late phase reaction. Inflammatory mediators from activated mast cells attract T-cells that together with mast cells induce other immune system cells, *e.g.* basophils, eosinophils and monocytes, to migrate into the affected tissue. The migrated cells then produce inflammatory substances on their own, sometimes leading to prolonged immune activity and tissue damage ⁶.

In conclusion, atopic disease is a hypersensitivity reaction in which the immune response reacts with up-regulated IgE antibody production upon exposure to innocuous antigens in the environment.

Immunological Mechanisms

T cells and cytokines

Atopy is characterised by Th2 deviated cytokine responses to allergens, with high levels of IL-4, IL-13, IL-9 and IL-5⁸, while Th1 cytokines, *e.g.* interferon (IFN)- γ , usually are observed in equal⁸ or lower⁹ levels. Atopic asthmatic children produce more IL-4 and less IFN- γ than non-atopic children with and without asthma¹⁰. This pattern is also noted in atopic dermatitis¹¹, thus suggesting that this imbalance in IL-4 and IFN- γ production is a feature of the atopic state. There are several factors influencing the specific cytokine profiles in T-cells (summarised in fig. 2), but the most important factor is what type of cytokines that dominates in the naïve T-cell environment¹². Presence of IL-4 is the most potent stimulus for Th2 differentiation, while IL-12 and IFN- γ favour Th1 development. Also, IL-4 inhibits Th1 development, while IFN- γ , IFN- α and IL-12 inhibit Th2 differentiation¹³. Interleukin-4 together with IL-13 promote B cell activation, plasmocyte differentiation and survival and isotype switch towards IgE synthesis¹⁴.

Once induced, the Th2 responses yield production of the typical cytokines sustaining the allergic response in different ways. Interleukin-5 is an important mediator of eosinophil differentiation and proliferation in bone marrow and also a chemotactic factor for their homing from bone marrow to inflamed tissues¹⁵. Interleukin-9 increases mast cell and eosinophil differentiation, proliferation, survival and homing¹⁶. Combined, IL-9 and IL-13 are involved in the allergic response promoting mucus secretion,

airway inflammation, hyperresponsiveness and tissue fibrosis^{17, 18}. Other Th2 associated cytokines are *e.g.* IL-16 that is chemotactic and induces activation and proliferation of CD4+Th2 cells, eosinophils and monocytes¹⁹ and IL-25 that induces IL-4, IL-13 and IL-5, thereby helping mast cells to enhance and sustain the Th2 activation²⁰.

It should be kept in mind that the Th1/Th2 dichotomy discussed above is a simplified working model. There are other subtypes of T cells that could be involved in the allergic process as well. T regulatory (Treg) cells assemble all T cell subtypes that may suppress immune responses via cell-cell interactions and/or production of suppressor cytokines. There are many different types of Treg cells. T helper 3 cells release transforming growth factor (TGF)-beta, while Tr1 cells are defined by their high production of IL-10 and TGF-beta. Another type, CD4+CD25+ Treg cells, also produce high levels of IL-10 and TGF- beta, have high expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and inhibit IL-2 production on target T cells²¹. During the early course of allergen-SIT, Tr1 cells are induced, suppressing allergen specific Th1 and Th2 responses via *e.g.* IL-10, TGF-beta and CTLA-4. If these suppressor functions of Tr1 are blocked, the activation of allergen specific Th2 cells is increased, suggesting that a balance between Tr1 and Th2 cells may control allergic disease²².

In conclusion, immune responses in atopic individuals are Th2 skewed with increased production of IL-4, IL-5 and IL-13. The most important factor influencing the specific cytokine profiles that develop in T-cells is the predominance of a given cytokine in the T-cell environment.

Immunoglobulins

Immunoglobulins (also called antibodies) mediate humoral immune responses and protect the human from infection in three main ways, *i.e.* neutralisation, opsonisation and activation of the complement system that may enhance opsonisation, and can directly kill certain bacterial cells ². There are five different isotypes of antibodies, IgM, IgA, IgG, IgE and IgD. The IgA and IgG antibodies can be further divided into different subclasses, IgA₁ and IgA₂ and IgG₁, IgG₂, IgG₃ and IgG₄, respectively.

Antigen-specific antibodies are produced by activated plasma cells. B-cells are produced in the bone-marrow, mature in the spleen and start thereafter to circulate in the body. Antigens that bind to the surface immunoglobulin receptor on B cells is internalised and processed into peptides that activate armed T cells. In order to proliferate and differentiate into plasma cells, the B cells also need accessory signals from helper T cells in a contact dependent pathway. The same effect can be achieved directly from repetitive epitopes of *e.g.* bacterial cell wall polysaccharides, so called T cell independent antigens. The most important component of contact dependent T cell help for B cells appears to be the CD40 ligand, which is transiently expressed in activated T helper cells. Depending on which type of cytokine signal that is present, different antibody isotypes are produced ²³.

If IL-4 and IL-13 are present in combination with CD40 stimulation, the production of IgE is initiated and enhanced ²⁴. The IL-4 stimulated IgE production is increased in the presence of *e.g.* IL-5, IL-6 and IL-9, in contrast to *e.g.* IFN- β , IL-12 and TGF- β which inhibit this IgE synthesis. The IgG₄ antibody production is also initiated by IL-4 ²⁴. On the other hand, IL-10 ²⁵ and IL-12 ²⁶ inhibit IgE production but up-regulates the secretion of IgG₄ ²⁷, although the data on IL-10 regulation of IgE synthesis

are contradictory. Immunoglobulin A is produced when CD40 signals are provided in combination with the presence of TGF- β and IL-10²⁸.

The basic Y-shaped structure is shared by all immunoglobulins and consists of four polypeptide chains, two heavy and two light chains connected by disulphide bonds (fig. 3). The five different types of mammalian heavy chains define the different classes of antibodies. A highly variable amino acid part of the Ig heavy and light chain determines the specificity of antigen recognition².

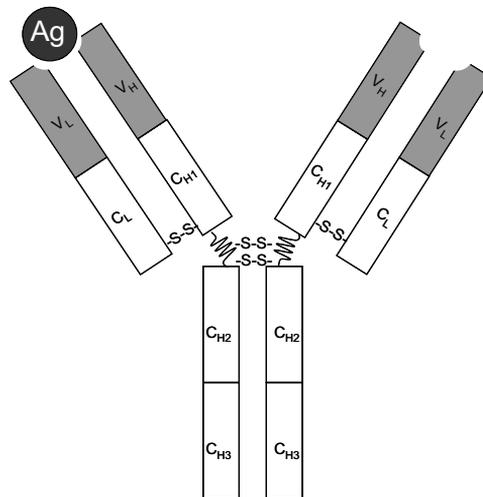


Figure 3. Principle structure of immunoglobulins.

VH, VL; portions of heavy (H) and light (L) chains with highly variable amino acid composition.

CH1-3, CL; constant parts of the heavy (H) and light (L) chains. They hold the molecule together and are involved in the binding to Fc receptors and complement.

Ag; antigen binding to the antigen binding site.

The different isotypes and subclasses of antibodies have different functions in the immune system. Immunoglobulin M is always the first antibody to be produced during a humoral immune response and can activate complement by the binding of the complement factor C1 to its Fc part.

Immunoglobulin G antibodies are the principal isotype in the blood and extracellular fluid, whereas IgA dominates in secretion. Immunoglobulin G may activate complement, except for the IgG₄ subclass. Immunoglobulin A is a weak activator of complement, having instead its chief function as a neutralising antibody. Immunoglobulin D occurs as a membrane bound antibody in B cells, and is co-expressed with IgM. However, IgD antibodies account for less than 1% of the total plasma immunoglobulin and their function is still obscure. As already mentioned, IgE antibodies are the most important antibodies in the allergic reactions.

IgG antibodies in allergic disease

During the foetal and newborn period, all IgG subclasses in the circulation are predominantly of maternal origin²⁹. The kinetics of IgG responses in the newborn child then vary depending on subclass and antigen specificity. However, the levels of serum IgG antibodies generally decrease during the first six months in life, increase thereafter and peak rather early in life³⁰,³¹. However, the levels of IgG antibodies differ between atopic and non-atopic individuals. The levels of IgG₄ antibodies to house dust mite (*Dermatophagoides pteronyssinus*), grass pollen, birch and cat dander are higher, while the levels of IgG₁ are reported to be similar or higher in sensitised, compared with non-sensitised individuals³¹⁻³³. IgG₄ switching is stimulated by Th2 type cytokines and this may be one explanation to the high IgG₄ antibody levels in atopics^{24, 34, 35}. Also, IgG antibodies to food allergens are produced in both atopic and non-atopic children, a production that peaks in early childhood but has declined by eight years of age^{30, 31}. Allergic symptoms and sensitisation are associated with higher levels of specific IgG subclass antibodies to food allergens, particularly IgG₄³¹.

High titres of IgG₄ are found in healthy individuals during chronic exposure to certain allergens³⁶. Also, previous studies on immunotherapy with inhalant allergens indicate a protective role of IgG₄ antibodies, as the

levels increase during treatment ^{25, 37}. Immunoglobulin G₄ antibodies to allergens have therefore been proposed to act as blocking antibodies by competing with IgE for allergen binding to IgE receptor expressing cells ²⁵. Competition between IgE and IgG₄ antibodies at the level of APC has been demonstrated in vitro as well ²⁵. However, the increase of IgG₄ during immunotherapy may reflect a change in T cell immunity with increased IFN- γ ³⁸ and IL-10 ³⁹ responses, leading to a more tightly regulated IgE antibody production compared with IgG₄ ⁴⁰. Modified Th2 immune responses that include high levels of IgG₄ antibodies in combination with a lack of IgE antibodies has been proposed to be associated with protection from allergic disease ⁴¹.

The expression of IgG₁ is promoted by IFN γ , and the antibody can form immune complexes with antigens, bind to Fc receptors on lymphocytes and activate complement ⁴². Cross-sectional studies have shown that IgG, IgG₁ and IgG₄ are associated with atopic dermatitis and increased levels of IgG₁ to OVA ⁴³ and BLG ⁴⁴ have been observed in children with persisting sensitisation to food. However, atopy is often most clearly associated with high levels of IgG₄ antibodies.

IgA antibodies in allergic disease

Immunoglobulin A is the predominant antibody isotype in humans. The IgA antibodies are either monomers, mainly present in serum, or dimers at mucosal surfaces. Both atopic and non-atopic individuals generate IgA antibodies to environmental allergens ^{45, 46}, although it has been reported that some atopics may lack allergen-specific IgA responses ⁴⁷. Antigen specific secretory (S)IgA blocks adherence and penetration of antigens, preventing immune inflammatory responses through the mucosal epithelium ⁴⁸. Thus, high levels of allergen specific IgA could, theoretically, prevent allergen absorption and thereby sensitisation and subsequent development of allergic disease. In mice, passive transport of allergen-

specific IgA antibodies may protect against airway hyperresponsiveness ⁴⁹. In humans, low levels of total SIgA or transient IgA deficiency are associated with increased risk of developing allergic diseases ^{50, 51}, although contradictory results have been reported ⁵². Local IgA response to cow's milk protein associates with the development of tolerance to that allergen ⁵³, low SIgA is associated with immediate reactions to foods ⁵⁴ and children with gastrointestinal symptoms to cow's milk allergy, have increased concentrations of IgA antibodies to bovine serum albumin (BSA) ⁵⁵. Moreover, it has been found that serum IgA is increased in anaphylactic mice, while β -lactoglobulin (BLG) specific SIgA was increased in faeces from tolerant mice ⁵⁶. Also, BLG-induced IL-10 and TGF- β levels were increased at IgA production sites *i.e.* Peyer's patches (PP) ⁵⁶. We have recently shown that the level of total (T)IgA in saliva is higher, whereas the levels of SIgA is lower in allergic, than in non-allergic children ⁵⁷. Furthermore, low levels of saliva SIgA in skin prick test (SPT) positive infants were associated with development of allergic symptoms ⁵⁷. Allergen specific IgA antibodies were more often detected in saliva from children with allergic disease compared to healthy children, which is in agreement with an earlier report ⁵⁸. Also, low serum IgA levels early in life are associated with food allergy at 18 months (Lundell A-C et.al, unpublished data). The levels of SIgA in saliva increase with age ⁵⁷, a development that has been suggested to occur more slowly in allergic than in non-allergic children. IgA antibodies to allergens are found in both allergic and non-allergic children, and the levels of IgA antibodies to foods are down-regulated with age ⁵⁷.

In conclusion, T helper cells regulate B cell antibody production. All isotype switching requires CD40 stimulation and cytokines confer isotype selectivity. IgG₄ may be up-regulated during allergen exposure and might have a protective effect against allergy development. IgA antibodies can theoretically prevent sensitisation, but might not have a decisive effect on the development of allergic diseases.

Mucosal immune responses

The gastrointestinal tract is the largest immunologic organ in the body and the gut mucosa has a surface of approximately 400 m² in humans and 80% of the body's activated B cells are located in the gut ⁵⁹. The gut lumen is constantly exposed to myriads of pathogenic microbes and dietary constituents. The gut must pursue a delicate balance between an effective barrier against pathogens and foreign structures and absorptive function for nutrients.

The surface epithelium of the mucosa is directly exposed to the environment in lumen, but the epithelial cell line constitutes an effective barrier as the cells are joined together by tight junctions, only allowing ions to pass through. There are several components in the mucosal barrier protective systems. The proteolytic enzymes in mouth, stomach, small bowel and colon break down polypeptides into smaller fragments making them less immunogenic. The low pH in the stomach is another protective tool. A key component of the mucosal defence is the production of mucus from goblet cells, creating a thick barrier covering the epithelial cells. Bacteria and viruses become trapped within the mucus particles, preventing access to the underlying epithelium ⁶⁰. Within the mucus layer, there are SIgA antibodies that may bind microorganisms, subsequently preventing epithelial attachment ⁴⁸. However, there are different routes for antigen uptake in the gut, *e.g.* via M cells covering organised lymphoid tissues like PP, via DCs interspersing the epithelial cells or by the epithelial cells themselves (fig. 4). Increased antigen uptake is also observed if the permeability of the gut is increased, *e.g.* during inflammation. Therefore, an abnormal intestinal permeability in food allergic individuals has been proposed ⁶¹. However, increased intestinal permeability in preterm babies seems not to be associated with increased risk of developing allergy ⁶².

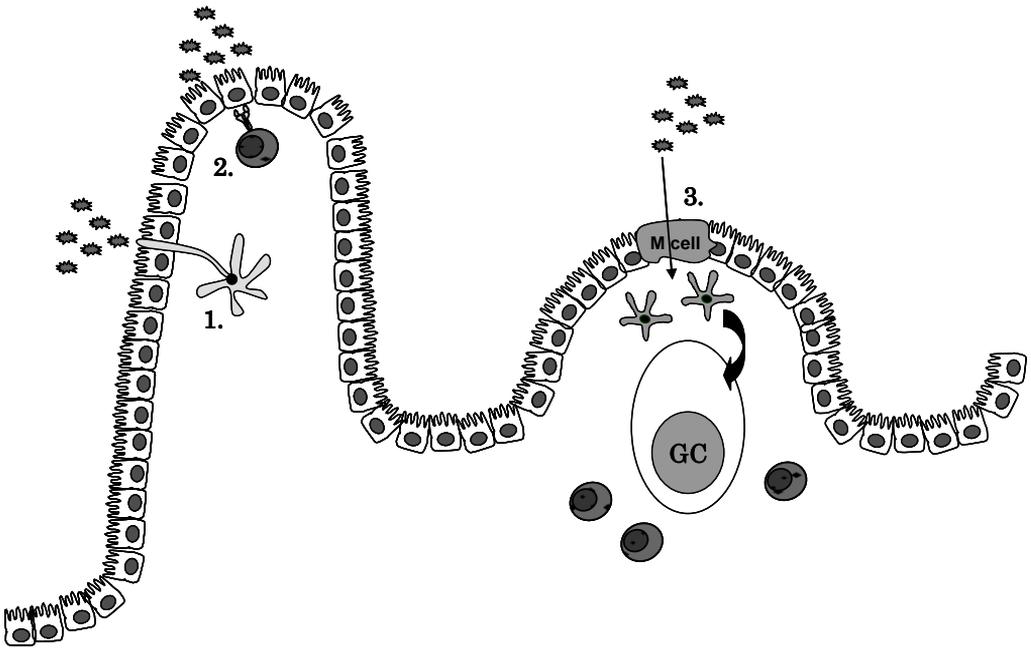


Figure 4. Different routes for antigen uptake in the gut.

1. Dendritic cells (DC) may send dendrites into the lumen and sample antigen directly from the lumen. Antigen-carrying DCs might then traffic through the lymphatics to the mesenteric lymph node.

2. Antigens can be taken up by endocytosis and cross the epithelium in small vesicles, or intestinal epithelial cells may express MHC class II molecules, capable to present antigens, and might then encounter T cells or macrophages in the lamina propria or might directly reach the circulation.

3. Antigens are taken up by M cells and ingested by DCs in Peyer's Patches. Antigen-carrying DCs then present the antigen for B cells in the germinal center (GC). B cells may, upon appropriate signals, migrate via the mesenteric lymph node to the intestinal lamina propria, differentiate and secrete dimeric IgA.

Modified from Chehade et.al. 2005

Secretory IgA

The most important antibody at mucosal surfaces is SIgA. Dimeric IgA antibodies consisting of two monomers joined by a J-chain are produced by B lymphocytes⁴⁸. Peyer's patches are organised lymphoid tissue that are covered with specialised epithelium *i.e.* follicle-associated epithelium,

including M cells that are epithelial cells specialised for antigen uptake. The PP consists of a germinal center comprised of B lymphocytes and to some extent also T lymphocytes. These B cells migrate, after appropriate signals, to the mesenteric lymph node, mature into plasma cell precursors and migrate thereafter further to the lamina propria for secretion of dimeric IgA⁶³. Dimeric IgA antibodies synthesised in plasma cells beneath the epithelial basement membrane, bind to the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of the epithelial cells at mucosal sites, such as the salivary gland. The complex is transported to the apical surface, where the pIgR is cleaved leaving the extracellular IgA-binding component, the secretory component (SC), bound to the IgA molecule (fig. 5).

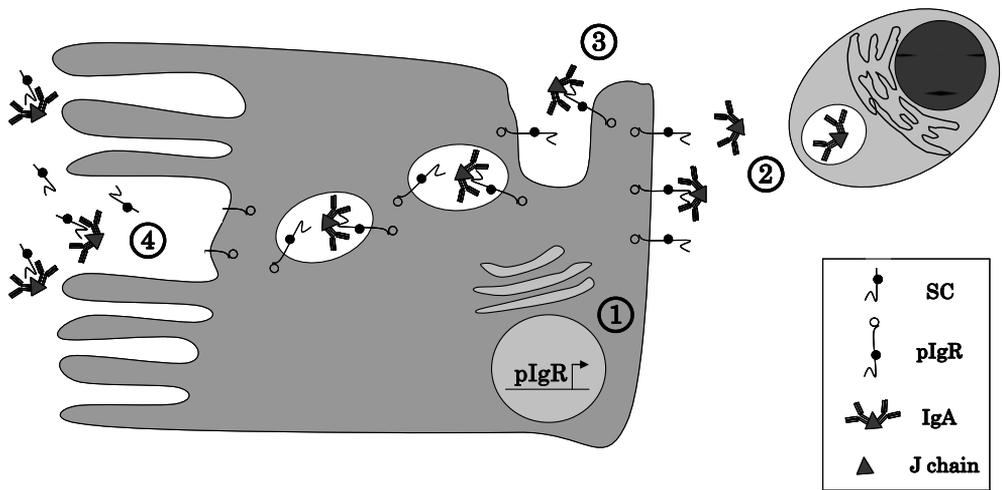


Figure 5. Transport of dimeric IgA over the epithelium.

Newly synthesised polymeric immunoglobulin receptor (pIgR) is transported to the basolateral surface (1), where dimeric IgA, produced by plasma cells in lamina propria, may bind to the receptor (2). The IgA-pIgR complex or pIgR alone, is then endocytosed (3) and transported through a series of intracellular vesicles to the apical surface (4). pIgR is then cleaved leaving a secretory component (SC) attached to the IgA dimer.

Modified from Kaetzel, 2005.

The SC makes the IgA molecule more resistant to cleavage by proteolytic enzymes and enhances both the stability and effector functions of IgA ⁶⁴. The pIgR-mediated transcytosis of IgA across the epithelium suggests that all IgA in the lumen of mucosal organs should be in the secretory form. Mice with pIgR deficiency have markedly reduced IgA levels in external secretions in combination with elevated serum IgA, showing the importance of pIgR in the transcytosis of IgA across mucosal epithelia ⁶⁵. The expression of pIgR is modulated by several different regulators *e.g.* cytokines (IFN- γ , TGF- β , IL-1, IL-6 and TNF), retinoic acid, hormones, microbes and microbial derived products (reviewed in ⁶⁴). Both bacterial LPS and double stranded RNA up-regulate the expression of pIgR ⁶⁶. Also commensal intestinal bacteria, *e.g.* *Bacteroides thetaiotaomicron*, have been shown to up-regulate the expression of the pIgR ⁶⁷.

Secretory IgA, and to a lesser extent, SIgM antibodies enhance the epithelial barrier function by a mechanism termed immune exclusion ⁶⁸. One of the main functions of SIgA is to protect against mucosal infections by preventing adherence and penetration of microbes, and therefore low levels of SIgA could theoretically impose an increased vulnerability for infections. Breast fed infants are protected against infections by high levels of IgA antibodies in breast milk ⁶⁹. Immunoglobulin A antibodies suppress neutrophil, eosinophil and monocyte chemotaxis and inhibit IgE-induced histamine release ⁴⁸. Thus, high levels of SIgA in the mucosa could possibly interfere with the interaction between allergen and IgE antibodies in sensitised individuals, thereby preventing the development of allergic symptoms.

Human B cells produce two subclasses of IgA *i.e.* IgA₁ and IgA₂. Immunoglobulin A₁ has an extended hinge region and is therefore more easily cleaved by proteases produced by certain bacterial pathogens, compared with IgA₂ that is more resistant against proteolytic degradation ⁷⁰. Serum IgA is predominantly of the IgA₁ subclass while the presence of

the two subclasses is rather similar at mucosal sites, except for the colon where IgA₂ is more common than IgA₁. Immunoglobulin A₂ is positively correlated with heavier microbial load.

In conclusion, the mucosa is constantly challenged by pathogens and antigens and has a very central role in protecting the humans from being infected. The most important antibody at mucosal sites is SIgA, enhancing the epithelial barrier function by immune exclusion.

Immune responses in children

The first years of life is a period with high risk for infections partly depending on the immature immune system in young children. The immune system at birth is not fully adapted for postnatal life in several respects, as neonates have poor cell-mediated immunity, poor inflammatory responses, impaired defences against intracellular pathogens and inability to produce certain immunoglobulin isotypes ⁷¹.

Infants have a high proportion of naïve T cells and a low proportion of memory T cells, not reaching adult proportions until 12-18 years ⁷². Proliferation of T cells and cytokine production are reduced in neonates and naïve T cells in neonates are more easily Th2 skewed than adult cells ^{73, 74}. This may be due to the proposed switch from Th1 to Th2 responses, supposed to characterise successful pregnancy aiming at reduction of the maternal reactivity against the foetal allograft ⁷⁵.

The APCs are central in induction of antigen specific responses and their function therefore contributes to the overall effectiveness of the defence mechanisms during infancy. Monocytes are rather mature at birth ⁷⁶, but they seem to have a reduced Th1 inducing capacity, possibly due to a low ability to produce IL-12 ⁷⁷ and IFN- α ⁷⁸ that induce IFN- γ synthesis. They

also have a reduced production of TNF- α ⁷⁹. Cord dendritic cells have lower capacity to produce IL-12 and IFN- γ in response to lipopolysaccharide (LPS) than peripheral blood DCs from adults ⁸⁰.

The ability to produce antibodies is impaired in newborns and they receive their humoral protection by active transfer of maternal IgG antibodies during pregnancy ⁸¹ and thereafter by IgA antibodies obtained through breast-feeding ⁸². The only antibody synthesised to a higher degree in newborns is IgM, while IgE, IgG and IgA are only found at minute levels ⁸³. Immunoglobulin M increases rapidly after birth ⁸⁴, adult levels of IgE ⁸⁴, IgG ⁸⁵ and IgA ^{84, 86} are not reached until 5 years of age, or even later. A reason for the impaired antibody production during infancy may be the immaturity of the Th cells, but it has also been shown that B cells are functionally immature ^{83, 87}.

Development of immune responses in allergic children

The immune system is influenced before birth by the Th2 skewed placental environment and the immune response may still be Th2 skewed at birth. It is possible that Th1 stimulating factors, *e.g.* from microbes, may be needed to redress this foetal Th1/Th2 imbalance ⁸⁸. During the first year of life a suppression of the Th2-like responses has been observed in non-atopic, but not in atopic children ⁸⁹, indicating that the postnatal maturation of immune functions may be delayed in children who develop allergy compared to those who not ⁹⁰. The reduced neonatal IFN- γ production is particularly pronounced in atopic children ^{91, 92} and mononuclear IFN- γ responses to mitogens are lower in children who have heredity for allergy compared to children who have not ⁹³.

Prospective studies have shown that IgE antibodies to foods are commonly detected both in atopic and non-atopic infants during their first year of life, although the magnitude of the responses is higher and of longer duration

in the former group ⁹⁴. In most cases, the initial IgE responses to foods are down-regulated at the age of 2-4 years ⁹⁴.

In conclusion, newborns have an immature Th2 skewed immune system with a reduced cytokine production and high proportion of naïve cells. Later on, the non-atopic children shift in favour of Th1-mediated responses to allergens, while the atopic children seem to fail to down-regulate the Th2-like response.

The influence of environmental factors on the development of allergic disease

There is a worldwide variation in prevalence of allergic diseases ⁹⁵ and the prevalence has increased over the last decades in industrialised countries with a market economy ⁹⁶⁻⁹⁸. However, recent data suggest that the prevalence has peaked in some regions ⁹⁹. Children raised in rural areas of developing countries have demonstrated a low prevalence of atopy and asthma compared to children from affluent countries ¹⁰⁰. Changes in the genotype can likely not account for such rapid increase, and therefore the explanation is sought in the environment. Several risk factors for allergic diseases have been proposed, including exposure to smoke and pollutions, poorly ventilated homes and reduced breastfeeding. However, none of these factors solely explain the large increase in prevalence of allergic diseases in some parts of the world. Instead the so called “hygiene hypothesis” has been proposed, suggesting that the increase of allergic diseases may be caused by a decreased and/or changed microbial pressure in the environment (discussed in more detail below).

Another possible explanation may be a change in the diet, towards a dominating intake of ω -6 fatty acids in relation to ω -3 fatty acids. The increase of allergic diseases in Western countries has occurred in parallel with a change in the consumed ω -6/ ω -3 ratio. There are several studies

indicating that a high intake of fish, rich in fatty acids, has a protective effect on the development of allergic diseases^{101, 102}. Moreover, from our group, very recent data from a randomised intervention trial featuring supplementation with ω -3 poly unsaturated fatty acids during pregnancy, indicate very promising results regarding protection from developing sensitisation to foods (Furuhjelm, C et.al, unpublished data). The composition of maternal serum phospholipids was affected in supplemented mothers and this was associated with a reduced production of prostaglandin E₂ that may influence the immature immune system of the child, possibly explaining the preventive effect of ω -3 supplementation (Warstedt, K et.al, unpublished data).

Microbial exposure

Several studies support the hygiene hypothesis. Epidemiological studies have generally found a lower prevalence of asthma, allergic rhinitis and inhaled allergen sensitisation in persons who have experienced infections of the respiratory tract *e.g.* measles and tuberculosis, or gastrointestinal tract (*Hepatitis A*, *Helicobacter pylori*, *Toxoplasma gondii*, hookworm)¹⁰³. The differences in prevalence of allergic diseases between Western and Eastern Europe may be caused by factors associated with a Western lifestyle, *e.g.* major improvements in public health and personal hygiene practice, diet changes, reduction of average family size and improvements of general living standards^{88, 104, 105}, resulting in a changed overall exposure to microbial stimulation. There is also a low prevalence of atopy in children from families with an anthroposophic lifestyle, characterised by a low use of antibiotics, few vaccinations and larger intake of food containing *Lactobacillus*¹⁰⁶. Environmental microbes are the major stimuli of the immune system and both internal and external microbial load may be of importance in the protection from allergic disease. Animal studies have shown that the gut flora is important for the development of the immune system and also for the induction of tolerance to food antigens¹⁰⁷, and it has also been shown that the microflora differs between allergic

and non-allergic children ¹⁰⁸. We have previously shown that the commensal gut flora differs between Estonian and Swedish populations, as Estonian individuals have a more diverse flora and are more often colonised with *e.g. Lactobacillus* and *Bifidobacteria* ¹⁰⁹. Several ongoing studies investigate the preventive effects of different strains of *Lactobacillus* species on development of allergic diseases. Although the outcome of these studies are conflicting, a very recent meta-analysis suggests a protective effect of probiotic supplementation during pregnancy regarding the outcome of atopic dermatitis in the children ¹¹⁰.

The importance of the external environment is supported by several studies showing that living on a farm during early childhood, particularly during the first year of life ¹¹¹, is associated with a reduced prevalence of allergic disease ¹¹²⁻¹¹⁴. Higher levels of endotoxin, the LPS component of the outer membrane of gram negative bacteria ¹¹⁵, were found in house dust from farming families compared with non-farming families ¹¹⁶. Consequently, it was hypothesised that exposure to endotoxin during early life may potentiate the Th1 maturation and thus protect against the development of allergic disease ¹¹⁴. Moreover, a cross-sectional study showed that exposure to low levels of endotoxin in house dust was associated with SPT reactivity in wheezing American children from families with a low socio-economic status ¹¹⁷. If the external microbial pressure fluctuates between countries with different prevalence of allergy, has not been elucidated before, however.

The potent immune stimulatory capacity of endotoxin is largely attributed to the lipid A moiety of endotoxin (fig. 6), which is highly conserved across different bacterial species. Endotoxin comprises most of the outer layer of the outer cell membrane of all gram-negative bacteria and very small amounts of endotoxin are needed to induce an immune response. Endotoxin is also remarkably resilient, suggesting a strong potential to persist as an immune modulator in our environment ¹¹⁸. The effect of LPS

is induced via toll-like receptors (TLR)4, which strongly influence innate APC, especially DC:s, to produce IL-12 and to costimulate T cells to become effector T cells that primarily secrete IFN- γ ^{119, 120}. Thus, memory T cells, generated after antigen and LPS stimulation, have been shown to be IFN- γ -producing effector T cells ¹²¹. Also, IFN- γ primes innate immunity cells to produce greater amounts of IL-12 in response to stimulation ¹²². It is therefore possible that endotoxin exposure, in combination with its capacity to drive the development of T cell memory to environmental allergenic proteins, also may push these memory T cells to produce IFN- γ , thereby inhibiting Th2 cytokine production and preventing atopic immune development ¹¹⁸. Moreover, LPS signalling via TLR on epithelial cells increase the capacity of DCs to sample antigens from lumen through dendrites crossing the epithelial tight junctions ¹²³. Thus, LPS stimulation may increase the antigen uptake leading to a more Th1 skewed response.

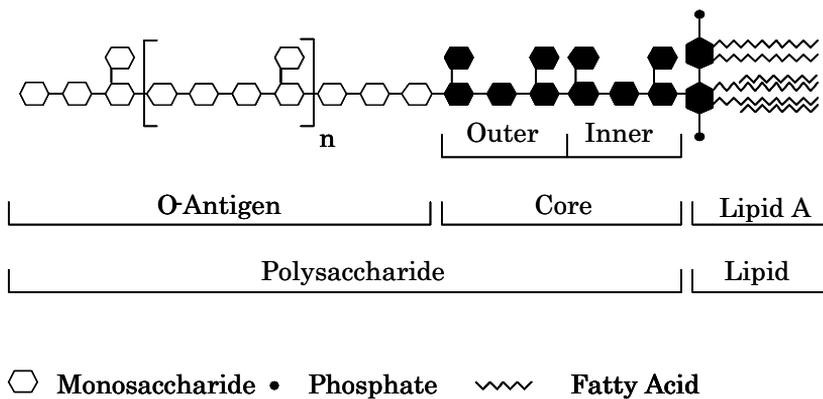


Figure 6. General chemical structure of bacterial lipopolysaccharide (LPS). It is composed of two chemically dissimilar structural regions, the hydrophilic repeating polysaccharides of the core and O-antigen structures and a hydrophobic domain known as lipid A. The O-specific chain, consisting of up to 50 repeating oligosaccharide units, is characteristic and unique for each bacterial strain. The lipid A component is responsible for the endotoxic activity of LPS.

Allergen exposure

Allergen exposure is obligatory to trigger an allergic reaction. The first encounters with ubiquitous allergens may occur already before birth. Allergen-specific immune responses have been detected in foetal blood already after 22 weeks of gestation¹²⁴ and proliferative responses to *e.g.* cow's milk protein (CMP) has been shown in cord blood from both atopic and non-atopic children^{125 126}. The presence of specific IgE antibodies in cord blood has also been demonstrated¹²⁷ and it has been hypothesised that allergen exposure during foetal life may be a risk factor for later sensitisation. However, several studies investigating allergen avoidance during pregnancy have been performed^{128, 129} without showing any preventive effects against development of allergic disease. In fact, in one study an increased risk of prolonged egg intolerance was shown in children whose mothers had been on egg elimination diet during pregnancy¹³⁰. Small amounts of allergens are also present in breast milk and may stimulate IgE production and evoke allergic symptoms during lactation¹³¹. Strict allergen avoidance of highly allergenic foods by lactating mothers decreases allergen levels in the milk and delays the onset of sensitisation and atopic symptoms^{132, 133}. However, when the children reached 10 years of age no differences in sensitisation or allergic symptoms could be observed¹³⁴. There is also an ongoing discussion whether introduction of food allergens during breast feeding could be of benefit for the child¹³⁵.

The role of allergen avoidance as a primary preventive strategy in childhood asthma is debated¹³⁶ and there are conflicting data regarding any relationship between allergen exposure during childhood and sensitisation¹³⁷. One reason for the controversy may be that any association between allergen exposure and sensitisation may be strongly influenced by genetic¹³⁸⁻¹⁴⁰ and other environmental factors¹⁴¹⁻¹⁴³. Moreover, any association may vary depending on which allergen that is considered¹⁴⁴ and may not be linear. For cat allergens, an increased risk of

sensitisation is observed at exposure to low and medium levels, whereas the risk is reduced at high exposure levels ^{41, 145}. The mechanism behind this phenomenon may be that high exposure to some airborne allergens may favor immunological tolerance development to such allergens through a modified Th2 response characterised by a high IgG₄/IgE ratio ⁴¹. However, any protective effect of cat exposure might be related to concurrent endotoxin exposure ¹⁴⁶.

Treatment with immunotherapy has proven that increasing doses of an allergen can induce tolerance in patients already suffering from allergic disease. Although the mechanisms behind this are not fully known, one essential step in allergen-specific immunotherapy or sublingual immunotherapy (SIT) is the induction of a tolerant state in peripheral T cells ²². Interleukin-10 and TGF- β are increasingly produced by antigen-specific T cells, mediating several different regulatory effects ¹⁴⁷⁻¹⁴⁹. The production of the Th2 cytokines IL-4 and IL-5 is inhibited by IL-10 and TGF- β . The release of proinflammatory mediators is suppressed by down-regulation of IgE dependent activation of basophils and mast cells and by decreasing survival and activation of eosinophils. Moreover, the production of IgE is decreased while IgG₄ and IgA production is increased by IL-10 and TGF- β , respectively. The increased IgG₄ antibody levels during treatment ^{25, 37} may have a protective effect as IgG₄ antibodies to allergens have been proposed to act as blocking antibodies (discussed in more detail on page 23). Furthermore, Treg cells could inhibit Th1 and Th2 cells by cell-cell contact or by decreasing the antigen presenting function of DCs.

In conclusion, the rapid increase in prevalence of allergic disease in affluent countries may be caused by changes in the environment due to a changed life style, resulting in a reduced internal and external microbial exposure. Allergen exposure is obligatory to trigger an allergic reaction, but allergen avoidance as primary prevention and treatment strategy has been questioned.

Food allergy

Food hypersensitivity affect about 6% to 8% of children in countries with a Western lifestyle ¹⁵⁰. A variety of symptoms such as eczema, urticaria, gastrointestinal and respiratory problems are involved. The condition is caused by IgE-mediated or non-IgE-mediated *i.e.* cellular mechanisms ¹. A recent meta-analysis reported that the prevalence of food hypersensitivity reactions is divergent between studies ranging from 0.2%-7% for egg and 1.2%-17% for milk ¹⁵¹. Another study investigated IgE-mediated food allergy confirmed by oral challenge in an unselected group of 3 year old children and reported a prevalence of 2.3%. The major offending allergens were hen's egg (1.6%), cow's milk (0.6%) and peanut (0.4%) ¹⁵², but also wheat, fish and soy were causal ¹⁵³. A common feature of these food allergens is their resistance to heat and gastric digestion ^{154 155} and primary sensitisation to food proteins occurs in the gut ¹⁵⁶. Children with other atopic disorders have higher prevalence of food allergy. About 35% of children with moderate-to-severe atopic dermatitis have IgE-mediated food allergy ¹⁵³ and about 6% to 8% of asthmatic children have food-induced wheezing ¹⁵⁷. Infants who develop symptoms to milk, usually do so very early in life, often within one week from introduction of cow's milk based formula, or even during breast-feeding, and onset after one year of age is unusual ¹⁵⁸. Before the age of five almost 80% of the children have outgrown their food allergy, *i.e.* developed clinical tolerance ^{158, 159}. Clinical symptoms to egg develop later than milk, possibly because of later introduction. Clinical tolerance to egg is achieved in 30-44% of egg allergic children by school age ¹⁶⁰. Why some children outgrow their food allergy while others develop persistent allergy is not fully known and there are no clinical markers that can be used to predict the tolerance development.

Several different methods can be used to diagnose food allergy, *e.g.* thorough case history, SPT, circulating IgE to food, outcome during elimination diet and oral food challenges. However, the golden standard for diagnosis of food allergy is the double-blind placebo-controlled food

challenge (DBPCFC) ¹⁶¹⁻¹⁶³. A positive SPT does not necessarily prove that the food is causal and a negative SPT may not confirm absence of IgE antibodies. However, increasing SPT wheal size correlates with an increasing likelihood of clinical allergy ^{164, 165}, and with a skin prick wheal diameter from 5 mm for egg and 6 mm for milk in infants, all challenges were positive ^{164, 165}. Increasing concentrations of food-specific IgE also correlate with an increasing likelihood of a clinical reaction ¹⁶⁶ and 95% of infants with a diagnostic value of 2-4 kUA/l for egg ¹⁶⁷ and 5-7.5 kUA/l for milk ^{168, 169} respond with a positive DBPCFC. However, different cut-off values are presumably required for different subpopulations of children ^{170, 171}.

Cow's milk specific T cell responses are present in both patients with cow's milk allergy (CMA) and healthy individuals ¹⁷²⁻¹⁷⁴, and also in children that have outgrown their CMA ¹⁷⁵. Similar results are observed for ovalbumin (OVA)-specific T cells responses ¹⁷⁶. This suggests that the presence of a food-specific T cell responses *per se* does not cause food allergy ¹⁷⁷. However, the cytokine production by food-specific T helper cells is Th2 skewed ¹⁷⁸, a phenomenon not observed in tolerant patients ¹⁷⁴, although conflicting observations are reported ¹⁷⁶. Milk allergic children express higher levels of activation cell surface markers such as CD25 and CD30 on *in vitro* generated CMP-specific T cells, compared with children who have developed tolerance ¹⁷⁵, suggesting a strong food specific T cell response in children with persistent food allergy.

Duodenal lymphocytes from food allergic children did not show the same Th2 skewing that was observed in T cells isolated from the circulation ¹⁷⁹, but the intestinal expression of TGF- β 1 was reduced suggesting an impaired generation of Th3 cells ¹⁷⁹. Also, it was shown that T cells in the gastrointestinal mucosa of patients with CMA and gastrointestinal symptoms produced virtually no TGF- β ¹⁸⁰. The proliferative capacity of CMP-specific T cells is suppressed by TGF- β . Thus, TGF- β may be

important for the development of tolerance to food proteins. Patients with hazelnut allergy have lower serum IL-10 levels than tolerant patients with a former hazelnut allergy, ¹⁸¹. Interleukin-10 has been shown to increase during and after immunotherapy ¹⁸² and the cytokine cooperates in the regulatory T-cell response to allergens ¹⁴⁷. In concordance with this, tolerance to milk has been shown to associate with high frequency of circulating IL-10 producing CD4+CD25+ T reg cells. They may suppress effector T cells, leading to a decreased *in vitro* proliferative responses to milk allergens ¹⁸³.

The recommended treatment for food allergy is avoidance of the offending food, unfortunately often leading to a decreased quality of life ¹⁸⁴. Milk and egg, the most common offending foods are also important sources of nutrients in childhood ^{185, 186}. It has been shown that children on elimination diet have significantly lower intake of nutrients like fat and proteins, thereby increasing the risk for malnutrition ¹⁸⁶ and impaired growth ^{185, 187}. Thus, all unnecessary elimination diets should be avoided and the benefit of dietary strategies on allergy preventive effects has recently been questioned ¹⁸⁸. Moreover, recent studies have suggested that administration of increasing oral doses of the offending food can promote faster tolerance induction in food-allergic children ^{189, 190}. However, further studies are needed to clarify which children that could benefit from continuing food allergen exposure.

In conclusion, food allergy and eczema are common in infants. Although most children outgrow their food allergy before three years of age, there are no clinical markers that can predict the tolerance development. Immunological differences between children with and without persistent food allergy are poorly investigated.

AIM OF THE THESIS

The overall aim of this thesis was to investigate humoral, mucosal and cell-mediated immunity in association to allergy and allergy development in young children and to relate this to environmental factors, *e.g.* living conditions, microbial exposure and allergen-free diet. The specific aims of the individual papers were to investigate the levels of endotoxin in two countries with high (Sweden) and low (Estonia) prevalence of allergic diseases (paper I) and to characterise the mucosal immune response in relation to microbial exposure and atopic development in those populations (paper III). The aim was also to study the levels of antibodies (paper II) and cytokines (paper IV) in relation to tolerance development in food sensitised eczematous children and to investigate the effect of allergen-free diet on the development of immune responses (paper II).

MATERIAL AND METHODS

Study subjects

Two cohorts have been investigated in this thesis.

Cohort 1

Pregnant women and their families were invited at the maternity ward in Tartu (Estonia) and Linköping (Sweden) to participate in a study regarding immunological and allergy development in children. Between February 1997 and June 1998, 115 Estonian families agreed to participate in the study, while 149 Swedish families approved to join between March 1996 and April 2000. Atopic heredity in the families was defined as a typical clinical history of allergic disease, *i.e.* allergic rhinoconjunctivitis, allergic asthma or flexural itchy dermatitis. Atopic heredity was not an inclusion criterion, but 68% of the participating children from Sweden and 30% of the Estonian children had atopic diseases within the families.

The children were examined at 3, 6, 12, 24 months and at 5 years of age (except that Swedish children were investigated at 3 or 6 months of age). At each visit, saliva, serum and faeces were collected, peripheral blood mononuclear cells (PBMC) were isolated and SPTs were performed. In Estonia, a clinical evaluation regarding atopic manifestations was done by a paediatrician at all visits, while this was done by an experienced allergy nurse in the Swedish children, except at the 24 months follow-up when a paediatrician performed the evaluation. To ensure clinical concordance between the two countries, paediatricians from Estonia visited Linköping several times, and participated in the follow-up examinations of the Swedish children. House dust samples from the homes were collected at one occasion, when the children were between 3 and 12 months of age. The families answered questionnaires regarding symptoms of allergy, infections, presence of pets at home, family size, dwelling space, damage

due to dampness and presence of fitted carpets, when the children were 3, 6, 12, 18 and 24 months and at 5 years of age.

Cohort 2

One hundred and twenty-three eczematous children under 2 years of age with suspected food allergy on referral from primary care were enrolled between June 1999 and September 2001 at the paediatric clinics of Linköping, Norrköping, Jönköping and Hudiksvall. The numbers of participating children and distribution of sex in the different study centres, as well as enrolment analysis from Linköping and Hudiksvall are presented in table 1. To assure clinical concordance and good cooperation in the study the authors visited the units to discuss the study design, before start of the study. During the study, all participating nurses and physicians met once or twice a year to discuss research questions and study progress.

Table 1. Numbers of participating children at the different units and distribution of sex. Analysis of declination for families in Linköping and Hudiksvall.

Unit	No. of patients	Female/male	Invited	Reason for non-participation
Tot	123	52/71		
Linköping	53	22/31	109	LP(n=12); P(n=8); D(n=3); NI(n=33)
Hudiksvall	12	3/9	18	NI(n=3); C(n=3)
Norrköping	14	6/8	-	
Jönköping	44	21/23	-	

LP, language/communication problems

P, participation in other studies

D, severe disease, other than eczema and allergy

NI, not interested

C, contact problem via phone and letter

At the first visit, eczema was diagnosed using Hanifin-Rajka criteria ¹⁹¹ and the severity was evaluated with the severity scoring of atopic dermatitis (SCORAD) ¹⁹². Before the start of the study the nurses practised scoring on children with eczema to reduce inter-observer variability.

Skin prick tests to egg and milk, the main food allergens in children of this age group, were performed at the first visit and at 4 ½ years of age.

Samples of blood, saliva, urine and faeces from the child were collected and if the child was breast fed, also breast milk from the mothers.

Questionnaires regarding other atopic manifestations, family history, environmental factors and nutritional supply were answered by the child's parents.

Parents of children with positive SPT to egg and/or milk (n=78) were instructed by a dietician to eliminate the offending food from the child's diet and from the mother's diet if she breastfed her child. The parents of all children were instructed how to treat eczema and dry skin with emollients and, if needed, with topical glucocorticoids.

After six weeks of elimination diet and/or skin care, all children were re-evaluated, and the SPT positive children were also investigated at 3 years of age. At 4 ½ years of age all children, both initially SPT positive and negative children, were summoned to a follow-up with assessment of eczema, SPT to egg, milk and aeroallergens, clinical examinations by a paediatrician and questionnaires about other allergic manifestations and clinical tolerance to foods. Children with an initially positive SPT to egg or milk underwent oral food challenge tests when their SPT were ≤ 10 mm and SCORAD were ≤ 25 . At the 4 ½ year follow-up 13 children were still excluded from oral food challenge tests due to SPT > 10 mm and/or SCORAD > 25 , or a recent allergic reaction after accidental exposure to the food, making a challenge too dangerous.

Paper II describes 89 out of the 123 children, including all 53 children from Linköping and all SPT positive children from the other centra with available serum samples from inclusion and at the 4 ½ year follow up (n=36). The results of SPTs and outcome regarding food tolerance in these

children are presented in figure 7. In paper IV we included all SPT positive children from which we had been able to isolate PBMC from at least three of the four visits (n=21). They were all from Linköping, since cell separations were performed only there.

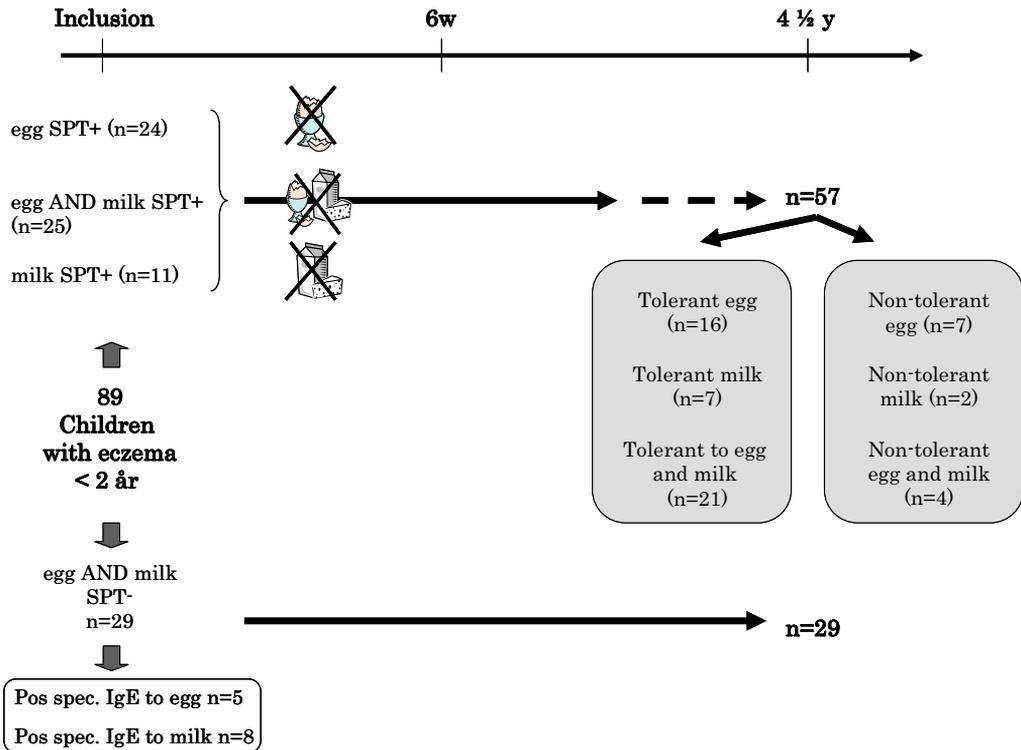


Figure 7. The study design of paper II. Skin prick tests (SPT) to egg and milk were performed on inclusion and at the 4 ½ year follow up. All children were treated with skin care and the SPT positive children with elimination of the offending food for at least 6 weeks, when they were re-evaluated. Eczema was estimated with SCORAD and blood and saliva samples were collected on inclusion and at both follow-ups. Data from the 4 ½ year follow-up were missing in 3 children.

Diagnostic criteria

In paper I and III, allergic symptoms was defined as atopic dermatitis, asthma and allergic rhinoconjunctivitis. Atopic dermatitis was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. Asthma was defined as three or more episodes of bronchial obstruction, at least one verified by a physician. Allergic rhinoconjunctivitis was defined as at least two occasions of rhinitis and conjunctivitis appearing after exposure to a particular allergen and not related to infection.

In paper II and IV, all children suffered from eczema according to the Hanifin-Rajka criteria ¹⁹¹. Very briefly, to diagnose eczema, 3 of 4 basic features and 3 or more minor features are needed (Table 2). Evaluation of the eczema was done using SCORAD ¹⁹². According to SCORAD classification, the children were subgrouped regarding the eczema severity; mild eczema (SCORAD ≤ 25), moderate eczema (SCORAD 26-50), or severe eczema (SCORAD >50).

Table 2. The Hanifin and Rajka criteria of atopic eczema in children. For diagnosis 3 of 4 basic and 3 or more minor features are needed.

Basic feature	Minor feature
Pruritus	Immediate (type 1) skin test reactivity
Typical morphology and distribution	Elevated levels of serum IgE
Chronic or chronically relapsing eczema	Early age of onset
Personal or family history of atopy	Tendency towards cutaneous infection
	Cheileitis
	Recurrent conjunctivitis
	Dennie-Morgan infraorbital fold
	Orbital darkening
	Facial erythema or pallor
	Itch when sweating
	Intolerance to wool and lipid solvents
	Food intolerance
	Course influenced by environmental or emotional factors

In paper I and III, sensitisation in the Swedish children was defined as positive SPT (≥ 3 mm) and/or detectable levels of circulating specific IgE antibodies. However, in the Estonian children, this definition is not considered reasonable to use as they often have low, but detectable, levels of circulating specific IgE antibodies that are poorly related to allergy and SPT positivity¹⁹³. In paper II and IV the definition for sensitisation was as mentioned above for the Swedish children, although the criterion for a temporary elimination diet was positive SPT to egg and/or milk.

SPT

In paper I and III, SPTs were done in duplicate on the volar aspects of the forearms with actual allergen. Histamine hydrochloride (10 mg/ml) was used as a positive control, and glycerol was included as a negative control. The test was regarded as positive if the mean diameter was ≥ 3 mm. Thawed egg white and fresh cow's milk (lipid concentration 0.5%) were used for SPT at 3 and 6 months of age. At 12 months a standardised cat extract was added to the panel and in Estonia also a standardised *Dermatophagoides pteronyssinus* extract. At 24 months and at 5 years the children were also tested with birch and timothy allergen extract and in Estonia also cockroach allergen (*Blattella germanica*).

In paper II and IV the SPTs were performed with single pricks on the volar aspects of the forearms. Histamine hydrochloride (10 mg/ml) was used as a positive control. As a negative control a prick without any allergen was performed. The test was regarded as positive if the mean diameter was ≥ 3 mm. Fresh or frozen hen's egg white and cow's milk (lipid concentration 0.5%) and wheat diluted in water were used at the first visit when the children were younger than 2 years. At the 4 ½ year follow-up aeroallergens (cat, birch) were added to the panel.

Food challenge tests

Children included in cohort 2 (paper II and IV) with an initially positive SPT to egg or milk underwent oral food challenge tests when considered safe, *i.e.* their SPT were ≤ 10 mm and SCORAD were ≤ 25 ¹⁹⁴. Successively increasing doses of the allergen (0.1-30 ml for milk and 0.1-10g for egg) were given in 5 steps every 20 minutes. The challenge was supervised by a doctor and a nurse and was immediately stopped if objective clinical symptoms arose. The challenges were performed in a double-blinded way in Linköping, and as open challenges at the other centers¹⁹⁴. The children were observed two hours after administration of the final dose and reactions within this time period were defined as early reactions. Late reactions were assessed by a nurse who contacted the families one day and one week after the challenge. If the challenge did not cause any early or late reactions, the families were instructed to carefully introduce the food into the child's diet. An evaluation of the child was done three months later to assess the introduction.

Laboratory analyses

The cut off value, as well as highest accepted CV and interassay CVs for the analysis of IgA and IgG antibodies, cytokines and endotoxin are presented in table 3.

Table 3. Cut off values, intra-assay variability (CV) and inter-assay variability (inter CV) for different methods included in this thesis.

Method	Cut off	CV	Inter CV
Secretory IgA	31 ng/ml	<15%	14%
Total IgA	31 ng/ml	<15%	10%
OVA IgA	OD above 0.10	<15%	25%
BLG IgA	OD above 0.10	<15%	25%
OVA IgG ₁	0.31 AU/ml	<15%	11%
OVA IgG ₄	0.31 AU/ml	<15%	21%
BLG IgG ₁	0.31 AU/ml	<15%	4%
BLG IgG ₄	0.31 AU/ml	<15%	21%
IL-4	6.2 pg/ml	<15%	15%
IL-5	6.3 pg/ml	<15%	10%
IL-10	4.6 pg/ml	<15%	15%
IL-13	2.0 pg/ml	<15%	10%
IFN- γ	15.6 pg/ml	<15%	5%
Endotoxin	50 EU/ml	9%	25%

ELISA

The general principle of enzyme-linked immunosorbent assay (ELISA) is presented in figure 8.

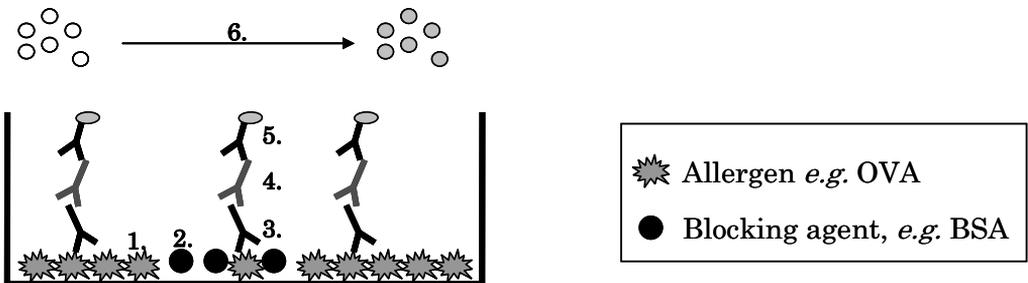


Figure 8. General principle of enzyme linked immunosorbent assay (ELISA).

1. Relevant allergen is added and attach to the plastic wells.
2. The blocking solution is added, occupying all empty spaces in the wells.
3. Samples or standard containing required antibody is added.
4. Addition of detection antibody.
5. Addition of conjugated antibody.
6. A substrate is added, forming a coloured product in the presence of conjugated antibody.

The levels of TIgA, SIgA (paper II and III), allergen specific IgA, IgG₁ and IgG₄ (paper II), as well as the cytokine production in PBMC (paper IV) and allergen analyses in house dust (paper I) were analysed using ELISA kits (antibody pairs) or in-house ELISAs, as described in the papers. Total IgG antibody levels in saliva (paper III) were analysed with a human IgG ELISA quantitation kit (Bethyl Laboratories, inc. Montgomery, USA), according to the manufacturer. The standard, a human reference serum, containing a known amount of IgG, was included in the kit. Two saliva samples were used to test that serum and saliva diluted in parallel (fig. 9). The allergen analyses were done in Estonia, while all other analyses were done in Sweden.

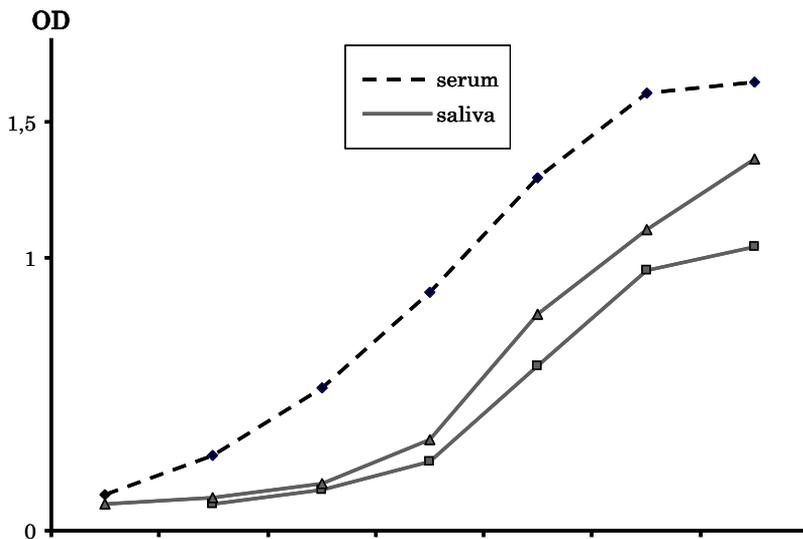


Figure 9. Human reference serum with known amount of IgG is diluted in parallel with saliva samples.

Endotoxin analysis

The levels of endotoxin in house dust samples (paper I) were determined by a chromogenic Limulus assay (fig. 10) according to the manufacturer's instructions. The method was optimized regarding incubation times, concentration of standard curves and optimal dilution of the samples. The influence of dust components on the assay was examined as described in paper I.

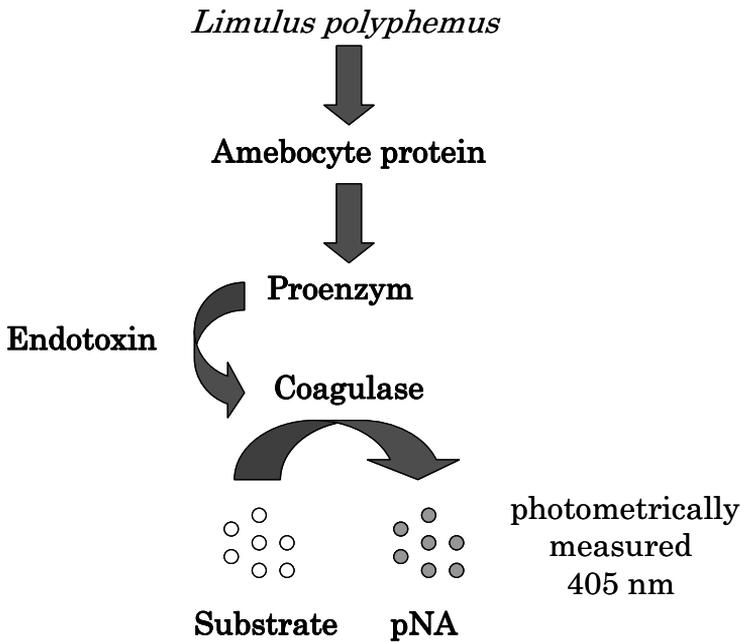


Figure 10. Mechanism for Chromogenic Limulus Amebocyte Lysate Test Method (QCL-1000®, Bio Whittaker, Walkersville, Maryland, USA). Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the Limulus Amebocyte Lysate. The activated enzyme catalyses the splitting of p-nitroaniline (pNA) from the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA. The released pNA is thereafter measured photometrically at 405 nm.

Total- and specific IgE

The levels of total IgE were analysed in paper II and IV using the fluoroenzyme immunoassay, UniCap®, according to the manufacturer's instructions. The levels of specific IgE antibodies to egg and milk in serum were analysed with UniCap® (paper II and IV), while the levels of specific IgE antibodies to egg white, β -lactoglobulin, cat and birch in paper III, were determined using a commercial chemiluminescence method, Magic Lite™. The cut off value for total IgE (UniCap®) was 2 kU/L and the specific IgE tests were regarded as positive at ≥ 0.35 kUA/l (UniCap®) and > 1.43 SU/ml (Magic Lite™), respectively.

Statistical methods

As the levels of antibodies, cytokines, endotoxin and allergens were not normally distributed, even after log transformation, nonparametric tests were used. Paired analyses between groups were performed with the Wilcoxon signed-rank test and unpaired analyses with the Mann-Whitney *U* test. Correlations were calculated with Spearman's rank order correlation coefficient test. The chi-square test was employed for categorical variables and the Fisher's exact test was used when the expected frequency for any cell was less than five. Differences together with a probability level of $< 5\%$ was considered to be statistically significant. The calculations were performed with a statistical package, StatView 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA) for paper I and StatView 5.0 for PC (SAS Institute Inc., Cary, NC, USA) for paper II-IV.

In paper I, a multivariate logistic regression was used to adjust for potential confounders, using the statistical package Statistica 7.0 for PC (Statistica Corporation, College Station, TX, USA). The endotoxin levels were included as a continuous variable in the multivariate logistic

regression model. This analysis was performed by a statistician, Mats Fredriksson, PhD.

To enable statistical analysis, samples with undetectable levels of the different parameters were assigned a value equivalent to half the value of detection limit for each assay.

Ethical considerations

The separate studies were approved by the Human Research Ethics Committee at the Faculty of Health Science in Linköping, Sweden (paper I-IV), the Medical Faculty at Uppsala University, Sweden (paper II) and the Ethics Review Committee on Human Research of the University of Tartu, Estonia (paper I and III).

Informed consent was obtained from the children's parents. To minimize the discomfort for the children, topical analgesic cream was used prior to the collection of blood samples.

RESULTS AND DISCUSSION

Clinical diagnosis and sensitisation

In paper I and III 16% of the children had allergic symptoms while 79% did not develop any allergic symptoms and 5% were classified as probably allergic. Our definitions have been employed in numerous prospective studies and are generally accepted for this purpose. However, due to the difficulties to define allergic disease, the children were also divided into more strict groups, *i.e.* sensitised symptomatic children (with positive SPT and/or circulating IgE antibodies to allergens, at least once) and non-sensitised, non-symptomatic children. As mentioned earlier, it is also important to note that in Estonia, sensitisation refers to positive SPT only, due to a rather high frequency of children with low levels of circulating specific IgE antibodies, but without symptoms and/or SPT positivity¹⁹³.

In cohort 2 (paper II and IV), 78 of the 123 children were SPT positive to egg and/or milk at the first visit. All of these children were recommended a temporary elimination diet after instructions by a dietician. In 31 children, the offending foods were re-introduced after a short period (at least 6 weeks) of elimination diet, either by parents at home, or after a negative open food challenge. In 47 children, early food challenge could not be performed due to severe eczema or large SPT. They were examined regularly and food challenge was performed when considered safe, *i.e.* when SPT was ≤ 10 mm and SCORAD ≤ 25 . At 4 ½ years of age, 13 of the initially SPT positive children still had not been able to re-introduce the offending foods due to recent allergic reactions at accidental exposure or positive DBPCFC. Further information is given in table 4.

Table 4. Clinical data from the 4 ½ year follow-up in children, initially eczematous and skin prick test positive to egg and milk.

Pat. no.	Do not eat	SPT (mm)	IgE (kUA/l)	Clinical reactions after exposure*
1	egg	11	4.4	Skin rash, gastrointestinal symptoms
2	egg	8	0.52	Urticaria
3	egg	23	1.4	Urticaria, obstructive, vomiting
4	egg/milk	13/7	missing	Skin rash
5**	egg/milk	14/10	0.78/0.35	Urticaria
6	egg/milk	7/11	2.2/1.3	Erythema, obstructive
7**	egg/milk	9/8	2.5/5.5	Generalized anaphylaxis
8	egg	0	2.1	Throat itching, difficulties to swallow
9**	egg/milk	8/9	19/2.2	Urticaria
10	egg	12	0.40	Urticaria, obstructive
11	egg	16	2.5	Urticaria, anaphylaxis
12	milk	0	1.4	Skin rash, gastrointestinal symptoms
13	milk	11	150	Aggravated eczema

*Double-blind, placebo-controlled, food challenge or accidental exposure at home.

**Children who have had a positive double-blind placebo-controlled food challenge.

All patients were included in paper II and patients 4-9 were included in paper IV.

The definitions of atopy and allergic diseases have been changed over time and although outlines have been defined by *e.g.* the Review Committee of the World Allergy Organization ¹, unequivocal criteria are missing. For definition of food allergy in young children DBPCFC has been proposed as the “golden standard” ^{162, 163}, although this has not always been feasible in clinical practice. A limitation of our study design in cohort 2, is that food challenges were not routinely performed on inclusion to substantiate a diagnosis of food allergy in the children. However, there are several factors supporting that the majority of the children in our study group were food allergic from the start. First of all, several studies have shown that SPT positivity is a good predictor for a positive outcome of food challenge ^{165, 195, 196}. Moreover, skin wheal diameters have been identified (5 mm for hen’s egg and 6 mm for cow’s milk), above which all food challenges were positive ¹⁶⁴. Most of the children in our study displayed rather large SPTs at inclusion (fig. 11). Thus, we assume that a large part of the children in our study would have had a positive food challenge on inclusion.

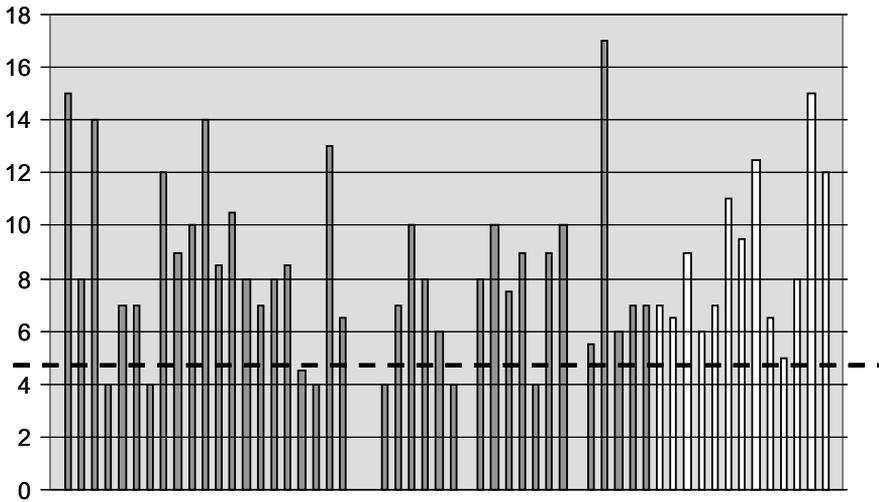


Figure 11a. Skin prick wheal size for egg (mm) on inclusion. The broken line indicates a skin wheal diameter, above which egg challenges tests have been shown to be positive. Children indicated in white are included in paper IV, while grey AND white bar charts indicate children who are included in paper II.

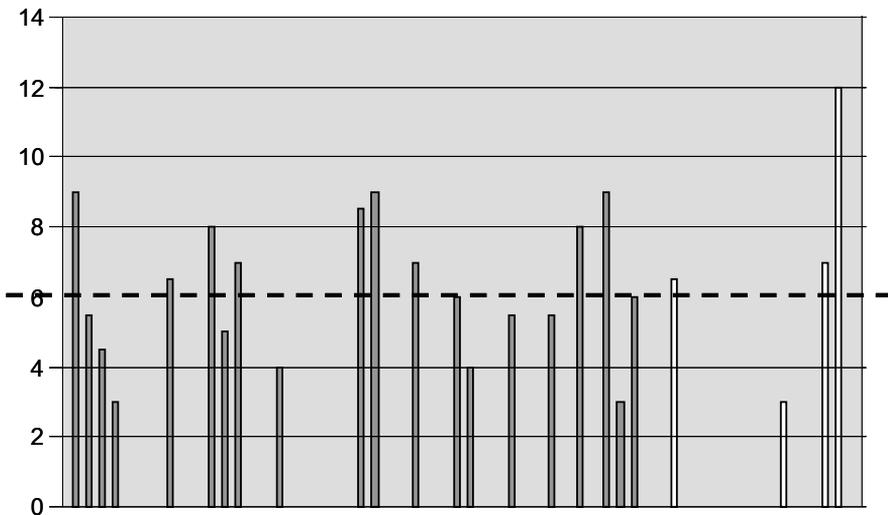


Figure 11b. Skin prick wheal size for milk (mm) on inclusion. The broken line indicates a skin wheal diameter, above which milk challenges tests have been shown to be positive. Children indicated in white are included in paper IV, while grey AND white bar charts indicate children who are included in paper II.

Methodological aspects

To overcome the difficulties of measuring the low levels of allergen specific IgA antibodies in saliva, we used a sensitive ELISA with an enzyme amplified system (AMPAK™). Analysis of IgA in saliva is complicated by high unspecific binding, necessitating correction for the large individual variations of the background. Therefore, in all analysis of IgA antibodies to OVA and BLG, uncoated rows have been used and the optical density (OD)s of the uncoated wells were subtracted from the ODs of the coated well. This has not been done in previous studies, which may explain some of the diverse results regarding the development of mucosal immune responses and their importance in the development of allergic disease. The system with uncoated rows was used in specific IgA analysis only, as total and SIgA analysis were not associated with unspecific binding (data not shown).

There were no commercially available standards to be used in the allergen specific IgA analysis of saliva and no samples were found that could be properly diluted and used as a standard curve. Therefore all samples were referred to a reference breast-milk sample with high levels of IgA antibodies to OVA and BLG and low background. Both the reference sample and all saliva samples were diluted 1:25. Antibody levels in the samples were calculated as a ratio between the OD of the sample and the OD of the reference, after subtracting the OD of the blanks and the OD values for the individual controls (uncoated wells). The ratio was then expressed in arbitrary units (AU). The specificity of the allergen specific IgA antibody assay was confirmed with inhibition ELISAs. The saliva samples, but not the reference sample, were pre-incubated with increasing concentrations of relevant antigens (OVA or BLG) as well as an irrelevant antigen (birch). Due to the binding of IgA antibodies to the added relevant allergens, the measurement of OVA- and BLG specific IgA antibodies was inhibited in a dose dependant manner (data not shown).

The method for analysing allergen specific IgG₁ and IgG₄ has previously been developed by our group ¹⁹⁷. The more sensitive AMPAK™ system was required for the measurements of IgG₄ antibodies in serum and this subclass restriction is common for allergens ¹⁹⁸. Immunoglobulin G₁ and IgG₄ antibodies to BLG and OVA in serum were determined as described earlier ¹⁹⁷, except that blocking was performed with BSA (Fraction V, Sigma-Aldrich) instead of human serum albumin (HSA). As BSA is an allergen occurring in cow's milk occasional subjects may demonstrate specific antibodies to BSA, as well as to the major cow's milk allergens, casein. However, the effect of BSA as a blocking agent has been extensively examined in our laboratory, with the same or better results in blanks and individual controls when compared with HSA. Besides that, uncoated wells were used in the analysis of 25 samples during the analysis of BLG specific IgG₄ antibodies without finding any unspecific BSA binding (data not shown).

The endotoxin analysis was optimised using dust samples collected from mattress and carpets from one household that did not participate in the study. The analysis was performed as described in paper I. Different incubation times (10, 15, 20, 25 and 30 minutes) after the addition of LAL were tested and 15 minutes was found to be optimal. The standard curve ranged between 0.05 and 2.0 EU/ml. In this interval, the curve was linear.

Microbial exposure in relation to environmental factors and development of allergy

In paper I, we report that Sweden and Estonia, two countries with markedly different socio economic structures, differ with respect to endotoxin levels in house dust. Higher concentrations of endotoxin were found in house dust collected from both mattresses and carpets in Estonian than Swedish households. Endotoxin is a component of gram negative bacteria and mirrors the microbial load in the environment. Although the

difference in microbial load between Sweden and Estonia has been proposed to be caused by different lifestyles, we could not define any specific factor explaining the higher load in Estonia (paper I). It has been shown that farming environments is associated with high levels of endotoxin ¹¹⁶, possibly because of the presence of animals ¹⁹⁹. Some investigators have shown that furry pets may increase the endotoxin levels in households ^{200, 201}, while others show that this effect is minor ²⁰². In our study, no differences in endotoxin levels were observed between homes with and without pets. Also, there were only weak correlations between endotoxin and the levels of cat and dog allergens, Fel d 1 and Can f 1, respectively. Other potential sources of endotoxin in homes include air conditioning ²⁰⁰, tobacco smoke ²⁰³, number and age of children in the homes ²⁰⁴, humidity ²⁰⁵ and seasonal differences ²⁰⁶. However, the influences of those contributions have not been fully clarified ^{200, 205, 207}. In our study we investigated seasonal differences, family size, dwelling space, dampness and fitted carpets, but none of these factors could explain the higher levels of endotoxin in Estonian compared to Swedish households. However, there was a weak inverse correlation between dwelling space and endotoxin levels in carpets ($\rho=-0.28$, $p<0.01$) and mattresses ($\rho=-0.20$, $p<0.01$), although this correlation disappeared when analysing the two countries separately. In Swedish households there was a correlation between family size and endotoxin in carpets ($\rho=0.34$, $p<0.0001$). The high prevalence of atopic heredity in the Swedish children may be a confounder, possibly explaining some of the differences in endotoxin levels between the two countries. However, the influence of heredity has been controlled for in different ways. In paper I, heredity was included as a variable in a multivariate logistic regression model. Moreover, when analysing children with or without heredity separately, there were still marked differences in house dust endotoxin levels between Sweden and Estonia (fig. 12).

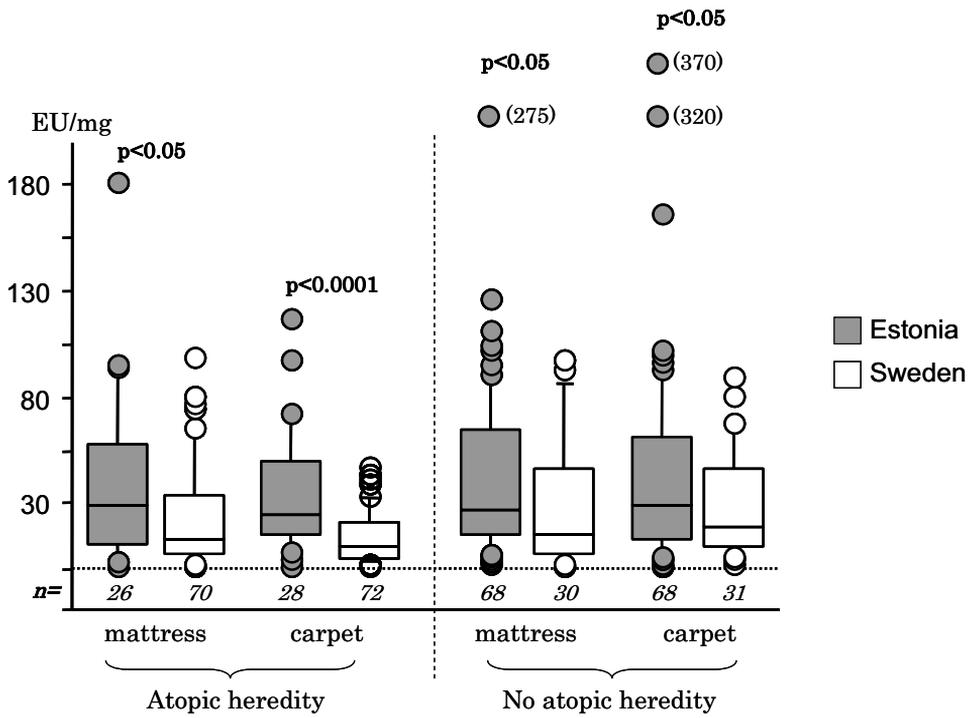


Figure 12. Endotoxin levels in house dust from homes of children with and without atopic heredity.

In Sweden but not in Estonia, low levels of endotoxin were associated with allergic disease (*i.e.* sensitisation in combination with allergic symptoms). Low endotoxin levels seemed to be associated with both allergic symptoms and SPT positivity as such (fig. 13). This is in line with several other studies showing that children growing up in farm environments with high levels of endotoxin have a reduced risk of developing allergic diseases ^{111, 116}. Moreover, low levels of endotoxin in house dust were associated with sensitisation to allergens ¹¹⁷ and endotoxin exposure also decreased the risk of atopic eczema in infants up to 6 months of age ²⁰⁸. One mechanism by which endotoxin may protect from allergic disease is by its Th1-inducing properties. The effect of LPS that is induced via TLR, strongly influences innate APC, especially DCs, to produce pro-inflammatory Th1-promoting cytokines *e.g.* IL-12, IL-6 and TNF ^{119, 120}.

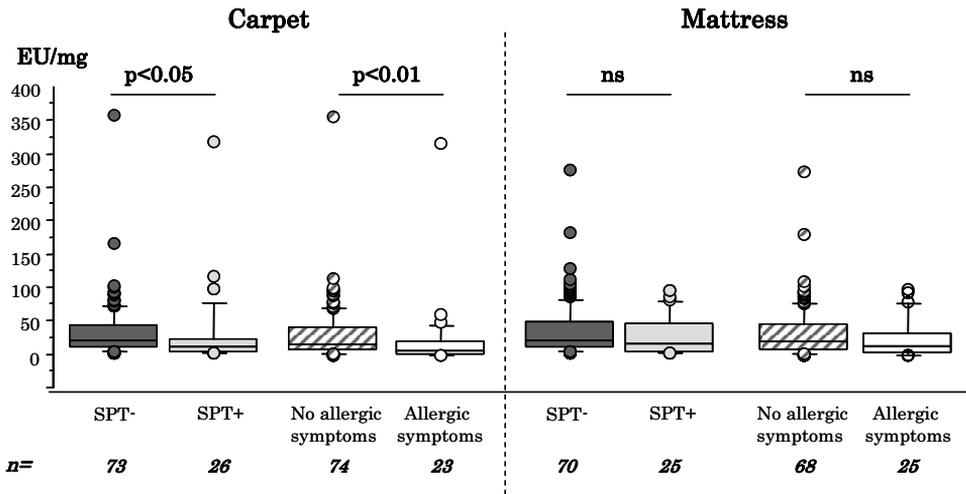


Figure 13. House dust endotoxin levels from homes of Swedish children, with and without positive skin prick test (SPT) or allergic symptoms.

The LPS receptor TLR4 is expressed on the apical surface of mucosal epithelium and signalling via this receptor enhances the extension of DC dendrites across epithelial tight junctions into the intestinal lumen ²⁰⁹. This enables the DCs to sample antigen directly from the lumen and brings them in direct contact with microbes and their products, possibly affecting the Th cell differentiation as high antigen load and DCs triggered by microbe-associated molecular patterns (MAMPs), preferably skew the T-cells immunity away from Th2-like responses ¹²³.

We can of course not exclude the possibility that endotoxin is a marker for something else in the environment causing this protective effect. Heat shock protein (HSP)60, a Th1 inducing microbial component, correlates with endotoxin levels in dust and HSP60 was much higher in barn dust than in dust samples collected in households ²¹⁰. Furthermore, an immune stimulatory cell-wall component of fungi, yeast and plants (β -(1→3)-glucan), also correlates with house dust endotoxin levels ²¹¹. Thus, endotoxin may mirror the exposure of various microbial components with possible immune modulatory effects. Also, recent data from our laboratory

shows that the levels of endotoxin are associated with numbers of strains of faecal *Bifidobacteria* (unpublished data), suggesting an additional explanation to the association between low levels of endotoxin and development of allergic disease. The gut microflora differs between Estonian and Swedish children with a more frequent colonisation of *e.g. Lactobacillus* in the former country, while strains of *Clostridium difficile* are more common in Swedish children ²¹². Moreover, healthy and allergic children differ in their composition of the gut microbiota ^{213, 214}. Microbial exposure provides a strong environmental signal for normal postnatal maturation of the immune system and also induces the maturation of APCs and Treg cells, which are essential for programming and regulating the T-cell response ²¹⁵. This is presumably of greatest relevance in early life when immune programming is initiated and less significant in relation to mature immune system in older children and adults ²¹⁵. As the microbial flora is established early in life and thereafter is relatively stable, the environment during infancy is of great importance. In concordance with this, recent data from our group show a reduced prevalence of IgE-mediated eczema in children to mothers supplemented with probiotics during pregnancy ²¹⁶.

Mucosal immunity in relation to environmental factors and development of allergy

There is ample evidence for a common mucosal immune system ⁴⁸. Primed B- and T-cells from gut-associated lymphoid tissue migrate to other secretory tissues, including the mucosa of lacrymal, salivary and lactating mammary glands ⁴⁸. Analysis of antigen specific antibodies and determination of cytokine profiles in different secretions, *e.g.* in saliva, thereby reflects the immune responses induced in the gut. Total IgA and SIgA (paper II and III) as well as IgA antibodies to OVA and BLG (paper II) were analysed in saliva, attempting to mirror the mucosal immune responses in the gut.

Detectable levels of IgA antibodies were found in the children from both cohorts (paper II and III). The levels of TIgA and SIgA increased with age in all children (fig. 14), and had reached near adult levels at 4 ½ years (paper II) and at 5 years of age (paper III), results that are supported by other investigators ^{84, 86}. IgA is only found in small amounts at birth, and the baby is initially supplied with IgA antibodies via breast milk, rich in SIgA.

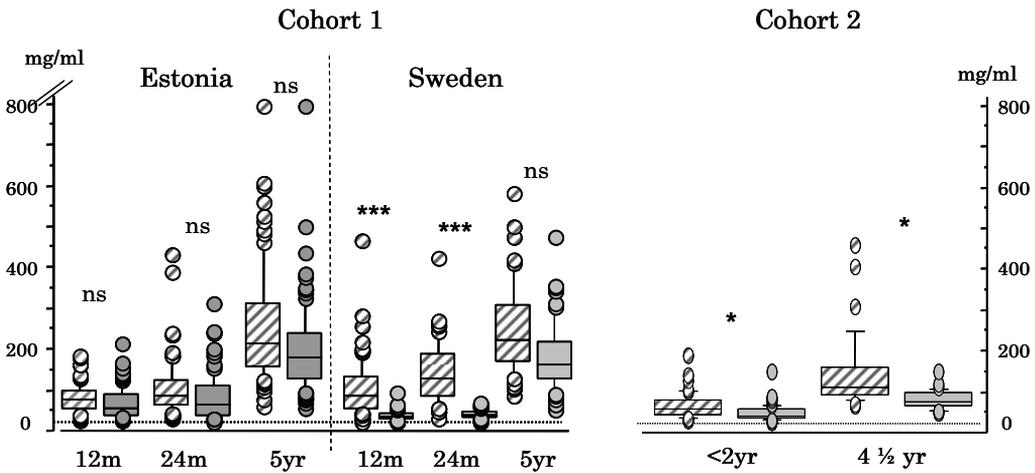


Figure 14. Total (striped boxes) and secretory (filled boxes) IgA antibody levels in Estonian and Swedish children from the two cohorts included in the thesis. There is a statistically significant increase in both total and secretory IgA between 12 months and 5 years in Estonian and Swedish children ($p < 0.001$), as well as between <2 years and 4 ½ years in cohort 2 ($p < 0.01$). * $p < 0.05$, *** $p < 0.0001$, ns=non significant.

To get into the salivary glands the IgA antibodies, produced by plasma cells at mucosal membranes, have to be actively transported via a pIgR serving as an epithelial cell receptor for the binding, uptake and transport of the IgA dimer across the epithelial cell (for more details, see figure 5, page 27). Inside the gland, this receptor is cleaved, but leaves a glycoprotein called the secretory component attached to the IgA dimer. In Estonia the levels of TIgA and SIgA were generally similar, indicating an

efficient pIgR transport. Thus, almost all IgA antibodies in the saliva from Estonian children are actually in the secretory form (fig. 14). In a very detailed analysis, even higher levels of SIgA than TIgA antibodies were observed for some Estonian children. We suggest this phenomenon is due to a methodological problem, however. As the levels of IgA antibodies were very high, many samples had to be diluted more than 1/100 000, causing a small dilution mistake to generate great variations. Surprisingly, the Swedish children showed a completely different pattern regarding SIgA antibody profile with much lower SIgA antibody levels than TIgA antibody levels (fig. 14), at least up to 24 months of age. This indicates that saliva from these children contains a large part of IgA antibodies missing the secretory component. This difference between SIgA and TIgA was observed in all the Swedish children (paper II and III, fig. 14) and it has also been shown in another cohort from Sundsvall (Anna Sandin, *et.al*, unpublished data).

The high salivary SIgA levels in Estonian children could possibly be due to contamination from breast-milk as SIgA are present in higher levels in breast-milk from Estonian than Swedish mothers (unpublished data). However, this is a rather unlikely explanation for the differences in salivary SIgA levels between Estonian and Swedish children, as sampling of saliva has been done in the same way in the both countries, 2 hours after the last meal. Moreover, the differences in SIgA levels were still present at 24 months of age when breast-feeding had ended in almost all children (paper III).

It is not known why Swedish children have such large differences between SIgA and TIgA levels in saliva, at least up to 2 years of age, but there are some different possibilities, as discussed in paper III. The synthesis of pIgR may be delayed in Swedish infants, possibly allowing dimeric IgA to passively diffuse over the epithelium. In a pIgR knockout mice model it was found that the small intestinal IgG and albumin levels were higher

than in wild-type mice, suggesting leakage of serum proteins across the epithelium ²¹⁷. In our study, there were no differences between Estonian and Swedish children regarding total IgG antibody levels at 12 months and 5 years of age, that could indicate a more leaky epithelial membrane in Swedish children. However, we found a correlation between total IgG and non-SIgA, *i.e.* the levels of IgA antibodies lacking the secretory component, in the Swedish children, possibly indicating IgA breaking through the mucosa.

Another possible explanation of the low SIgA proportion in saliva from Swedish children may be that the SC has been cleaved of the IgA dimer by proteases produced by different human pathogens ²¹⁸. There are two different IgA subclasses, IgA₁ and IgA₂, and the SC attaches to the two subclasses in different ways. The binding to IgA₁ is covalent, whereas the binding to IgA₂ is non-covalent ²¹⁸, indicating that the SC can be more easily liberated from IgA₂ than IgA₁. At mucosal surfaces the concentration of IgA₁ and IgA₂ is supposed to be similar, in contrast to the IgA in serum, which is predominantly of the IgA₁ subclass. Thus, another explanation to the high proportion of non-SIgA found in saliva from Swedish children could be that the SC is more easily cleaved from the SIgA complex in these children, possibly due to the presence of proteolytic enzymes or a different ratio between IgA₁ and IgA₂. A possible method to investigate this would be to analyse the levels of free SC in saliva or the levels of IgA₁ and IgA₂. Unfortunately, inadequate sample size made it impossible for us to test this hypothesis.

We studied the association between salivary IgA levels, microbial load (paper III) and allergen free diet (paper II). There was no association between salivary TIgA or SIgA and endotoxin levels in house dust at any age, but there was an inverse correlation between endotoxin and the levels of non-SIgA. This association was only observed in Swedish children as non-secretory IgA antibodies generally not existed in the Estonian

children. This inverse correlation between endotoxin and non-SIgA, may indicate that microbial load stimulates the transport of IgA antibodies, possibly due to an up-regulated expression of pIgR ⁶⁶.

In paper II, no association between allergen free diet and TIgA, SIgA or allergen specific IgA antibody levels could be found when analysing the levels on inclusion and after 6 weeks of elimination diet. Six weeks may be too short to ensure any differences but when analysing the children who still were on an elimination diet at 4 ½ years of age, the only differences observed were the expected increase in TIgA and SIgA with age (data not shown), that also was observed in all children in both cohorts.

In paper III, low levels of SIgA in Swedish children were associated with symptoms in sensitised children while children with high SIgA levels seemed to be protected from developing symptoms, at least up to 12 months of age. At 2 years of age there still tended to be a difference between sensitised children with or without expression of clinical symptoms. In Estonia, there are very few children with clinical allergic symptoms making this type of analysis impossible. However, the high SIgA antibody levels in the Estonian children might partly explain the poor conformity between circulating specific IgE and symptoms in that country ¹⁹³.

Skin prick test positive Swedish children had higher levels of non-SIgA than SPT negative children. The same phenomenon was also observed in children from cohort 2 (fig. 15) at the first visit, when the children were younger than 2 years and also at the six week follow-up. A similar pattern was also observed at 4 ½ years of age, although not statistically significant. High levels of allergen specific SIgA could, theoretically, prevent allergen absorption and thereby sensitisation and subsequent development of allergy. Supporting this possibility, low levels of SIgA have been associated

with an increased risk for allergy. This is controversial, however, as a tendency towards the opposite has also been reported. Possibly, our new findings concerning great differences between TIgA and SIgA in Swedish children may shed new light on the contradictory results regarding the protective effects of SIgA. Many investigators have measured IgA antibodies in saliva, not SIgA *i.e.* with a coating antibody directed against the secretory component, possibly resulting in misinterpreted levels of SIgA in some studies.

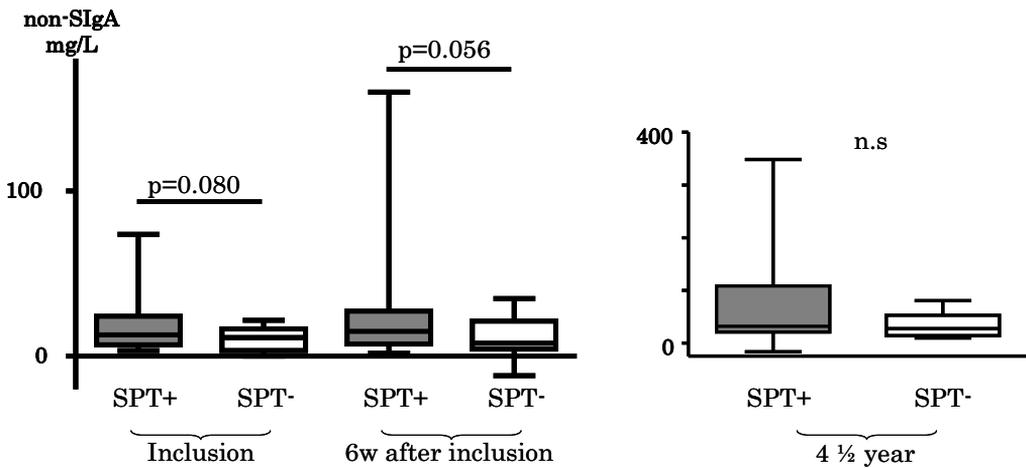


Figure 15. Non-secretory IgA, *i.e.* IgA antibodies lacking the secretory component, in skin prick test (SPT) positive and negative Swedish children from cohort 2.

Immunological factors in relation to eczema and persistent food allergy

The eczematous children followed in cohort 2 are thoroughly described by Norrman et.al ²¹⁹. All children, both SPT positive and negative, improved their eczema significantly during the 6 weeks treatment with skin care in combination with elimination diet (SPT positive) and skin care only (SPT negative). Recommended treatment for eczematous children with suspected food allergy has traditionally been elimination of the suspected foods. However, elimination diet may be negative for children in several

ways. Milk and egg are important sources of nourishment and the children on elimination diet are in risk of nutritional problems. It has been shown that growth can be impaired in children on a milk free diet ^{185, 187}.

Moreover, there is an impaired risk of acute allergic reactions after accidental exposure of the eliminated foods after a diet period ²²⁰. Due to recent research stressing tolerance induction, the benefit of elimination diet has become questioned. The advantage of allergen exposure, rather than avoidance, has been known for several years regarding air-borne allergens used in immunotherapy studies. The benefits of exposure to food allergens have not been that clear, although animal studies have indicated similar mechanisms. However, recent studies have shown that specific oral tolerance induction is possible in children with food allergy ¹⁸⁹.

Paper II describes a subgroup consisting of food sensitised children *i.e.* with detectable levels of food specific circulating IgE antibodies in serum samples, which were SPT negative to the same allergen. They improved their eczema during the treatment period although they did not eliminate food from their diet (fig. 16). This small group of sensitised children improved their eczema despite continued allergen exposure, suggesting that exposure is possible in sensitised children and possibly also in some food allergic children.

The levels of IgG₄ antibodies early in life were observed to have an impact of the allergic status of the children later in life (paper II), indicating that high IgG₄ antibody levels early in life in food allergic children are associated with tolerance development. However, we can not prove that the children were food allergic as we did not perform DBPCFC in the beginning of the study to substantiate food allergy. Several studies support the diagnostic value of SPT for food allergy in children ^{164, 165}, especially large SPTs in combination with detectable levels of circulating food specific IgE antibodies.

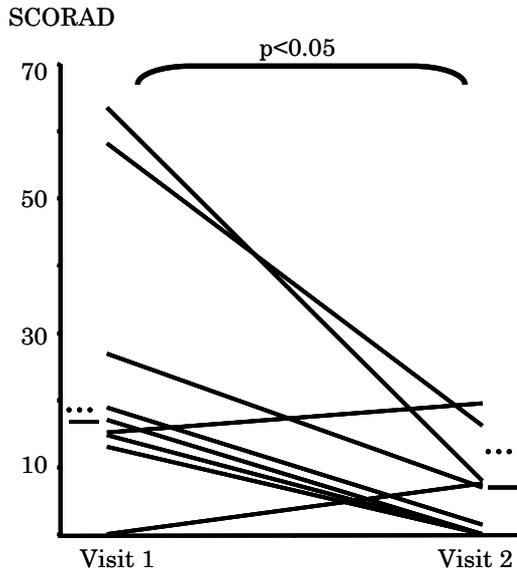


Figure 16. Eczema improvement in egg and milk sensitised children without positive SPT to corresponding allergen, after 6 weeks of treatment with skin care. The children were not treated with elimination diet. Median SCORAD values are indicated for this group of children (black line), as well as for 76 SPT-positive children treated with skin care AND elimination diet (dotted line).

It is possible to create more strict groups in our study to improve the diagnoses of food allergy. In figure 17, I have selected children with moderate or severe eczema, with large SPT (≥ 10 mm for egg and ≥ 7 mm for milk) and detectable levels of circulating specific IgE antibodies to egg (median and range; 3.98 (0.38-38.4)) and milk (median and range; 3.14 (0.4-23.4)). Also with this more strict classification of the groups there are marked differences in food allergen specific IgG₄ and IgG₄/IgE ratios between the two groups, supporting our belief that high IgG₄ or IgG₄/IgE ratios early in life in food allergic children with eczema, may predict faster tolerance development.

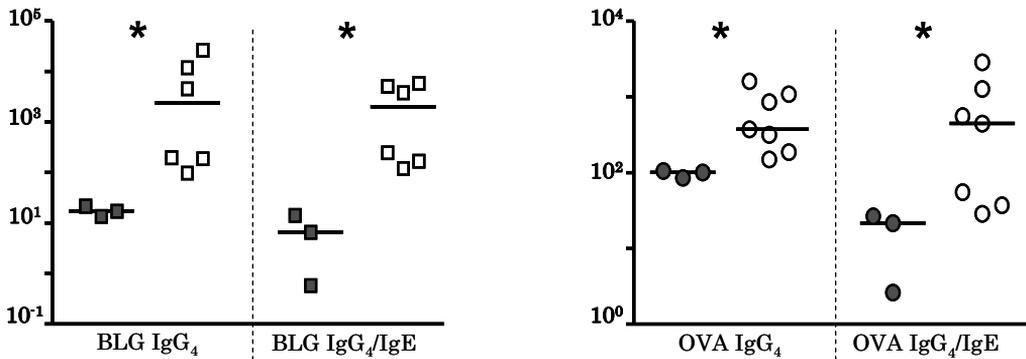


Figure 17. Levels of β -lactoglobulin (BLG) and ovalbumin (OVA) specific IgG₄ antibody levels and IgG₄/IgE ratios in food allergic infants who develop tolerance before 4 ½ years of age (white) or not (grey).

The y-axis are logarithmic and all differences are significant ($p < 0.05$), indicated with *.

In cohort 2, 21 SPT positive children from Linköping were followed with blood samples from all visits. As reported in paper IV, the levels of both Th1 and Th2 cytokines after food allergen stimulation of PBMC tended to increase with age, and were present in higher levels at 4 ½ years of age in children with persistent food allergy compared to children who could tolerate food. However, the differences were observed for IFN- γ and IL-5 only and not for IL-13, IL-4 or IL-10. The percentage of children who responded with detectable levels of cytokines after food allergen stimulation in tolerant and non-tolerant children, as well as median and range of the cytokine levels are presented in table 5. Why the observed pattern is true only for IFN- γ and IL-5 is unknown.

As discussed in paper IV, it is impossible to conclude if the increase and higher levels of IL-5 and IFN- γ in children who still are food allergic at 4 ½ years of age, is caused by the food allergy *per se* or due to the excluded food allergens. From the literature it is hard to evaluate the impact of elimination diet on allergen specific cytokine production. Many studies

investigating food allergic children do not control for the effects of elimination diet. In an attempt to investigate the effect of food elimination in our study the cytokine responses were analysed on inclusion and after 6 weeks of food elimination diet and also in a few children that ingested egg or milk (fig. 18). Interestingly, increase of egg and milk allergen induced IL-5 and IFN- γ production were only observed in children who had excluded these food items. Unfortunately, the children who ingested egg or milk were rather few and had no positive SPT to the ingested food. However, before the elimination diet was initiated, the cytokine levels were similar in the groups, suggesting a minor importance of SPT. Thus, further studies are needed to clarify the effect of elimination diet.

Table 5. Percentage of children who produced detectable levels of cytokines after OVA and BLG stimulations of PBMC. Levels are also presented as median and (range).

		Non-tolerant			Tolerant		
		Inclusion	Visit 2	4 ½ yr	Inclusion	Visit 2	4 ½ yr
OVA	IL-13	80%	67%	60%	42%	69%	60%
		12.1	3.7	1.8	0.0	2.7	0.9
		(0.0-100)	(0.0-10)	(0.0-33)	(0.0-30)	(0.0-150)	(0.0-23)
	IL-10	25%	40%	67%	29%	36%	67%
		0.0	0.0	6.0	0.0	0.0	5.8
		(0.0-2.5)	(0.0-4.2)	(0.0-32)	(0.0-5.6)	(0.0-4.0)	(0.0-11)
	IL-5	20%	50%	50%	14%	23%	0%
		0.0	2.2	5.2	0.0	0.0	0.0
		(0.0-8.7)	(0.0-11)	(0.0-130)	(0.0-3.9)	(0.0-35)	(0.0-0.0)
	IFN- γ	40%	50%	50%	21%	36%	20%
		0.0	2.6	72.9	0.0	0.0	0.0
		(0.0-16)	(0.0-40)	(0.0-250)	(0.0-53)	(0.0-54)	(0.0-59)
BLG	IL-13	100%	80%	100%	79%	85%	100%
		17.9	16.5	79.4	14.6	21.2	49.6
		(2.7-120)	(0.0-260)	(0.0-380)	(0.0-390)	(0.0-380)	(0.0-130)
	IL-10	30%	75%	100% ^A	64%	50%	80%
		0.0	2.3	57.2	1.5	0.0	3.4
		(0.0-4.4)	(0.0-16)		(0.0-13)	(0.0-33)	(0.0-29)
	IL-5	50%	60%	80%	21%	38%	80%
		2.4	0.0	54.4	0.0	0.0	15.1
		(0.0-15)	(0.0-16)	(0.0-130)	(0.0-12)	(0.0-45)	(0.0-42)
	IFN- γ	40%	80%	80%	36%	50%	80%
		2.1	22.6	127.2	0.0	11.1	38.6
		(0.0-16)	(0.0-71)	(0.0-250)	(0.0-84)	(0.0-142)	(0.0-63)

^A only one available sample

OVA, ovalbumin BLG, β -lactoglobulin

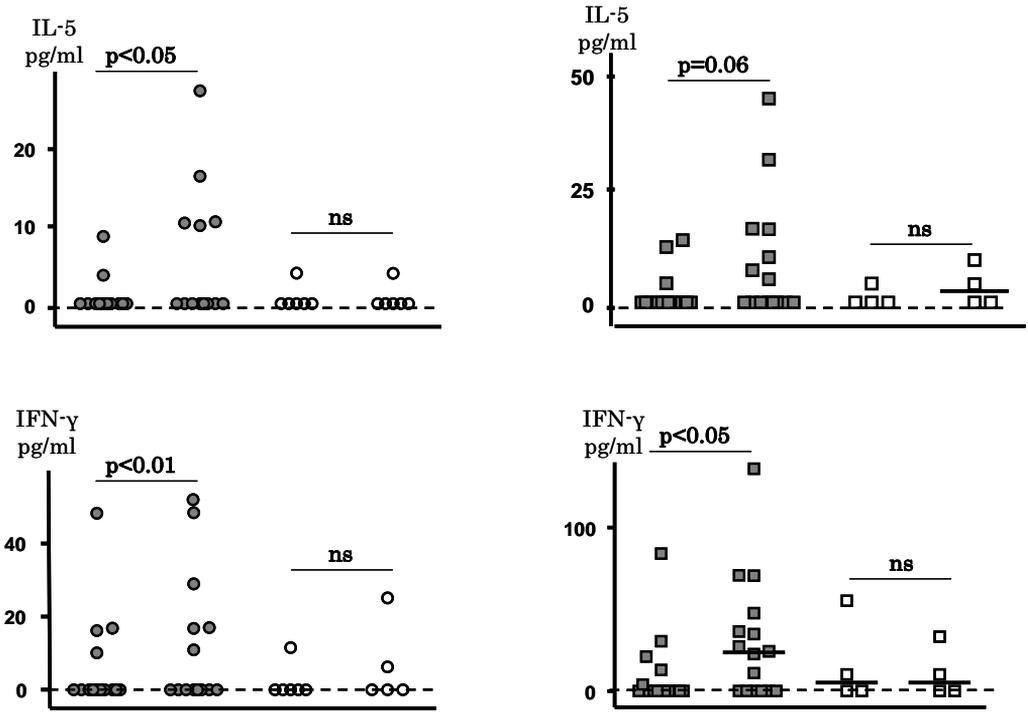


Figure 18. The effect of 6 weeks of elimination diet on ovalbumin (dotts) or β -lactoglobulin (squares) stimulated IL-5 and IFN- γ secretion in peripheral blood mononuclear cells from eczematous children with (grey) or without (white) egg or milk elimination diet, respectively. At the first visit none of the children were on an elimination diet.

The levels of IL-10 were expected to be higher in the tolerant group due to its anti-inflammatory properties. Moreover, IL-10 up-regulates the secretion of IgG₄ and the observed differences early in life regarding IgG₄ and IgG₄/IgE ratios between children who developed food tolerance or not were also true in this selected group of children (data not shown). However, there were correlations between IgG₄ and un-stimulated IL-10 production as well as between the ratios of IgG₄/IgE and IL-10 production (table 6), supporting that IL-10 stimulates the release of IgG₄ antibodies. Even though no differences in levels of IL-10 were observed between food-allergic and tolerant children, these results give a possible explanation to the observed IgG₄ pattern. It is possible that the low numbers of children preclude any statistically significant differences in IL-10 levels.

Table 6. Correlation between unstimulated IL-10 production from PBMC and OVA and BLG specific IgG₄ antibody levels or egg and milk specific IgG₄/IgE ratios.

		rho	p-value
IL-10 inclusion	OVA IgG ₄	0.304	0.09
	BLG IgG ₄	0.733	<0.01
	OVA IgG ₄ /egg IgE	0.300	ns
	BLG IgG ₄ /milk IgE	0.597	<0.05
IL-10 visit 2	OVA IgG ₄	0.469	ns
	BLG IgG ₄	0.400	0.09
	OVA IgG ₄ /egg IgE	0.554	<0.05
	BLG IgG ₄ /milk IgE	0.424	<0.05

The anti-inflammatory property of IL-10 has earlier been shown by a correlation between size of SPT and IL-10 levels²²¹. However, in this selected group of children no such correlation could be found. Instead a correlation between SCORAD and IL-10 was found at the second visit (fig. 19). At the first visit there was only a tendency to a weak correlation (rho -0.335, p=0.09).

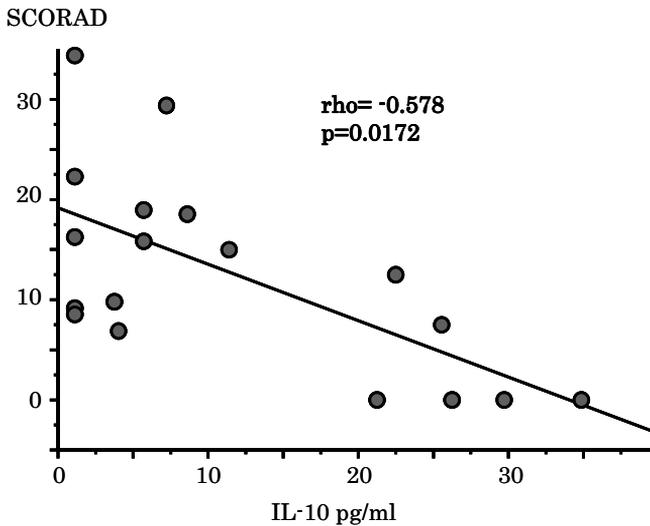


Figure 19. Correlation between eczema severity measured with SCORAD, and unstimulated IL-10 production in peripheral blood mononuclear cells from children with eczema and suspected food allergy.

The low numbers of IL-10 responding children at the first visit and few children with SCORAD above zero at 4 ½ years of age, might explain why a statistically significant correlation was observed at the second visit only. This result is supported by Dunstan *et.al*, who showed that children with atopic dermatitis had lower secretion of IL-10 in un-stimulated mononuclear cells compared with children without eczema ²²².

Summary and concluding remarks

In this thesis the association between allergic disease in children and different environmental and immunological factors has been elucidated. It has been stated that the microbial load, represented by endotoxin levels in house dust, is higher in Estonia, a country with high prevalence of allergic disease, compared with Sweden, a country with low prevalence of allergic disease. The higher microbial load in Estonia could not be completely explained by any of the parameters investigated in our study, although the dwelling space and levels of cat and dog allergens correlated with the endotoxin levels. It is possible that the lower prevalence of allergic disease in Estonia, at least partly, may be explained by the high microbial load as a negative association between allergic symptoms and sensitisation and endotoxin levels was found. This is supported by the immune modulatory effect of endotoxin, possibly directing the immune response in infants away from Th2 skewed responses.

The different exposure to microbes in Sweden and Estonia might have an impact on the mucosal immune responses in the children as a marked different IgA pattern was observed in the two countries. The Estonian children demonstrated similar levels of SIgA and TIgA in saliva from birth up to five years of age, while the Swedish children showed much less SIgA compared with TIgA, at least up to 2 years of age. This unexpected IgA pattern in Swedish children may be caused by a non-working pIgR transport of the IgA dimer across the epithelial or by permeable

membranes. The consequence of this unexpected pattern in Swedish children is unknown, but the non-SIgA antibody levels were associated with sensitisation in the Swedish children. Further, the sensitised children with high SIgA antibody levels seemingly developed allergic symptoms to a lesser extent than sensitised children with low SIgA levels. This might be one possible explanation to the unsolved conundrum why some sensitised children do not develop allergic symptoms, and a protective effect of SIgA might be proposed.

Most food allergic children develop tolerance with age, but a traditional treatment strategy during the reactive phase is to eliminate the offending food from the diet. The impact of this elimination diet on the immune system in the children is not fully elucidated and there are no predictive factors to foretell which children that will develop tolerance. However, in this thesis, food specific IgG₄ or the ratio between food specific IgG₄ and IgE, have been proposed as a possible predictive factor for tolerance development. The levels of IgG₄ to OVA and BLG and the ratios IgG₄/IgE to OVA/egg and BLG/milk respectively, were higher during infancy in individuals who developed tolerance before the age of 4 ½, compared with children who still were food allergic at 4 ½ years. Moreover, the ratios of IgG₄/IgE were highest in sensitised children without positive SPT, and these children improved their symptoms (eczema) although they did not try an elimination diet. Thus, IgG₄/IgE might be a good predictor to foretell which children that will benefit from continuing food allergen exposure, as well.

The levels of both Th1 and Th2 cytokine production by food allergen stimulated PBMC, increased with age and were at 4 ½ years of age higher in children who still were food allergic, compared with children who were tolerant at 4 ½ years. If this increase was caused by their food allergy *per se* or if it was affected by the elimination diet is not known. However, the levels of cytokines did not differ between the two groups before the

elimination diet were introduced. Moreover, SPT positive children increased both Th1 and Th2 responses during six weeks of elimination diet, while SPT negative children without elimination diet had similar cytokine levels during the same time period. Before the six weeks period the cytokine production were similar in both SPT positive and negative children. Taken together, these results indicate that allergen exclusion might affect the immune system, resulting in increasing allergen specific cytokine responses.

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