ANALYSIS OF T CELL DIFFERENTIATION DURING THE DEVELOPMENT OF ATOPY IN CHILDREN

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ANALYSIS OF T CELL DIFFERENTIATION DURING THE DEVELOPMENT OF ATOPY IN CHILDREN

ANALYSE VAN T CEL DIFFERENTIATIE TIJDENS DE ONTWIKKELING VAN ALLERGIE BIJ KINDEREN

PROEFSCHRIFT

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I want to travel as far as I can go
I want to reach the joy that's in my soul
and change the limitations that I know
and feel my mind and spirit grow

I want to live, exist, 'to be',
and hear the truths inside of me

D. Warshay

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GENERAL INTRODUCTION

CHAPTER 1

THE IMMUNE SYSTEM DURING THE DEVELOPMENT OF ATOPIC DISEASE

THE IMMUNE SYSTEM DURING THE DEVELOPMENT OF ATOPIC DISEASE

In the past 20-30 years, there has been a substantial increase in the prevalence of atopic disease in the Western world. This increase was observed particularly among children (1) and concerns asthma (2,3), atopic dermatitis (AD) (4,5), and allergic sensitization (6). The definitions for the different clinical expressions of atopic disease are described in Chapter 2. The prevalence of asthma, for example, increased from 4% in 1973 to 9% in 1988, and the prevalence for AD increased from 5% in 1973 to 16% in 1988, as reported by Burr et al. (7). For allergic sensitization the prevalence increased from 19% to 27% between 1991-92 and 1995-96 (6). One of the consequences of the increase in prevalence of atopic disease is the spiraling costs of treating established atopy (8). This provides an urgent imperative for the development of effective preventive measures to limit further increases in atopy prevalence. Prevention aims to identify at risk children at an early stage and to begin prophylactic therapy to prevent or diminish the clinical expression of different atopic diseases, such as AD and allergic asthma. Targets for this early prevention should include the underlying immunopathology of the allergic reactions to allergens (9,10). Therefore, knowledge of the dysregulations in the immune system of young children who develop atopy is essential.

1.1. THE NATURAL COURSE OF ATOPIC DISEASE IN EARLY CHILDHOOD

The natural course of atopic disease gradually evolves from AD with or without food allergy in the first 3-4 years of life, to asthma (>6 year) and rhinitis (>15 year) in the years thereafter (Figure 1). Allergic sensitization in children below the age of 4 years occurs predominantly against foods. Most children outgrow their food allergy (to cow's milk and egg) by the age of 2 to 4 years. After 6 years of age allergic sensitization to inhalant allergens such as house-dust mite and pollen is more frequently diagnosed than food sensitization (11,12).

During the first year of life, AD is the most common atopic disease. Sixty percent of children who will develop AD already have symptoms during the first year of life and 85% during the first 5 years of life (13). Of children with AD approximately 20% have allergic reactions to food constituents compared to 8% of the general population of children (14,15). Peanuts, cow's milk and eggs account for approximately 80% of adverse reactions to foods in AD (14). The combination of AD and food allergy in the first 12 months of life increases the risk from 10% to 40% to develop asthma at 5 years of age (16). Eighty percent of all childhood asthma has its onset before 6 years of age (17). The large majority (±80%) of childhood asthma is associated with atopy, manifested by a positive skin test, a clinical history of

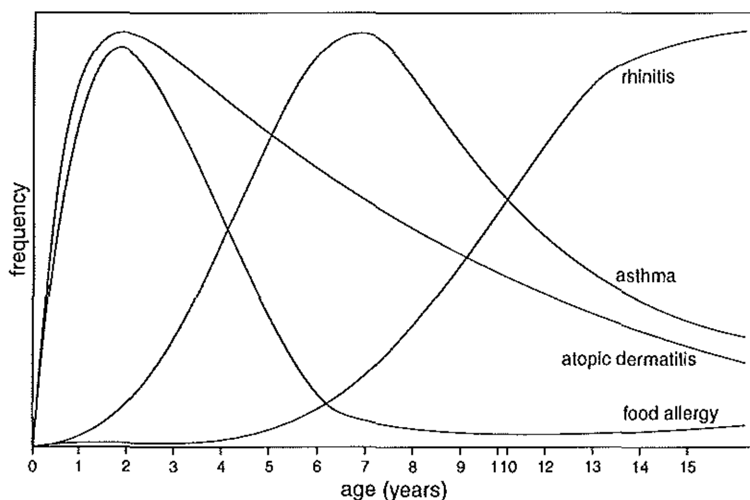

Figure 1. The natural course of atopic disease in childhood.

allergen-induced wheezing, concomitant eczema or allergic rhinitis (18) (Chapter 2).

1.2. RISK FACTORS FOR THE DEVELOPMENT OF ATOPIC DISEASE

The development of atopic disease in early childhood is increased by numerous factors, called risk factors. One of the most relevant risk factors is the atopic inheritance. There is increasing evidence, however, that environmental factors in infancy may affect an individual's symptomatic expression of atopy.

1.2.1. Genetic factors

The role of genetic influence, relative to those of the environment, in atopic disease is difficult to ascertain. Theoretically, twin studies provide the best insight. However, comparisons of monozygotic (MZ) twins and dizygotic (DZ) twins are based on the assumption that both sets of twins share roughly the same environmental exposures and thus differ only in their genetic background. The results of twin studies showed significant differences between MZ and DZ twins, providing evidence of a genetic component in the development of asthma and atopy (19,20). A positive family history is frequently reported as an important risk factor for the development of atopic disease in children (21-23). The risk for a child to develop atopy increases with the proportion of affected family members. A child without atopic parents has a risk around 10% to develop an atopic disease during his/her

life. This risk increases to 20% in the case of one atopic parent and to 30% in the case of one atopic parent and an atopic sibling. If a child has two atopic parents, the risk to become atopic is increased to 50%, which can further increase to 70% when both parents have the same atopic disease (24).

Many genes were proposed to be candidates for a role in the heredity of atopic disease (reviewed in 25,26). There are multiple regions in the genome containing susceptibility genes that could affect the expression of atopy or the response to therapy. The most frequently reported genes are located on chromosomes 5q, 6, 11q, 12q, 13 and 14q. On chromosome 5q the cytokine gene cluster, encoding for e.g. interleukin (IL)-4, IL-5, IL-9 and IL-13 is located. These cytokines play an important role in the pathogenesis of atopic disease (see section 1.3.1.1 and 1.4.1). For most chromosomal regions only associations, but not genetic linkages, were observed with specific immunoglobulin (Ig)-E levels.

In addition to the reported chromosomal areas, polymorphisms in the promoter regions of cytokine genes predisposing to the development of asthma and atopy have recently been described. These polymorphisms are located in the IL-4, IL-9, IL-13, IL-10 and transforming growth factor (TGF)-β promoter regions (27-31). IL-4, IL-13 and IL-9 are candidate genes to be involved in asthma. The upregulation of IL-4 and IL-13 production correlates with an increase in IgE, while upregulation of IL-9 production is correlated with enhancing T cell and mast cell growth and amplifying IgE synthesis (27,28,30,31). The transcription of the IL-4 gene is regulated by multiple promotor elements that either induce or suppress transcription. In asthmatic subjects a polymorphism in the IL-4 promotor region was found resulting in increased IL-4 transcription and consequently elevated IL-4 production. Also a polymorphism in the intracellular signaling part of the IL-4-receptor has been identified whereby normal levels of IL-4 may exert stronger biological effects resulting in higher IgE antibody levels. Particular polymorphisms in the TGF-β and IL-10 promoter are associated with elevated total IgE levels (27,29). Therefore, these polymorphisms in cytokine promotors may be important regulators of the IgE levels and the expression of asthma or atopy. Possible clinical implications of the recognition of polymorphisms in cytokine promotors remain to be studied further.

In summary, the candidate genes and genetic polymorphisms described above could contribute to the predisposition of susceptible subjects to develop atopic disease. Although new techniques of gene mapping may improve diagnostic methods, it is unlikely that such techniques will have direct applicability for preventive strategies in the next few years. Besides the genetic components of atopy, there are multiple environmental factors, which influence the development of atopy. Preventive interventions involving environmental risk factors are therefore generally preferred.

1.2.2. Environmental factors

Environmental factors (like presence of pets, strategies for house-dust mite (HDM) avoidance, parental smoking, bottle or breast-feeding, infections and air pollution) are currently believed to influence allergic sensitization and the development of atopic disease in young children. In Table 1 several important risk factors are summarized. A selection of the most documented factors, which are important for the early allergic sensitization in the first 2 years of life, are discussed below.

Passive smoking (maternal or paternal) is the best documented environmental risk factor for the development of atopic disease (21-23,32-39). Numerous epidemiological studies have found an association between exposure to tobacco smoke and recurrent wheezing, asthma, AD and an increased risk of sensitization to environmental allergens (23,33-36). Compared with the large numbers of studies conducted to demonstrate the harm caused by passive smoking, relatively few studies have tested the beneficial effects of reducing passive smoking (39). Maternal smoking during pregnancy is associated with increased cord blood IgE levels (37) and an increased risk for the development of AD and asthma in their children (35,36). Although, the effect of maternal smoking can be confounded by factors such as low birth weight and low social class (23,38), there is an undisputed consensus of the negative influence of passive smoking on the risk to develop atopy in children.

Epidemiological studies to the relation between infections and manifestation of atopic disease are complicated by the fact that symptoms such as runny nose, wheezing and cough may be caused by either an infection or an allergic reaction. Certain infectious agents, e.g. Respiratory Syncytial Virus (RSV), Epstein-Barr virus and *Bordetella pertussis* are reported to enhance allergic sensitization (40-43). RSV is the predominant virus isolated from children of age 2 years or less (44). Data obtained from animal, but more recently also from human studies, propose a role for $CD4^+$ (T helper) cells, $CD8^+$ cells and natural killer (NK) cells in RSV-induced enhanced

Table 1. Risk factors for the development of atopy.

Risk factor	References
Passive smoking	21-23,32-39
Maternal smoking during pregnancy	32,35-37,39
Particular infections in early childhood	40-52
Formula feeding	22,23,32,53-55
Exposure to allergenic foods	11,21,56
Elevated cord blood IgE levels	21,32,37,57,58
Exposure to household pets	23
Month of birth	59,60
Exposure to HDM	61,62
Small family size	32
Low birth weight	23

IgE antibody secretion and other components of the allergic inflammation (41,45-47). Although the mechanism of RSV infection in the development of atopy is not yet clear, most studies indicate a link between RSV and atopy development. In contrast to the association between RSV infections and increased risk to develop atopy, it was proposed that tuberculin and BCG infections in early childhood might protect against atopy in later life (48-50). These infectious agents were postulated to downregulate the IgE formation to allergens, encountered at the time of infection, and bias cytokine profiles towards T-helper-1 (section 1.3.1.1). Reports of fewer atopic disease among school children in the former socialist countries of Eastern Europe (more tuberculin infections) compared to Western Europe support this finding (51). In summary, the role of infections in the development of atopy during early childhood is dualistic. Infections can be divided in two groups: those who protect (BCG, tuberculin) and those who do not protect (RSV, Epstein-Barr virus and *Bordetella pertussis*) the development of atopy in children.

Reported associations between breast-feeding and the subsequent development of atopy have been conflicting: several studies showed a protective effect (20,53), some showed no effect (21,32), while a single study showed even a stimulatory effect (54). The mechanism by which breast-feeding can possibly protect against the development of atopy is poorly understood. Breast-feeding may promote the natural maturation of the intestinal mucosal barrier. Moreover, breast-feeding could reduce the exposure to food antigens through inhibiting their absorption and give local protection of the immature mucosa by secretory IgA and IgG in human milk. Björksten et al. showed that the composition of breast-feeding differs between various mothers (55). So, the composition of breast-feeding may determine whether it is a risk factor or a protection factor for allergic sensitization in early life.

The avoidance of exposure of infants to allergenic foods (cow's milk, and egg) during the first months of life is temporarily effective with regard to prevention of food allergy and AD during the first year of life, but fails to prevent the development of respiratory atopy at later ages (11,19,56).
Established atopic disease in children is associated with increased total serum IgE antibody levels. Several studies found that elevated IgE levels in umbilical cord blood is a risk factor for atopy (19,37), while others did not (32,57). The second group of studies showed increased cord blood total IgE levels only in a minority of children who developed atopy. Mainly due to its low sensitivity, cord blood IgE is not suitable for allergy-risk screening meant to give advice on an individual basis (58).

Summarizing, various environmental factors have been suspected to influence the risk of developing atopy, although much of the evidence is still conflicting. This makes it difficult to use these factors as predictors for the development of atopy. Genetic linkage and twin studies have shown that genetic factors are involved in the development of atopic disease (63). In

contrast to the genetic components, the environmental factors are easier targets for prevention of the development of atopic disease in young children. Observations from a range of sources, such as geographic variations of disease prevalence within countries, links with feeding-method, increased prevalence amongst migrants and the rapid rise in disease prevalence, all indicate that environmental factors may be critical in determining disease expression (5,64). The clinical expression of atopic disease is mediated by the immune system, especially the T cell response and IgE production.

1.3. THE IMMUNE SYSTEM IN RELATION TO ATOPY

Gell and Coombs defined four types of allergy. Types I, II and III are antibody-mediated and are distinguished by the different types of antigens recognized and the different classes of antibodies involved. Type IV allergy, also called delayed type hypersensitivity (DTH) is T lymphocyte-mediated. In this thesis we only focus on type I allergy, also called atopy and type I hypersensitivity. In type I allergy, IgE antibodies are produced in response to exposure to antigens, in atopy called allergens. Atopic, in contrast to non-atopic, children develop a strong and persisting IgE mediated response against these relatively 'harmless' protein antigens, resulting in a chronic inflammation with tissue damage. Allergic reactions occur when an already sensitized child, having developed allergen-specific IgE antibodies, is re-exposed to the same allergen. Once antibodies or T cells recognizing the specific allergen have been produced, further exposure will result in clinical symptoms of atopy. In general, the specific immunity towards allergens is exerted by T cells and antigen-presenting cells (APC), such as dendritic cells, macrophages and B cells, and results in the formation of allergen-specific IgG4 and IgE antibodies, from which IgE antibodies are potentially most harmful.

1.3.1. T cells subsets and cytokines

T cells play a central role in the specific immune response as both regulators and effectors of immune functions. The expression of specific cell surface molecules and the selective production of cytokines largely mediate these functions. T cells can be divided in $CD4^+$ helper-T cells and $CD8^+$ cytotoxic T cells. From these, the $CD4^+$ T cells are considered as the most important in atopy.

1.3.1.1. T helper cell subsets

Two subpopulations of $CD4^+$ T cells, namely T helper-1 (Th1) and Th2 were first identified in mice by Mosmann and Coffman (65). Similar subpopulations have now also been identified in humans (66,67). These Th cell subsets differ in their cytokine production profile after activation by

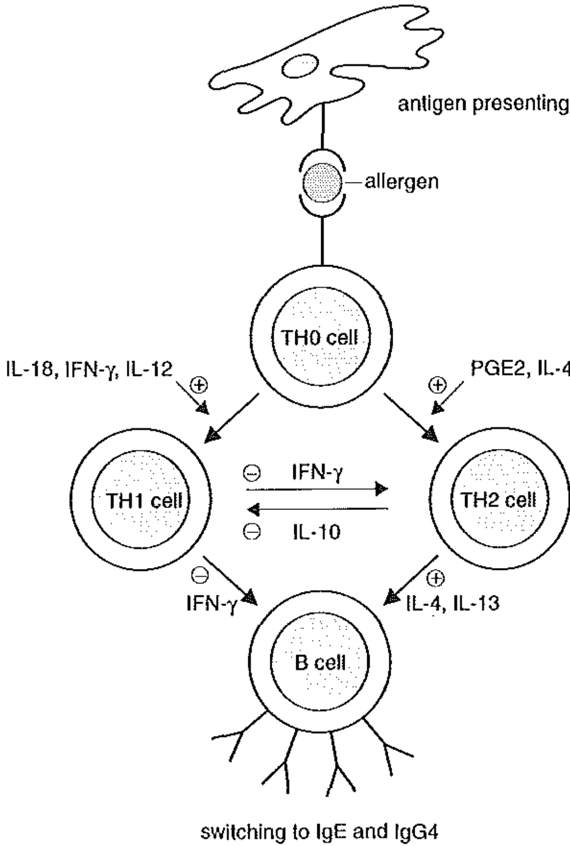

Figure 2. T helper cell subsets and the cytokines they produce. After allergen exposure by APC, Thp cells can differentiate into Th0 cells. Subsequently, Th0 cells can differentiate into Th1 and Th2 cells depending on different cytokines present in the local micro-environment. IL-12, IFN-γ and IL-18 strongly promote the outgrowth of Th1 cells, while PGE2 and IL-4 promote the generation of Th2 cells. Th2 cells and the cytokines they produce stimulate isotype switching in B cells to IgE and IgG4. IFN-γ produced by Th1 cells inhibits this process. The ratio of Th1 *vs* Th2 cytokines determines the final outcome of the B cell response. Cross-regulation between Th1 and Th2 cells by IFN-γ and IL-10 further modifies this ratio.

antigen. Th1 cells produce mainly IL-2 and interferon (IFN)-γ and induce DTH. Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, which promote immunoglobulin production and isotype switch to IgE and IgG4 in B cells. Th1 and Th2 cells represent extremes of differentiation and develop from common precursors: Thp and Th0 (Figure 2). Thp cells secrete only IL-2 (68), and can differentiate in Th0 cells after allergen-specific stimulation. Th0 cells have been described to produce an unrestricted cytokine profile; i.e. they produce both type 1 and type 2 cytokines (69).

A number of factors affect the differentiation of naive CD4 precursors

into type 1 and type 2 effector cells. These include the affinity of the T cell receptor (TCR) for a particular immunodominant peptide (70), the dose of peptide presented by the APC (71), and the presence of type 1 or type 2 cytokines (72-76). When present in the early immune response, IL-4 drives naive CD4 cells to become Th2 cells (72,73). Also prostaglandin E2 (PGE2) promotes the generation of Th2-like cells (74). The presence of IL-12 and IFN-γ strongly promotes the Th1 phenotype (73,75) and IL-10 inhibits the IL-12 production effectively. Since IL-10, IL-12 and PGE2 are derived from APC, the balance between different APC populations might determine the selective outgrowth of Th1 and Th2 cells. As said before, the dose of antigen can also determine to some extent the preferential outgrowth of Th1 versus Th2 type cells. Very low doses of peptide presented by dendritic cells are suggested to cause selective priming of Th2 cells. Priming with much higher doses of antigen could lead to selective outgrowth of Th1 cells (71,77).

Differentiated Th1 and Th2 cells need cytokines and proper expression of co-stimulatory molecules in order to become activated efficiently. In addition to its Th1 phenotype-promoting action, IL-12 is needed in combination with the co-stimulatory molecules CD80/86 for the induction of maximal IFN-γ production in fully differentiated Th1 cells (78). In contrast, IL-10 inhibits IL-12 production and CD80/86 upregulation in APC and, as a consequence, inhibits IFN-γ production by established Th1 cells (78,79). Unlike IFN-γ, which partially inhibits Th2 cell proliferation, IL-2 and IL-4 have no suppressive cross-regulatory effect on Th2 and Th1 cell activation, respectively. Besides the inhibition of cytokine synthesis by Th1 cells (80), IL-10 has been suggested to downregulate proliferative responses and Th2-mediated inflammatory processes (80,81). IL-2 is the most potent growth factor for both subsets, but the relative efficacy of IL-4 in comparison to IL-2 is increased in Th2 cells (82). Most recently, it was demonstrated that IL-18 and IL-12 act synergistically to promote differentiation of Th1 cells (83). So, the differentiation of Th0 cells into Th1 or Th2 cells is largely influenced by cytokines present in the local microenvironment.

A disturbed balance of the Th1 and Th2 cell subsets is involved or accompanies an ever increasing list of human diseases. Atopy is classified as a 'Th2 disease', in which overexpression of the Th2 cytokines contributes to the major characteristics of atopic disease: induction of IgE production and eosinophilia. IL-4 is a key cytokine for the production of IgE by B cells (84). Next to the enhancing effect of IL-5 on IL-4 induced IgE synthesis, IL-5 promotes the differentiation (85), recruitment (86), activation and survival of eosinophils (87). Therefore, IL-5 is considered a pivotal cytokine in the allergen-induced eosinophilic response (88). IL-10 has been described to play an important regulatory role in the removal of eosinophils from the inflammatory site (81) and to prevent IL-5 synthesis in resting $CD4^+$ T cells by interfering with CD80/86-CD28-dependent signals (89). Recently, an important role for IL-

13 has been suggested in the IgE synthesis in atopic disease (30,90,91). IFN-γ has a broad spectrum of effects on cells of the immune system, including anti-viral activities, activation and growth enhancement of cytotoxic T lymphocytes and NK cells and the induction of major histocompatibility complex (MHC) class I and class II antigen expression. Defective production of this cytokine may have serious immunological consequences that may be related to the pathology seen in atopy.

1.3.1.2 Other T cell subsets

The process of allergic sensitization is currently only discussed in the light of the Th1-Th2 dogma. Recently, other T cell subsets were described, including Th3 and T-regulator (Tr)-1 cells, displaying regulatory properties and which could potentially play a role in atopic disease. Th3 cells produce mainly TGF-β and few IL-4, while Tr1 cells produce high amounts of IL-10 and little IL-4, TGF-β and IFN-γ (92-94). The role of these cell populations in atopy in humans is currently under investigation.

In parallel to $CD4^+$ T cells, also $CD8^+$ T cells can be subdivided in T-cytotoxic (Tc)-1 and Tc2 cells (95,96). Tc1 cells secrete IL-2 and IFN-γ, while Tc2 cells secrete IL-4, IL-5 and little or no IFN-γ. Tc2 cells are a relatively recent discovery, and little is known about how these cells fit into the classic type1/type2 network. In atopic subjects Tc2 cells are observed at sites of allergic inflammation (97,98), but it is not yet clear whether sufficient allergen is available there to activate these cells. Moreover, more data is becoming available that $CD8^+$ T cells can also play a role in the IgE synthesis. Secretion of IFN-γ by $CD8^+$ T cells can inhibit the IgE production. Alternatively, $CD8^+$ T cells may stimulate macrophages to produce nitric oxide that in a non-specific manner inhibits IgE production. In contrast to the role of $CD8^+$ T cells in inhibiting the IgE synthesis, there is also some evidence that under specific conditions $CD8^+$ T cells can stimulate IgE synthesis, e.g. *in vitro* differentiated Tc2 cells were shown to promote IgE class switching (99-101).

1.3.1.3. Naive and memory T cells

T cells can be further subdivided in naive and memory/activated cells. Naive T cells have never encountered antigen after their exit from the thymus and only release IL-2 when activated (102). Three cell-surface markers, L-selectin, CD44 and CD45 are generally used to distinguish naive and memory T cells. L-selectin is lost, while CD44 levels increase after antigen priming. Other markers frequently used as activation markers for T cells are CD25 (IL-2 receptor), CD69 and HLA-DR.

CD45 represents a family of membrane glycoprotein isoforms. Naive T cells express the CD45RA and memory/activated T cells the CD45RO isoform (103). As a result of repeated antigen exposure (104), the CD45RA marker is

gradually replaced by the CD45RO marker. The phenotypic change from CD45RA to CD45RO is associated with functional changes, such as an increased poliferative response to IL-2 and an increased production of IFN-γ (105). CD45RO$^+$ T cells show *in vitro* enhanced help for IgE synthesis (106). Confusingly, memory CD4$^+$ T cells have been reported to convert into a naive phenotype, since CD45RA tends to be re-expressed (107).

CD4$^+$CD45RA$^+$ cells produce high levels of IL-2 mRNA upon polyclonal stimulation, but they express trace quantities of mRNA for IL-4 and IFN-γ. In contrast, CD4$^+$CD45RO$^+$ cells produce high levels of IL-4 and IFN-γ mRNA and less IL-2 mRNA (108). In contrast to the other markers for naive or memory/activated T cells, CD45 is considered to be an important regulatory protein that controls activation of T and B cells following ligation of the antigen receptor (109).

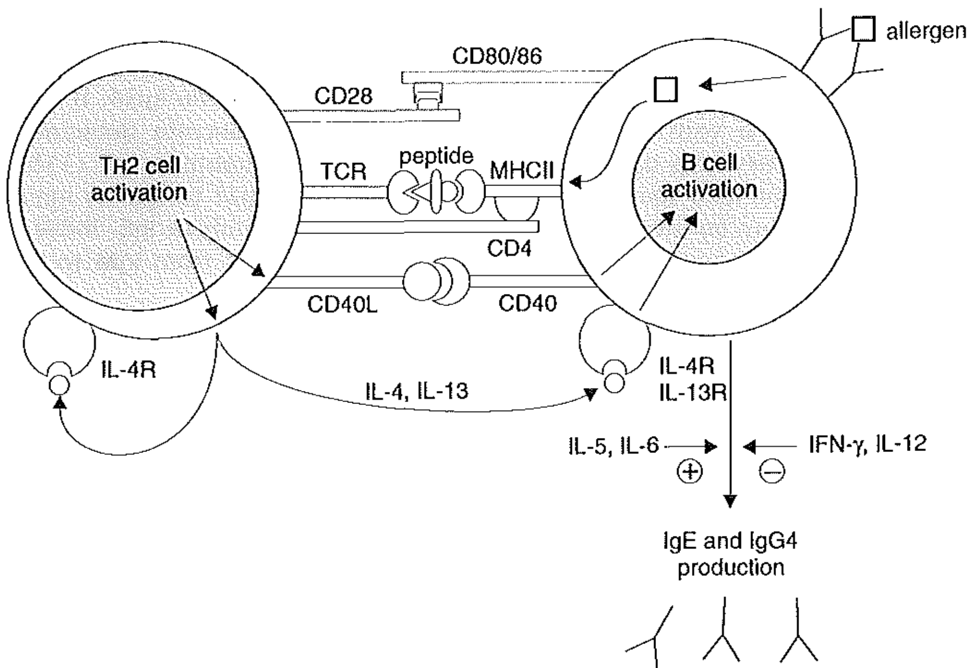

Figure 3. Cognate interaction between T and B cells. B cells acting as APC, present peptides of the allergen in the groove of MHC class II molecules to the specific TCR on Th2 cells. Subsequently, CD80/86 expression on B cells is upregulated and interacts with CD28 expressed on Th cells. Upon activation the Th2 cell transiently express CD40L and secrete IL-4. Binding of IL-4 to the IL-4 receptor on B cells provides signal 1, while interaction between CD40L and CD40 provides signal 2. These two signals result in B cell activation and subsequent switching from IgM production to the synthesis of IgE and IgG4. This process of isotype switching can be modulated by several cytokines interacting with the IL-4 directed processes.

1.3.2. Induction of IgE and IgG4 synthesis

Naive B cells express IgM and IgD antibodies on their membrane. Antigen binding leads to uptake, processing and presentation of antigenic peptides in the groove of MHC class II molecules on B cells. Th cells can recognize the antigenic peptides by their specific TCR, resulting in cognate interaction between B and T cells (Figure 3) (84). Via membrane-bound and soluble signals, the T cell allows the B cell to mature into an Ig-producing plasma cell. Cognate interaction between T and B cells results in upregulation of CD80/86 expression on the B cell (110), which interacts with CD28, constitutively expressed on the T cell. Signals through the TCR and the CD28 co-stimulatory molecule are obligatory for the activation of the T cell. Upon activation, Th2 cells transiently express CD40L and secrete IL-4 (111,112). Binding of IL-4 to the IL-4 receptor on B cells provides signal 1, while interaction between CD40L and CD40 expressed on the B cell provides signal 2 (113). These 2 signals result in B cell activation and subsequent isotype switching to the synthesis of IgE and IgG4 (114). The isotype switching from IgM to IgE could be direct or indirect by sequential switching via IgG4. B cells stimulated *in vitro* with IL-4 plus anti-CD40 antibodies secrete detectable IgG4 approximately two days earlier than they secrete detectable IgE (115). This supports the idea that in the switch process from IgM to IgE an initial switch to IgG may be involved, followed by a second switch to IgE. Indeed, in atopic dermatitis patients sequential switching of B cells from IgM to IgE via IgG4 has been demonstrated (116).

This process of isotype switching can be modulated by several cytokines. Similar activities as for IL-4 in the regulation of IgG4 and IgE synthesis (115-118) have been reported for the closely related IL-13 (119). The IgE synthesis is largely dependent on the presence of IL-4 and IL-13, while the synthesis of IgG4 is less dependent on these cytokines (120). IL-13 is two- to five-fold less potent than IL-4 in inducing IgE synthesis (121), but is produced earlier and for longer time periods than IL-4 (91,122). In T and B cell cocultures of atopic asthmatics, the IgE production was more dependent on endogenously produced IL-13 than the IgE production by healthy subjects. Neutralization of IL-4 resulted in equal inhibition of IgE production in asthmatics and healthy subjects.

The IgE synthesis is inhibited by IFN-γ (114). A possible route via which IFN-γ may inhibit IgE synthesis is downregulation of CD40L on T cells (123). The main activity of IL-10 is to downregulate IFN-γ production, and therefore IL-10 is implicated in the IgE and IgG4 synthesis. IL-10 might have differential effects on these isotypes (124). IL-10 can decrease the IL-4 induced IgE switching and IgE production when added during the first 3 days of *in vitro* culture of peripheral blood mononuclear cell (PBMC). In contrast, if added later to B cells that are already switched to IgE, IL-10 potentiates the IgE production. Independent of the time of addition to the culture, IL-10 augments the

expression of IL-4 induced γ4-germline transcripts with subsequent IgG4 synthesis (124,125). Additionally, IL-10 is a potent inhibitor of B cell proliferation (126). In summary, the induction of IgE and IgG4 synthesis is dependent on cognate interaction between T and B cells. The production of IgE and IgG4 is influenced by several cytokines, such as IL-4, IL-10, IL-13 and IFN-γ. Therefore, a disturbed cytokine regulation as described in atopic disease must have consequences for the production of IgE and IgG4 by B cells.

1.3.3. The allergen-specific immune response

The first exposure of an atopic child to an allergen generally does not result in allergic symptoms. In subsequent exposures, when an allergen enters the body of a sensitized atopic child, symptoms occur within a few minutes, the so-called early allergic response. The allergen interacts with allergen-specific IgE antibodies bound to the high affinity IgE receptor (FcεRI) present on, for example mast cells (127). Cross-linking of IgE antibodies triggers mast cell activation, resulting in the release of several stored and newly synthesized mediators, such as histamine, leukotrienes and cytokines (128,129). This allergen-specific response is accompanied by a non-specific response based on bystander B cell activation, resulting in an increase of the total IgE production.

Several hours (4-8hr) after the allergen enters the body, a late response may become manifest. The mediators released by the mast cell during the early response mainly initiate the late response and the chronic-allergic inflammation. These mediators and cytokines result in infiltrates of eosinophils, basophils, monocytes and lymphocytes.

1.4. THE IMMUNE SYSTEM IN ESTABLISHED ATOPIC DISEASE IN CHILDREN

Elevated IgE synthesis in atopic disease is the result of T cell dysregulation (130,131). In particular, the Th2 cytokines IL-4, IL-5 and IL-13 are responsible for the IgE antibody response (65). In contrast, the Th1 cytokine IFN-γ inhibits IgE responses and the development of Th2 cells (132). The concept that children with atopic disease have a disturbed balance towards the Th2 phenotype, is supported by the presence of Th2-like cells in peripheral blood and in the local infiltrate as found in various atopic diseases (133,134).

1.4.1. Cytokines in children with established atopic disease

Several studies investigated Th1 and Th2 cytokines in children with established atopic disease, before and after polyclonal or allergen-specific stimulation of PBMC or purified T cells, as summarized in Table 2. Polyclonal

stimulation results in non-specific stimulation of PBMC or purified T cells and is used to study the maximum ability of cells to produce cytokines and thus permits analysis of possible intrinsic differences between cells of different patient groups. Allergen-specific stimulation reflects more closely the *in vivo* situation and is used to study allergen-induced changes in cytokine profiles, for example. We can conclude from Table 2 that most of these studies concentrate on mRNA expression and protein production levels of IL-4 and IFN-γ. The combination of more cytokines, including IL-5, IL-10 and IL-13, in a single study dealing with children is not yet adequately investigated.

In general, in atopic children increased levels of Th2 cytokines, such as IL-4, IL-5 and IL-13, and decreased levels of IFN-γ were observed after polyclonal and allergen-specific stimulation of PBMC and purified T cells (Table 2). However, for all these cytokines analyzed the cytokine levels did not differ between atopic and non-atopic children. Moreover, in studies where several cytokines were investigated, the observed Th1-Th2 cytokine dysbalance could be different. These different results may be due to details in the techniques employed and the definitions of the different patient groups.

For Th2-derived IL-10, both increased (139,140) and decreased (145) levels were described in atopic children. IL-10 is an immuno-suppressive cytokine and low levels of IL-10 could result in more extensive allergic inflammation. On the other hand, high IL-10 levels induce a general inhibition of cytokine release both by Th1 and Th2 cells, but resulting in a preferential inhibition of IFN-γ production (80,81). Therefore, increased levels of IL-10 in particular result in a downregulation of the Th1 response.

For IFN-γ, a discrepancy between equal or even elevated mRNA expression levels and reduced protein production after polyclonal stimulation of PBMC was reported in children with AD (136,148,152). This discrepancy between IFN-γ mRNA expression and protein production is attributed to a post-transcriptional defect in AD (148,152), as these children have the same percentage of IFN-γ producing cells as healthy children (152). However, the difference between equal mRNA expression and reduced protein could also be due to an increased autocrine receptor-mediated consumption or proteolytic cleavage of secreted IFN-γ (R. de Waal-Malefyt, personal communication).

Similar cytokine profiles were observed in skin biopts and in peripheral blood lymphocyte supernatants of AD patients and healthy subjects. In addition, a correlation between locally and systemically produced cytokines was reported (153). Therefore, the systemically detected cytokines produced by cells isolated from the blood are probably a reflection of the cytokines produced in the local infiltrate.

In summary, the current concept that atopic children have a disturbed cytokine balance towards the Th2 phenotype is supported by an increase in IL-4, IL-5 and IL-13 levels and a decrease in IFN-γ levels after in vitro stimulation of PBMC or purified T cells. However, for all cytokines there are

Table 2. Literature data about cytokine production by stimulated cells from children with established atopic disease[1] .

Cytokine	Atopy[2]	Stimulation[3]	Cells tested	Expression/protein	Reference
IL-4 ↑	atopy, asthma, AD, FA	polyclonal, ag-spec	PBMC, purified T cells	mRNA and protein	135-141
IL-4 =	asthma, AD	polyclonal, ag-spec	PBMC, purified T cells	mRNA and protein	136,142-144
IL-5 ↑	asthma, AD	polyclonal, ag-spec	PBMC, purified T cells	mRNA and protein	139,145,146
IL-5 =	asthma	polyclonal	PBMC, purified T cells	protein	143-145
IL-10 ↑	astma, AD	polyclonal, ag-specific	PBMC	protein	139,140
IL-10 =	atopy, astma, AD	polyclonal, ag-specific	PBMC, purified T cells	mRNA and protein	141,142,145
IL-10 ↓	AD	polyclonal	purified T cells	protein	145
IL-13 ↑	atopy, astma	ag-specific	PBMC	protein	140,147
IL-13 =	AD, astma	polyclonal, ag-specific	PBMC, purified T cells	mRNA and protein	136,142
IFN-γ ↑	AD	polyclonal, ag-specific	PBMC, purified T cells	mRNA and protein	136,148
IFN-γ =	atopy, AD, astma	polyclonal, ag-specific	PBMC, purified T cells	mRNA and protein	136,141,142,149
IFN-γ ↓	atopy, AD, astma, FA	polyclonal, ag-specific	PBMC	mRNA and protein	135,138,139,143, 144,148,150-152

[1] The age of the children in the different studies ranged between 2 and 16 years. [2] Different studies compared children with different atopic diseases, such as asthma, AD, food allergy and some studies did not distinguish between these different atopic diseases. [3] PHA, PMA plus Ca-ionophore and Con A were the polyclonal stimuli used in the various studies. Ovalbumin and house-dust mite were the allergenic stimuli used in the different studies. The studies summarized in this table can not be easily compared to each other as the studies used various protocols and different patient groups. AD= atopic dermatitis, FA= food allergy.

studies that could not show a difference in cytokine levels between atopic and non-atopic children while for some cytokines (IL-10) even opposite results were reported. In order to get a detailed overview of the dysregulation of the different cytokines that are involved in the allergic process, more studies are needed in which both cytokine mRNA expression and protein production as well as cytokine profiles after polyclonal and allergen-specific stimulation are investigated.

1.4.2. IgE and IgG4 antibodies in atopy

IgE is generally associated with atopic disease. Serum total IgE levels are elevated in 80-85% of children with AD (154). In addition, in 20% of these children specific-IgE antibodies with a broad spectrum of reactivities against food and inhalant allergens were described (155,156). Moreover, some studies also described increased total and allergen-specific IgG4 levels (156-158). The role of IgG4 in allergic disease is less clear than the role of IgE antibodies. The presence of IgG4 antibodies is often considered to be the result of chronic exposure to allergen (158,159). IgG4 antibodies may have a protective effect in the allergic response. This might be due to an effective interference with the allergen-induced triggering of IgE positive cells (160). On the other hand, small amounts of IgG4 are reported to be able to sensitize mast cells and basophils to release histamine (161,162), although others were not able to repeat this (163).

The production of IgE and IgG4 can be modulated by several cytokines that also influence the process of isotype switching (see section 1.3.2.). In short, IL-4 and IL-13 stimulate and IFN-γ inhibits the production of IgE and IgG4.

1.4.3. Adhesion molecules in atopy

Leukocyte-endothelial adhesion molecules are thought to be involved in the initial stages of the selective recruitment and migration of inflammatory cells from the circulation to the sites of inflammation. E-selectin (CD62E) and P-selectin mediate the first attachment of the leukocytes to the endothelial layer, resulting in rolling along the endothelium. This weak binding promotes stronger interactions between intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) on the endothelium and their leucocyte surface ligands, lymphocyte function associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), respectively (164). Several cytokines, including IL-4 and IFN-γ, promote the induction and upregulation of these adhesion molecules, both on endothelium and on leucocyte surfaces (165). At sites of inflammation, increased expression of ICAM-1, VCAM-1, and E-selectin on vascular endothelium in the lung and skin have been demonstrated (166). In skin biopsies from patients with respiratory allergy, an allergen-induced upregulation of E-selectin on endothelial cells was reported during the late phase reaction after intradermal allergen (ragweed or house-dust mites) injection (167). In allergic asthma patients an increased

expression of ICAM-1 was shown on bronchial epithelial cells compared to patients with chronic bronchitis or healthy individuals (168,169). Moreover, in these asthmatic patients an increased expression of both ICAM-1 and E-selectin on bronchial endothelium was observed (169). These results indicate that adhesion molecules are up-regulated in allergic inflammation, most likely through the presence of inflammatory cytokines.

During inflammation not only the expression of adhesion molecules is increased, but also soluble adhesion molecules are released (170-172). The presence of soluble adhesion molecules in the circulation of patients with a variety of inflammatory disease states has raised the possibility that plasma levels may reflect disease activity. This is supported by the finding that the amount of sE-selectin and sICAM-1 released was correlated directly with the cell surface expression *in vitro* (173). Thus, the concentration of soluble adhesion molecules may reflect the intensity as well as the extent of the inflammation in atopic disease.

1.5. THE IMMUNE SYSTEM DURING THE DEVELOPMENT OF ATOPIC DISEASE

1.5.1. Immaturity of immune system

There has been accumulating evidence that the immune system of newborns is relatively immature compared to adults. Neonates are immunologically naive, as exposure to microorganisms and foreign antigens is prevented to some extent by the placental barrier. The capacity of cord blood T cells to produce cytokines, such as IFN-γ and IL-4, is diminished (174,175), the secretion of Ig by B cells is strongly decreased (176) and antigen-presenting cells are functionally immature (177).

The IFN-γ production by neonatal T cells is decreased to as low as 10% of the adult IFN-γ production, while the production of IL-2 is comparable to those in adults (178). Several causes have been suggested for this low IFN-γ production by T cells: an increased sensitivity of newborn T cells to the inhibitory effect of PGE2 (175), an intrinsic deficiency in the capacity of newborn T cells to synthesize IFN-γ (178) or an inefficient stimulation of T cells by newborn macrophages (177). The reduced production of IFN-γ coincides with the time when the baby's gastrointestinal and respiratory tract is first exposed to environmental antigens and this may be important in the development of specific atopic disease (179). The IL-4 production is also strongly decreased by neonatal T cells (174). However, given the appropriate stimulation, *in vitro* neonatal T cells were able to express the cytokine mRNA repertoire characteristic for adult T cells (IL-3, IL-4, IL-6 and IFN-γ) (180). This suggests that T cells generated from neonatal blood have the intrinsic capacity to express all cytokines *in vitro*, indistinguishable from T cells

isolated from adult blood (180).

In contrast to adult B cells, neonatal B cells secrete minimal amounts of Ig in response to *in vitro* stimulation. This deficiency could be overcome by the addition of IL-2, IL-4, or IL-6 to the cultures, resulting in the secretion of all Ig isotopes (176). The deficient production of these cytokines by neonatal T cells is likely to contribute to the decreased capacity of neonatal B cells to produce Ig.

Phenotypic studies of T cells have shown lower percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in cord blood than in adult peripheral blood samples (104,181). The percentage of $CD45RO^+$ T cells, a marker for activated or memory T cells, is lower (104,182), and the percentage of $CD45RA^+$ T cells is higher compared to adults (104,181,183). Most (91%) neonatal $CD4^+$ T cells express the CD45RA marker.

During the first two years of life, many important natural maturational changes occur in the immune system of children. This time period is also a critical period for allergic sensitization. The simultaneous processes of maturation of the immune system and allergic sensitization compromise studies to the development of atopic disease in children below two years of age. A study to the development of atopic disease suggests that while many immune functions are normally less mature at birth, these maturational deficiencies (see below) may be more marked in newborns who develop atopy (142). To conclude that the immune system of newborns prone to develop atopy is more immature compared to that of newborns who will not develop atopy, requires further insight in the "natural" development of the immune system, including T cell subset cytokine profiles.

1.5.2. Cytokine profiles and proliferative responses by cord blood cells of children who developed atopy

Expression of specific cell surface markers, proliferative responses and selective cytokine production profiles after allergen-specific and polyclonal stimulation of PBMC were suggested as immunological risk factors for the development of atopy (184-190). Some cross-sectional studies analyzed these immunological risk factors in T cell clones (184) or cord blood mononuclear cells (CBMC) of high-risk children (185,186). However, only few studies described the analyses of immunological factors during a follow up of children starting at birth (142,187-190) (Table 3).

Studies analyzing phenotypes of CBMC revealed lower percentages of $CD25^+$ T cells and $CD45RO^+$ T cells in children who developed atopy at 12 months of age (189). Also decreased percentages of T cells and NK cells were described in children born to atopic parents compared to those born to non-atopic parents (185,191).

Proliferative responses of CBMC to a polyclonal stimulus are increased in children who developed atopy later in life (188,192). In addition, both newborns who did and those who did not develop atopy are capable of

Table 3. Literature data about abnormalities in the immune system of newborns who are to develop atopy relative to those who did not[1].

Immunological marker	Abnormalities	References
Phenotype of CBMC	Decreased percentage of T cells*, NK cells*, CD25+ T cells and CD45RO+ T cells	185,189,191
Proliferative response of CBMC[2]	Increased after polyclonal stimulation	188,192
	Increased after allergen-specific stimulation	188
Cytokine expression and production	Production IFN-γ reduced after polyclonal stimulation	147,185*,186*, 192,194
	Production IFN-γ reduced after allergen-specific (HDM,ovalbumin, β-lactogloubulin) stimulation	147,185,192 195
	Increased cord blood serum IL-4 levels	187
	Decreased IL-4 mRNA, IL-10 protein and IL-13 protein after HDM stimulation	142
	Increased IL-5 mRNA and decreased IL-13 protein after egg stimulation*	193*,196*

* newborns born to atopic compared to non-atopic parents. [1]The follow-up period for the various studies ranged between 12 months and 6 years of age. [2]In some studies (147,190,193) allergen-specific proliferative responses were observed at birth, but were not different between children who did and did not develop atopy.

lymphoproliferative responses to allergens at birth (147,190,193), suggesting that in utero allergen priming has occurred, which is not restricted to newborns who will develop atopy. However, one study described increased allergen-induced proliferative responses in children who had developed atopy at 12 months of age compared to those who did not develop atopy by that age (188).

The intrinsic capacity of CBMC to produce IFN-γ is reduced in children who are to develop atopy, as shown by measuring IFN-γ production after polyclonal stimulation of CBMC (147,192,194). Borres et al. (187) found that cord blood serum IL-4 levels were associated with atopic disease in infancy. These studies suggest that an altered balance between IL-4 and IFN-γ protein produced by cord blood cells could represent a primary abnormality in newborns prone to develop atopy. After allergen-specific stimulation of CBMC with different allergens, such as betalactoglobulin, ovalbumin and house-dust mite, also a reduced (192,195) or even undetectable (147) IFN-γ production was observed in newborns who will develop atopy. The Th2 cytokines IL-4, IL-10 and IL-13 were all decreased after house-dust mite stimulation of CBMC of children who develop atopy compared to those who did not (142). In contrast, after egg-specific stimulation an increased IL-5 mRNA and a decreased IL-13 protein level was observed in high-risk children compared to low-risk children (193,196).

These data suggest that children who will develop atopy fail to mount a profound Th1 response. Based on the reduced cytokine production in cord

blood, especially IFN-γ (193-195), a delayed prenatal maturation of cellular immune functions could be responsible for the failure to develop a profound Th1 response in children who develop atopy. However, this hypothesis is only based on studies that focussed on the expression or production of IFN-γ alone (193-195). Only Holt et al. studied a panel of different cytokines (such as IL-4, IL-5, IL-13 and IFN-γ) after allergen-specific stimulation on consecutive time points, during the development of atopy in children (9). No longitudinal studies have been described yet, that studied cytokine profiles both after polyclonal and allergen-specific stimulation on consecutive time points in children who did and did not develop atopy.

1.6. MECHANISMS OF ALLERGIC SENSITIZATION

Evidence is steadily accumulating which indicates, that the patterns of T cell reactivity against allergens, determining the allergen responder phenotype in adulthood are, in many cases, established during early childhood. Several mechanisms were proposed to explain the underlying regulatory processes, which determine the nature of long-term immunological memory against these allergens.

1.6.1. Intrauterine sensitization

Ruiz et al. (197) showed that significantly more children with an atopic mother developed AD than those with an atopic father. In addition, cord blood T cells are capable to proliferate *in vitro* in response to both food and inhalant allergens (147,190,193). This suggests that the initial T cell priming may occur in utero as a result of transplacental transfer of low levels of the relevant allergens, to which mothers are exposed during pregnancy. It appears that the 2nd trimester of pregnancy is already an important time period for the initiation of T cell priming, as maternal food-allergen avoidance in the 3rd trimester of pregnancy is ineffective in the prevention of infant atopy (198,199). Some data indicated that this priming process may be strongest in infants with a positive atopic family history (192,196), and this priming may be influenced via the transplacental transfer of maternal allergen-specific IgG subclass antibodies (200).

In summary, allergen-specific proliferative responses of cord blood T cells suggest that the initial allergic sensitization can already start in utero.

1.6.2. A reduced production of IFN-γ protein

Although newborns have a reduced capacity for mitogen induced IFN-γ production (174,175,178), this appears more marked in newborns either at risk for atopy and those who later develop atopic disease (147,185,186,192,194). In non-atopic children, the IFN-γ production after

mitogenic stimulation gradually increases over the first years of life and by the age of 4 to 5 years most children have reached adult IFN-γ production levels (201). In contrast, children with atopic heredity showed a more delayed increase in IFN-γ production in the early post-natal period. In those children the capacity for IFN-γ production remained significantly reduced until at least 5 years of age compared to low risk children (184). During the first years of life, responses to vaccine antigens are also weaker in children at high risk for the development of atopy (202). It is likely that the IFN-γ production during this period is important for the normal post-natal immune maturation (see also General discussion). Furthermore, in children and adults with established AD a discrepancy is described between equal IFN-γ mRNA expression levels and reduced IFN-γ protein production (136,148,152). So, both during the allergic sensitization and in established atopic patients reduced IFN-γ protein is observed. Reduced IFN-γ levels, which could indicate a reduced Th1 cell function, result in decreased inhibition of Th2 cell development and/or function. In this way IFN-γ facilitates the development of a Th2 cytokine profile and thus indirectly stimulates the development of allergic sensitization and finally clinical manifestation of atopy. These findings indicate an important role for IFN-γ in the development and maintenance of allergic sensitization and atopy.

1.6.3. Proposed model for allergic sensitization

The pioneering work of Holt and Macaubas has led to the following model explaining allergic sensitization at the perinatal age (203). In utero, T cell priming can occur in response to low levels of transplacental

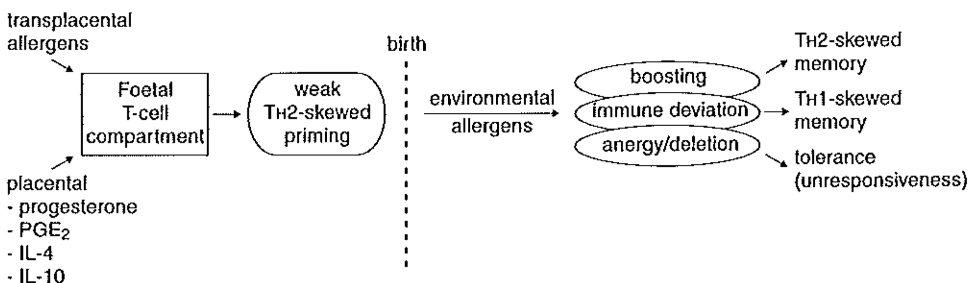

Figure 4. Schematical presentation of the model for allergic senstization as proposed by Holt and Macaubas (202). *In utero*, initial exposure of foetal T cells to environmental allergens, which cross the placenta in combination with a milieu, which is strongly inhibitory to Th1-associated functions, will result in a weak Th2 skewed T cell population in newborns. After birth, stimulation by the same environmental allergens can modulate this weak Th2 skewing. In newborns at low risk to develop atopy, high levels of inhalant allergens lead towards deviation from the weak Th2 response to a progressively increasing Th1 skewing, while in response to food allergens tolerance by T cell anergy or T cell deletion is induced. In atopy-susceptible individuals, on the other hand, exposure to food or inhalant allergens fails in tolerance induction or immune deviation, but will result in a boosting of the Th2-polarised immunity.

environmental allergens. In the presence of a range of placental Th1-inhibitory factors, such as a high production of IL-10, IL-4 and PGE2 (204,205), a weak Th2 skewed T cell population can gradually develop. It has been proposed that this weak Th2 skewing is present in all children at the time of birth (203). During early infancy, these Th2 skewed responses are subject to modulation via stimulation with incoming environmental allergens (Figure 4). In newborns at low risk to develop atopy high levels of inhalant allergens lead towards deviation from the weak Th2 response to a progressively increasing Th1 skewing, while in response to sustained high levels of food allergens tolerance by T cell anergy or T cell deletion is induced (205). In atopy-susceptible children, on the other hand, the combination of a relatively immature T cell compartment being skewed towards a Th2 phenotype can easily lead to failure of tolerance induction or of the development of a profound Th1 response. In these children exposure to high levels of food- or inhalant allergens results in the development of a strong Th2 polarized response and finally in clinical manifestation of atopic disease.

The basic processes underlying the apparent failure of immune deviation in atopics remain to be defined. These are probably related to developmental defects during the process in which the imbalance in fetal Th cell function (skewing towards Th2 phenotype) is established during early childhood.

In the General discussion (Chapter 11) the role of these proposed mechanisms for allergic sensitization and the development of atopy will be discussed in more detail, considering our results described in this thesis.

1.7. AIM OF STUDY

As discussed before, in young children various compartments of the immune system (including T and B cells) are relatively immature compared to adults (section 1.5.1). This immature immune system in young children could contribute to the widely held view that during the first years of life there is an enhanced risk of allergic sensitization (section 1.5.1), leading to the development of atopic disease in childhood. After birth, the T cell function and especially the capacity to produce cytokines are immature (section 1.5.2).

Our hypothesis underlying the experimental work of this thesis is that children at high risk to develop atopy have a deviant capacity to generate the various T cell subsets and allergen-specific responses within the first months of life. As a result, a disturbed balance will be generated in Th1-Th2 subsets and the cytokines they produce, leading to a characteristic aberrant generation of allergen-specific IgE antibodies. Consequently, clinical expression of IgE-mediated atopic disease will occur within the first 2 years

of life. These disturbed cytokine profiles persist and are still detectable after the establishment of atopy later in childhood.

From this hypothesis the following research questions were formulated:

1. How do immune parameters implied in the development of atopic diseases develop under natural conditions? What is the time course of allergen-specifically and polyclonally-induced Th1 and Th2 cytokine mRNA expression and protein production levels in high-risk children that will not develop atopy within the first 2 years of life?
2. What are the differences in the development of these immune parameters and the cytokine profiles between children who will or will not develop atopy within the first 2 years of life?
3. Are the dysregulations in the T cell response and cytokine production profiles similar between children who develop atopy before 2 years of life and older children (mean age of 4 years) with established atopy?
4. What are the associations between the changes in immune parameters and the clinical manifestations of the atopic disease? Could any one of the immunological markers analyzed be used as a predictor for atopy development in children below 2 years of age?

We addressed these research questions by combining a longitudinal and a cross-sectional study in young children. In the longitudinal study, the development of allergen-specifically and polyclonally-induced T cell responses, in particular cytokine mRNA expression and protein production profiles were defined in children whether or not prone to develop atopy within 2 years of life. In the cross-sectional study, differences in allergen-specifically and polyclonally-induced T cell responses were studied between children with established IgE-mediated atopy and healthy children. In addition, the *in vitro* observed T cell responses in the children with an established atopy were associated with those T cell responses in the children enrolled in the longitudinal study.

In Chapter 2 we describe the different patient groups used in our studies and define the various atopic diseases in early childhood as well as the employed clinical tests. In chapter 3 we describe the development and optimization of different techniques for the analysis of proliferative responses, cytokine mRNA expression and protein production, after both polyclonal and allergen-specific stimulation. The relevance to investigate cytokine mRNA expression and protein production both after polyclonal and allergen-specific stimulation was discussed in section 1.4.1.

1.7.1. T cell response during childhood development of atopy

Early diagnosis of children at high risk for the development of atopic disease may help preventing symptomatic atopy. Therefore, we tried to correlate the clinical expression of atopy (as defined in Chapter 2) with changes in the immune system.

For the longitudinal study presented in this thesis, we selected 133 newborns at high risk for the development of atopy. Children from mothers with an atopic disease were selected only, to increase the genetic risk to develop an atopy from 10% to at least 30% (section 1.2.1). These high-risk children were followed from birth to 2 years of age, as this was considered the most sensitive time window to develop atopic disease (203). At 5 different time points (birth, 6, 12, 18 and 24 months) physical examinations (except at birth) were performed, questionnaires concerning environmental factors and histories of symptoms of atopy were filled out (except at birth), and venous blood samples were obtained. PBMC, isolated from blood obtained at the different time points, were stimulated polyclonally (PMA and Ca-ionophore) and allergen-specifically (cow's milk, egg and house-dust mite). Before and after stimulation, proliferative responses, cytokine mRNA expression (IL-4 and IFN-γ), cytokine protein production (IL-4, IL-5, IL-10, IL-13 and IFN-γ) and IgE antibody levels were investigated. The time course of total- and specific-IgE levels, allergen-induced proliferative responses and skin-test positivity, plasma sE-selectin levels and blood eosinophil count in relation to the development of atopic disease during the first 2 years of life are described in Chapter 4. In order to get more insight into the process of allergic sensitization, we examined whether environmental and genetic risk factors and immunological responses at birth could contribute to the development of clinical symptoms of atopic disease at 12 months of age (Chapter 5). The proposed weak Th2 priming at birth in all children (section 1.6.3) has made it challenging to investigate whether the intrinsic capacity of T cells to give rise to Th1 or Th2 cytokine profiles was different between atopy-prone children and children who did not develop atopy within their first year of life (Chapter 6).

1.7.2. Analysis of T cell response in children using a cross-sectional study

We were interested to which extent the dysregulation in immune functions as observed during the development of atopic disease persisted in children with established atopy. During the first years of life we expected AD as the most common atopic disease (section 1.1). Therefore, in the cross-sectional study children were selected who suffered from AD.

The most frequent food allergies in the Netherlands are directed against cow's milk, egg and peanut (14). In contrast to cow's milk and egg allergy, peanut allergy does not resolve. Moreover, peanut allergy could result in severe reactions as anaphylaxis (206). Therefore, we expected to find the most profound differences in T cell responses after stimulation of PBMC with peanut allergen. Therefore, we chose peanut-allergen to study allergen-induced T cell responses and used this as a model to study food allergy.

PBMC of children suffering from atopic dermatitis with or without peanut-sensitization and healthy age-matched children were polyclonally stimulated to study the intrinsic capacity of the cells to proliferate, and to

express and produce cytokines. Both, Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10 and IL-13) cytokines were analyzed. In addition, proliferative and cytokine responses were measured after allergen-specific stimulation with a commercial peanut-extract. Chapter 7 describes the optimization of the peanut-specific stimulation. Proliferative responses are described in Chapter 8, and cytokine profiles were described in Chapter 9.

Adhesion molecules are also thought to play a role in the pathogenesis of atopy. We therefore investigated possible differences in concentrations of soluble adhesion molecules in the plasma of children with AD and healthy control children (Chapter 10).

Finally, in the General discussion (Chapter 11) we integrated and evaluated the data obtained in the experimental studies in the context of relevant literature, with emphasis on the different cytokine production profiles during the development of atopic disease in young children.

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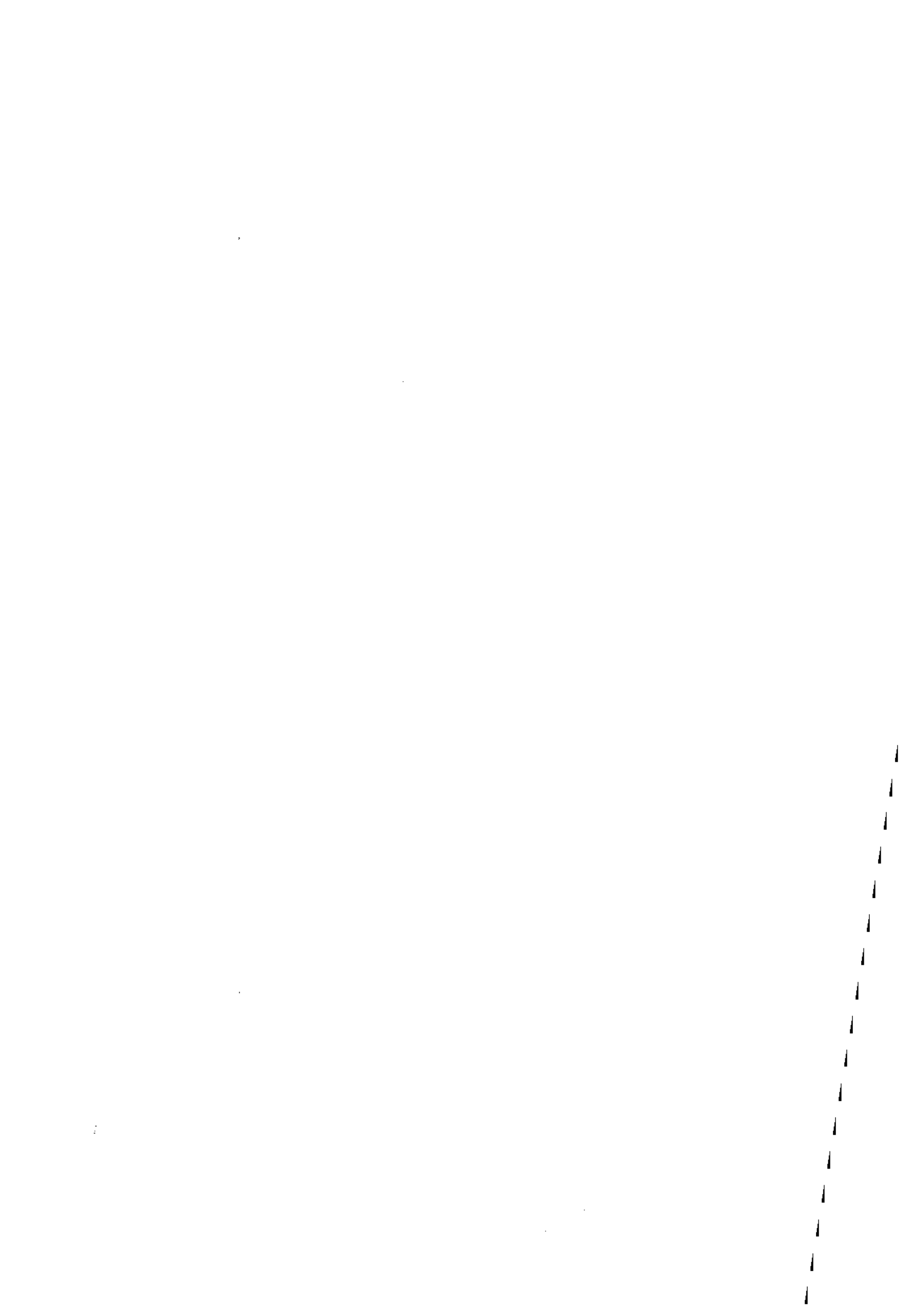

PATIENT GROUPS AND METHODOLOGY

CHAPTER 2

THE DIFFERENT PATIENT GROUPS STUDIED AND THE CLINICAL TESTS USED

2.1. PATIENT GROUPS

Two groups of children were investigated: children included in the cross-sectional study and children included in the longitudinal study. The first group of children consists of children between 1-10 years of age suffering from atopic dermatitis (AD) with (n=24) or without (n=11) a peanut-allergy. They were compared with healthy non-atopic children (n=21). The second group consists of 133 newborns that were followed for the development of atopic diseases (AD, food or upper-airway allergy, asthma) from birth to 2 years of age. Below are the characteristics of these diseases presented, with emphasis on early childhood.

2.2. ATOPIC DISEASES

2.2.1. Atopy

In this thesis atopy was defined as type I allergy. The term atopy was used for high-risk children who developed clinical manifestations of allergy, such as AD, asthma-like disease, food or upper-airway allergy.

2.2.2. Atopic dermatitis

AD or atopic eczema is an intensely pruritic inflammatory skin disorder, especially common in infants (1). In this thesis we investigated children between 0-10 years suffering from AD. AD is characterized by a large number of clinical symptoms. None of these symptoms is pathognomic. Therefore a combination of different symptoms is necessary for the diagnosis. Children with AD older than two years were selected according to the diagnostic criteria of Hanifin & Rajka (2), while for children below two years of age the criteria of Sampson et al. (3) were used.
The main criteria according to Hanifin & Rajka are:
- Pruritis
- Typical morphology and distribution; in children: facial and extensor
- Chronic or relapsing dermatitis
- Personal or family history of atopy

The main criteria according to Sampson are:
- Family history of atopic dermatitis
- Evidence of pruritic dermatitis
- Typical facial or extensor eczematous or lichenified dermatitis

In the longitudinal study children were only diagnosed as AD when they fulfilled the criteria of Sampson and when the duration of the AD symptoms were lasting for longer than four weeks.

The severity of the eczema was measured using the objective SCORAD-criteria (4).

These criteria include scoring of the involved area with eczema (A) and the intensity of the eczema (B). Five criteria were used for scoring the intensity: erythema, edema/papulation, oozing/crust, excoriation and lichenfication. The SCORAD index was calculated using the following formula: A/5 + 7B/2. An index below 20 was classified as light AD, an index between 20 and 40 as mild AD and above 40 as severe AD.

2.2.3. Food allergy

Food allergy was defined as a clinical manifestation of erythematous, papular, macrovesicular skin eruptions, diarrhea, vomiting, or respiratory problems in relation to food ingestion. For the definitive diagnosis a positive prick-prick test (5), a positive skin application food test (SAFT) (6,7) or specific IgE was necessary. All children suspected for food allergy were asked for an open oral provocation test to confirm the diagnosis.

2.2.4. Asthma

Asthma is difficult to define in young children. The current working definition is : "asthma is a lung disease with the following characteristics: (a) reversible airway obstruction, either spontaneously or with treatment; (b) airway inflammation; and (c) increased airway responsiveness to a variety of stimuli" (8).

For the work presented in this thesis, we introduced the term asthma-like disease to classify the complaints that indicate asthmatic symptoms before 2 years of age. This group of asthmatic children includes both children with ongoing and temporary respiratory problems after the age of 2 years. Based on criteria of the American Thoracic Society (9), asthma-like disease was diagnosed if children either had at least three separate wheezing episodes each lasting three days or more, or had more than two separate episodes of paroxysmal nocturnal cough with sleep disturbance for three consecutive nights, or required anti-asthma therapy during the last 6 months.

2.2.5. Upper-airway allergy

Upper-airway allergy was defined as seasonal rhinoconjunctivitis, symptoms of sneezing, redness, itching and tearing of the nose or eyes and/or respiratory complaints. Diagnosis was confirmed by the presence of specific IgE in the plasma.

2.2.6. Allergic sensitization

Sensitization to food or inhalant allergens was defined as a positive skin test or the presence of specific IgE without clinical manifestation.

2.3. CLINICAL TESTS

2.3.1. Total and specific IgE

In plasma samples of the children total IgE levels was measured using the Pharmacia CAP RAST (Kabi Pharmacia, Uppsala, Sweden). The total IgE concentrations in cord blood samples were determined with a modified radio-immuno assay to result in a lower detection limit of 0.2 kU/L. Analysis of specific IgE antibodies directed towards the most common inhalant allergens (house-dust mite (HDM), grass and birch pollen, cat, dog and moulds) and food allergens (peanut, chicken egg white (EW), cow's milk (CM), codfish, wheat and soy) were performed using the Pharmacia CAP system Phadiatop® RAST and the Pharmacia CAP RAST (Kabi Pharmacia), respectively (10).

2.3.2. Skin tests

In the *SAFT test* (6,7), the food was applied in the same condition as usually consumed, on the back of the child using large Finn chambers. The test was read after 10, 20, and 30 minutes. The results were scored as follows: 0 = no reaction; 1+ = erythema; 2+ = erythema and edema within the area of the Finn chamber; 3+ = eythema and edema up to the outer area of the Finn chamber. Reactions with a score of 2+ or 3+ were regarded as positive.

In the *prick-prick test* (5) a microlancet with a 1-mm needle with shoulders (ALK, Benelux) was used. The needle was first pricked in the food and subsequently vertically into the lower arm. A drop of histamine dihydrochloride solution (10 mg/ml, ALK, Benelux) was used as positive control and a drop of saline as negative control. The test was read after 15 and 30 minutes. The reaction to the food was evaluated by comparing it with the reaction to histamine. A positive reaction to food had an average diameter of the wheal that was at least 3 mm above the negative control.

2.3.3. Oral provocation test

The oral provocation test was performed in a standardized way at the day-care unit, under appropriate safety conditions. After examination and registration of the blood pressure and pulse rate, a small amount of the food was given to the child. All observed reactions were noted. Positive reactions consisted of an urticarial rash, perioral redness, flare-up of eczema, itching, increased pulse rate, depression of blood pressure, and respiratory or gastro-intestinal complaints. If no reactions were observed after 1 hour, a double amount of the food was given; after 2 hours, the amount was doubled again. After 3 hours, the food was given without restrictions. The total amount given correlated to age-related average 'daily intake'. If a positive reaction was observed, the test was stopped. If no reaction was observed, the child was dismissed after 8 hours. The parents were urged to contact the

dermatologist if any late reaction was observed, in order to evaluate this reaction.

2.4. QUESTIONNAIRES

The questionnaires obtained from the children included in the longitudinal study focussed on environmental factors and histories of symptoms of atopy. Questions about environmental factors concerned the presence of pets, strategies for HDM avoidance, parental smoking and feeding. Questions about possible symptoms of atopy concentrated on skin problems (frequency and duration of eczema, pruritis, itching and erythema), colds (frequency, bacterial or viral), upper-airway problems (sneezing, redness, itching and tearing of the nose or eyes), and respiratory complaints (frequency and duration of wheezing episodes, paroxysmal nocturnal cough and requirement of anti-asthma therapy).

The questionnaires obtained from the children included in the cross-sectional study focussed on environmental factors and family history of atopy.

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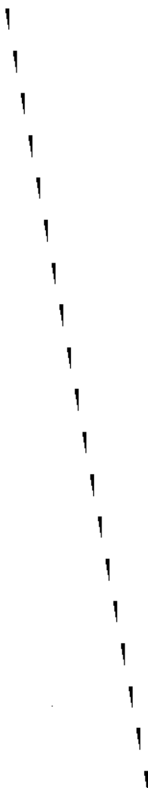

CHAPTER 3

DIFFERENTIAL mRNA EXPRESSION AND PRODUCTION OF INTERLEUKIN-4 AND INTERFERON-γ IN STIMULATED PERIPHERAL BLOOD MONONUCLEAR CELLS OF HOUSE-DUST MITE-ALLERGIC PATIENTS

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ABSTRACT

Optimal culture conditions were established for the analysis of interleukin-4 (IL-4) and interferon-gamma (IFN-γ) mRNA expression and protein production as well as proliferative capacity of peripheral blood mononuclear cells (PBMC). These culture conditions permitted the analysis of differences in the responses of house-dust mite (HDM) allergic patients and healthy controls after polyclonal and allergen-specific stimulation. Proliferative responses were optimal when PBMC were cultured in RPMI, whereas for studying mRNA expression by RT-PCR and protein production by ELISA, PBMC should be stimulated in Yssels's medium. Blood holding period influenced the cytokine mRNA expression and proliferative capacity of primarily the unstimulated cells. It is thus crucial to isolate PBMC as soon as possible, but not later than 7 hours after blood collection.

Proliferative responses to *Dermatophagoides pteronyssinus*-extract were observed in HDM allergic patients (mean SI = 5.3 ± 0.75), but not in non-allergic subjects (mean SI = 2.3 ± 0.21). After *D. pteronyssinus*-specific stimulation, IL-4 mRNA expression was significantly ($p = 0.03$) increased in HDM allergic subjects compared to non-allergic subjects. No significant differences were found in IFN-γ mRNA expression between HDM allergic and non-allergic subjects. On the other hand, both IFN-γ ($p = 0.04$) and IL-4 ($p = 0.06$) protein production were increased after *D. pteronyssinus*-specific stimulation in HDM allergic subjects compared to non-allergic subjects.

Our data suggest activation of both Th1 and Th2-like cells, as well as $CD8^+$ T cells in allergic patients. Furthermore, analysis of possible functional differences in PBMC between allergic and non-allergic patients, necessitates the stimulation of PBMC both under polyclonal and allergen-specific conditions. Moreover, proliferative responses as well as cytokine mRNA expression and protein production need to be studied under optimal culture conditions to evidence the often subtle differences.

INTRODUCTION

Many cytokines are involved both in regulation of IgE responses and in the inflammatory processes characteristic of allergic diseases [1-6]. T cells play an immunoregulatory and in some cases a causal role in these processes. Based on their cytokine production profile helper T cells (T_h) are generally divided in at least two effector populations: T_h1 cells producing IL-2 and IFN-γ and T_h2 cells producing IL-4, IL-5 and IL-13 [7-8]. Several factors, mostly cytokines themselves, may specifically promote T_h1 or T_h2 outgrowth from antigen-activated precursor cells. IFN-γ and IL-12 strongly promote the generation of T_h1-like cells, whereas IL-4, IL-10 and prostaglandin E_2 promote the generation of T_h2-like cells [9-11]. A predominantly T_h2 response leads to IgE production [1-3], whereas a T_h1 response inhibits IgE production [4-6].

Elevated serum IgE levels are generally considered to be a typical factor in the expression of allergic diseases [12-14].

Several studies investigated the cytokine mRNA expression and protein production profiles (T_h1 versus T_h2) of T cell clones [7,8,15-18] and of peripheral mononuclear cells (PBMC) [19-25] of allergic subjects. These studies showed that allergic diseases are associated with expression of T_h2-like cytokine responses. However, these studies showed conflicting conclusions as to the nature of the specific defect in cytokine synthesis. IFN-γ mRNA expression and production was reported to be reduced in atopic subjects [8,19-21,24], whereas in others it was normal [18,26] or elevated [15,23,27] compared to healthy controls. IL-4 production was usually [7,19-21,28,29], but not always [18,22], reported to be elevated. A cause of these discrepancies could be the frequent use of aspecific stimuli such as phorbol myristate acetate (PMA) plus Ca-ionophore, as surrogates for major histocompatibility complex (MHC)-restricted, antigen-driven activation of cytokine production. The use of these polyclonal activators stimulates especially $CD8^+$, but not $CD4^+$ cells, whereas allergen-specific stimulation preferentially activates $CD4^+$ cells [30]. Furthermore, the different cytokine patterns found in allergic subjects could be due to suboptimal stimulation conditions. For example, in most studies PBMC were cultured in RPMI medium [19,21,23,28], whereas few others used Yssel's medium (YM) [15,20].

In this study optimal culture conditions for PBMC for proliferative responses and cytokine gene expression and protein production were investigated both for allergen (house-dust mite)-specific and polyclonal (PMA + Ca-ionophore) stimulation. Finally, proliferation capacity and IL-4 and IFN-γ mRNA expression and production of PBMC of house-dust mite (HDM) allergic subjects were studied after polyclonal and allergen-specific stimulation.

MATERIAL AND METHODS

Subjects

Peripheral Sodium-heparinized venous blood was obtained from 8 subjects with a history of house dust mite-allergic rhinitis (HDM) (mean age 29 year, 4 males and 4 females) and 6 non-allergic subjects (mean age 28 year, 2 males and 4 females). All allergic subjects were skin prick test positive to *Dermatophagoides pteronyssinus* and/or had *D. pteronyssinus*-specific IgE as assessed by radio-allergo-sorbent test (RAST) (Phadiatop cap RAST, Pharmacia, Uppsala, Sweden) [31]. Healthy non-atopic volunteers had no history of allergic diseases. Informed consent was obtained from all participating subjects

PBMC from heparinized venous blood were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia) [32]. For several experiments heparinized whole blood samples were kept 4, 7 and 24 hours before processing as indicated in the Results section.

Lymphocyte proliferation assay

PBMC were washed two times and resuspended in different culture media: RPMI-1640 (Gibco Life Technologies B.V., Breda, The Netherlands) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 mg/ml streptomycin, 1 mM pyruvate (RPMI) and 10% heat-inactivated human serum (HS). Alternatively, Yssel's medium (YM) [33] containing 10% HS or serum-free AIMV medium supplemented with 2 mM L-glutamine (Gibco) were used. AIMV medium was tested because it was described that in serum-free medium more cytokines will be produced. Human serum, pooled from healthy donors, was previously shown not to induce any non-specific proliferative responses.

Proliferation assays were performed in quadruplicate in 96-wells round bottom or flat bottom plates (Falcon, Becton & Dickinson, Mountain View, CA) at $2x10^5$ cells/well in the presence of PMA (2 ng/ml; phorbol-12-myristate-13-acetate; Sigma, St.Louis, MO) and Calcium-ionophore (1 mg/ml; A23187, Sigma) or a lyophilized extract of *D. pteronyssinus* (50 mg/ml; ALK-Benelux, Groningen, The Netherlands) or without additions in a total volume of 200 µl/well. The amount of endotoxin in the *D. pteronyssinus*-extract was measured using the Limulus amebocyte lysate micromethod [34] and was below 12.5 EU/mg. At 3 days of polyclonal stimulation (PMA + Ca-ionophore) or 7 days of *D. pteronyssinus*-specific stimulation, proliferation was measured using tritium-($[^3H]$)-thymidine incorporation. These culture conditions were selected in previous dose titration and kinetic studies (data not shown). After addition of $[^3H]$-thymidine (0.5 mCi/well; Amersham, Aylesbury, UK) the plates were incubated for 8 hours before harvesting. $[^3H]$-thymidine incorporation was measured with a BetaPlate (Pharmacia LKB, Turku, Finland).

Results are expressed as mean cpm ± sem or as stimulation index (SI), equal to the ratio of mean counts per minute (cpm) in polyclonal- or allergen-stimulated cultures over mean cpm in unstimulated cultures.

Analysis of IL-4 and IFN-γ mRNA expression in PBMC cultures

PBMC ($1x10^6$ cells/ml) were cultured either with the addition of 2 ng/ml PMA and 1 mg/ml Ca-ionophore or the addition of 50 mg/ml *D. pteronyssinus*-extract at 37°C, 5% CO_2. Cultures were performed in 24-wells plates or 96-wells round bottom or flat bottom plates (Falcon). Different media were tested: RPMI containing 1% heat-inactivated HS, YM containing 1% HS and AIMV serum-free medium. PBMC stimulated with PMA + Ca-ionophore were cultured for 16-18 hr, and PBMC stimulated with *D. pteronyssinus*-extract were cultured for 4 days. Previous kinetic studies had shown that these stimulation periods for studying cytokine mRNA expression were suitable.

After harvesting the cells, supernatants were stored at -80°C and RNA was isolated from the cells by the RNAzol B method [35] (Cinna-Biotecx Laboratories Inc., Houston, TX). As described previously [32], 1 mg RNA was used for cDNA synthesis and amplification by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) using primers specific for either the housekeeping gene HPRT (human hypoxanthine phosphoribosyl

transferase), IL-4 or IFN-γ [32]. The PCR protocol started with a hot-start at 94°C, to prevent aspecific primer annealing. Subsequently, denaturation of PCR samples for 0.5 min at 94°C followed by annealing for 0.5 min at 55°C, and extension for 1 min at 72°C were performed. After PCR amplification, 3 samples taken after different cycle numbers, were loaded on a 1.2% agarose gel (Sea Kem Leagarose, FMC Bio Products, Rockland, ME), stained with 75 mg/500 ml ethidium bromide (Boehringer Mannheim, Germany). 0.5 ml PhiX174 (HaeIII digest, 0.5 mg/ml, New England Biolabs, Beverly, MA) was used as marker. The bands of the PCR products on the gel were visualized with UV light. A life-size photo was taken and used for scanning. Collected scanvalues were analyzed as follows: the mean scanvalues were calculated at two different cycle numbers (30-35 cycles for HPRT and IFN-γ, 35-40 cycles for IL-4), in the pre-established linear phase of the PCR reaction. In order to correct for inter-experimental variation a batch of cDNA of B21 (a T_h0 clone, Paliard [36]) was used for standardization of HPRT, IFN-γ and IL-4 mRNA expression. The scanvalues obtained were further corrected for differences in the quality of isolated RNA and efficacy of the cDNA reaction by dividing the corrected scanvalues of IFN-γ and IL-4 by corrected HPRT values of the same sample.

The results are expressed as mean scanvalue ± SD or stimulation index equal to scanvalue of cytokine gene expression of stimulated PBMC over scanvalue of cytokine gene expression in unstimulated PBMC. Some scanvalues of cytokine expression in stimulated PBMC were related to scanvalues of cytokine gene expression in unstimulated PBMC, which were put to 100%.

Assay for IFN-γ and IL-4 production

The levels of IFN-γ and IL-4 in the culture supernatants were determined by ELISA kits (Medgenix Diagnostics SA, Fleurus, Belgium) according to the manufacturers instructions. The lower detection limit was 30 pg/ml for IFN-γ and 3 pg/ml for IL-4.

Statistical analyses

Statistical analyses were performed with STATA™ (Computing Resource Center, Los Angeles, CA). Normally distributed parameters were compared using the Student's t-test and non-parametric parameters were tested using the Mann-Whitney test. In either test, P values smaller than 0.05 were considered significant.

RESULTS

Optimal culture conditions for allergen-specific and polyclonal stimulation in HDM allergic patients

Three different culture media: RPMI, YM and serum-free AIMV medium, were compared in their ability to support polyclonal (PMA+Ca-ionophore) and

D. pteronyssinus-specific proliferation assays. Cells were cultured in round bottom 96-wells culture plates. On day 3 baseline proliferation of PBMC cultured in YM (mean cpm = 676.1 ± 85.7) was significantly higher as compared to RPMI (mean cpm = 150.3 ± 26.4, $p < 0.0001$; Fig. 1A). After 3 days of culture of polyclonally stimulated PBMC no significant differences were observed between cultures in YM (mean cpm = 20298.0 ± 837.0) or in RPMI (mean cpm = 32256.7 ± 889.4). By 7 days of culture baseline proliferation of PBMC cultured in YM (mean cpm = 707.8 ± 164.7, $p = 0.0003$) was significantly elevated compared to baseline proliferation of PBMC cultured in RPMI (mean cpm = 158.1 ± 0.9; Fig 1B). After *D. pteronyssinus*-specific stimulation the proliferative responses were not significantly different between the media tested (mean cpm YM = 2401.8 ± 313.3, mean cpm RPMI = 2232.9 ± 711.9).

Next, the influence of round vs. flat bottom 96-wells culture plates on the induced proliferative responses was studied. After 3 days of polyclonal stimulation of PBMC no significant differences in proliferative responses were observed between cultures in round bottom (mean cpm = 33256.7 ± 889.4) versus flat bottom (mean cpm = 33757.1 ± 1714.0) 96-wells plates (Fig. 2A).

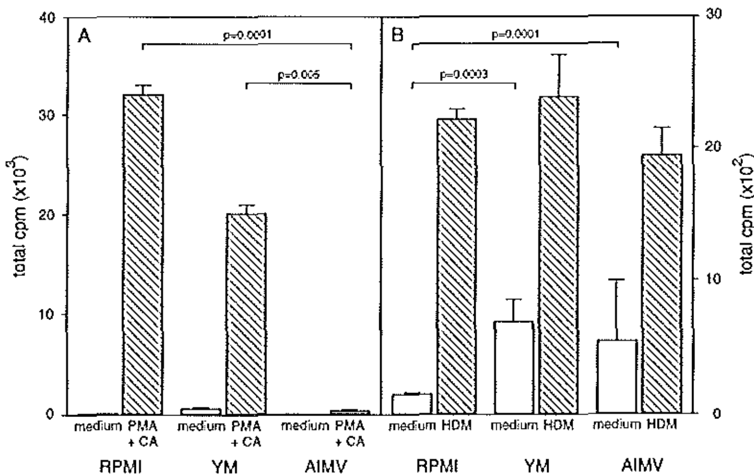

Figure 1. Influence of culture media on proliferative responses. PBMC were cultured in RPMI, YM or AIMV medium. The influence of culture media on proliferative responses was studied after polyclonal (PMA + Ca-ionophore) stimulation (a) and after *D. pteronyssinus*-specific stimulation (HDM) (b). Results are presented as mean total cpm ± sem or as baseline cpm ± sem. This experiment was repeated 8 times with PBMC from a different subject in every experiment.

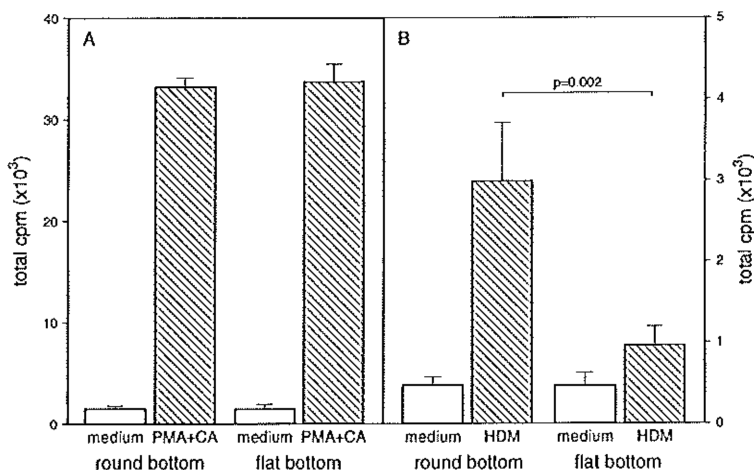

Figure 2. Influence of culture plates on proliferative responses. PBMC were cultured in 96-wells round and flat bottomed plates. (a) proliferative responses in round versus flat bottom plates after polyclonal stimulation of PBMC with PMA + Ca-ionophore and (b) after stimulation of PBMC with *D. pteronyssinus* (HDM). Results are presented as mean total cpm ± sem or as baseline cpm ± sem. This experiment was repeated 8 times with PBMC from a different subject in every experiment.

Table 1. Reproducibility of proliferative responses.

Stimulation	Baseline cpm mean ± sd	Total cpm mean ± sd	SI
Polyclonal	127.9 ± 4.6[1]	47786.8 ± 1391.1	373.6
(n=4)	134.3 ± 18.5	41685.1 ± 2084.4	310.4
	156.7 ± 20.0	41291.2 ± 2597.0	262.5
	129.5 ± 38.9	38708.5 ± 3509.5	298.9
Total mean	137.1 ± 13.4	42367.9 ± 3846.3	311.6 ± 45.9
CV (%)[2]	9.7	9.1	14.7
Allergen-specific	441.6 ± 217.2[3]	20093.6 ± 7843.5	45.5
(n=5)	505.4 ± 264.1	17328.0 ± 2979.6	34.3
	409.9 ± 130.1	14997.0 ± 4293.3	36.6
	306.6 ± 79.1	12682.8 ± 2574.7	41.4
	431.6 ± 117.0	15767.9 ± 2345.5	36.5
Total mean	419.0 ± 72.2	16173.9 ± 2758.3	38.9 ± 4.5
CV (%)	17.2	17.1	11.7

[1] Each experiment was performed in quadruplicate. Numbers respresent mean ± sd.
[2] CV = coefficient of variance (sd/mean x 100%).
[3] Baseline cpm on day 3 of culture were significantly (p=0.0001) lower than baseline cpm on day 7 of culture.

After 7 days of *D. pteronyssinus*-specific stimulation, mean proliferative responses were significantly ($p=0.002$) higher in round bottom plates (mean cpm=2992.9 ± 712.0) compared to flat bottom plates (mean cpm= 463.1 ± 48.9). Baseline proliferative responses, however, were comparable between the two different culture plates (Fig. 2B).

Furthermore, the reproducibility of proliferative responses was determined after *D. pteronyssinus*-specific and polyclonal stimulation (Table 1). Baseline proliferation of cells cultured in medium alone was significantly ($p=0.0001$) lower after 3 days compared to 7 days of culture. The coefficient of variance of the stimulation index after polyclonal or allergen-specific stimulation were virtually equal (14.7% vs 11.7%).

Next, we determined the influence of holding time on the proliferative responses obtained. Proliferation assays were performed with PBMC fractions of 5 subjects after they were kept for several hours at RT before processing. During that holding time, no significant fluctuations in the degree of proliferative responses were found both after polyclonal and allergen-specific stimulation. There was a consistent, but not significant trend, of increasing baseline proliferative responses with increasing storage time of blood samples (data not shown).

Thus, for the analysis of proliferative responses, induced after either polyclonal or allergen-specific stimulation, PBMC should preferably be cultured in RPMI medium in combination with 96-well round bottom microtiter culture plates. Moreover, cultures should be started as soon as possible as holding the blood tended to increase baseline proliferative responses.

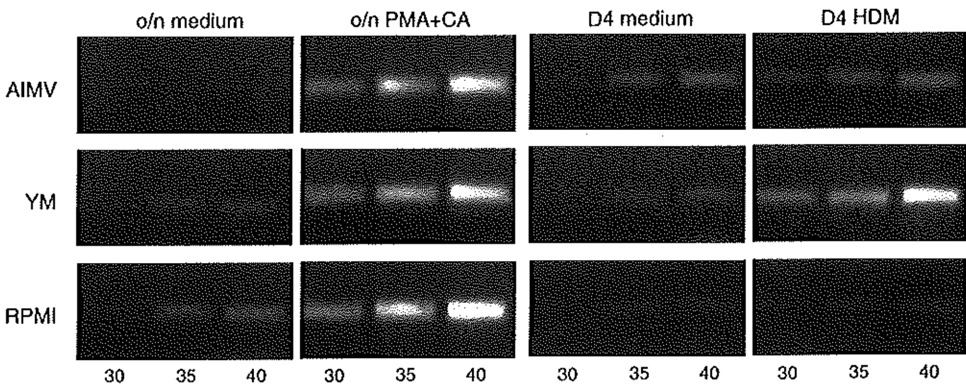

Figure 3. IFN-γ mRNA expression in PBMC stimulated in different culture media. PBMC of a *D. pteronyssinus* allergic individual were stimulated for 16-18 hours with PMA + Ca-ionophore or for 4 days with *D. pteronyssinus*-extract (HDM). IFN-γ mRNA expression was determined by RT-PCR at 30, 35 and 40 cycles. PBMC were stimulated in different culture media: RPMI, YM and AIMV media.

Table 2. Comparison of PBMC of one subject cultured in different media used for cell proliferation and cytokine expression.

		Media	
		RPMI	YM
Proliferation	medium	122.7	3213.5
	HDM	1091.6[1]	19356.2
	SI	8.9	6.0
IFN-γ mRNA	medium	3.4[2]	3.6
	HDM	1.5	38.4
IL-4 mRNA	medium	26.2[2]	28.2
	HDM	12.6	33.8

PBMC of one subject were cultured in YM and RPMI. Subsequently, IL-4 and IFN-γ mRNA expression and proliferative responses were determined after *D. pteronyssinus*-extract stimulation.
[1] Results of proliferative responses are expressed as mean counts.
[2] Results of cytokine mRNA expression are presented as scanvalues.

Cytokine gene expression and production by PBMC from HDM allergic patients

Polyclonal stimulated and allergen-specific stimulated PBMC cultured in the different media were used to analyze cytokine mRNA expression and protein production levels. After polyclonal stimulation IFN-γ mRNA expression in PBMC were comparable when cultured in all three media tested (Fig. 3). After polyclonal stimulation IL-4 mRNA expression and IL-4 protein production were detectable, but not significantly different between the media tested.

After allergen-specific stimulation no IFN-γ mRNA expression was detectable in cells cultured in RPMI in contrast to cells cultured in YM. The strongest upregulation of IL-4 mRNA expression occurred when YM was used in these cultures. After *D. pteronyssinus*-specific stimulation no IL-4 production was detectable, except for a few cases when PBMC were cultured in YM. For IFN-γ production no significant differences were found between the different media tested after allergen-specific stimulation of PBMC (data not shown).

In order to directly compare the media used for cell proliferation and for evaluating the cytokine expression PBMC of one subject were cultured in all three media and studied both for IL-4 and IFN-γ (Table 2). Cells cultured in RPMI resulted in lower baseline proliferative responses as compared to cells cultured in YM. After *D. pteronyssinus*-specific stimulation maximal SI were obtained by culturing PBMC in RPMI. To study cytokine mRNA expression after allergen-specific stimulation, the strongest upregulation of IL-4 mRNA expression (28.2 to 33.8) and IFN-γ mRNA expression (3.6 to 38.4) occurred when YM was used in these cultures. Also from these results we again concluded that optimal proliferative responses were obtained when PBMC were cultured in RPMI, whereas for studying cytokine expression PBMC should be cultured in YM.

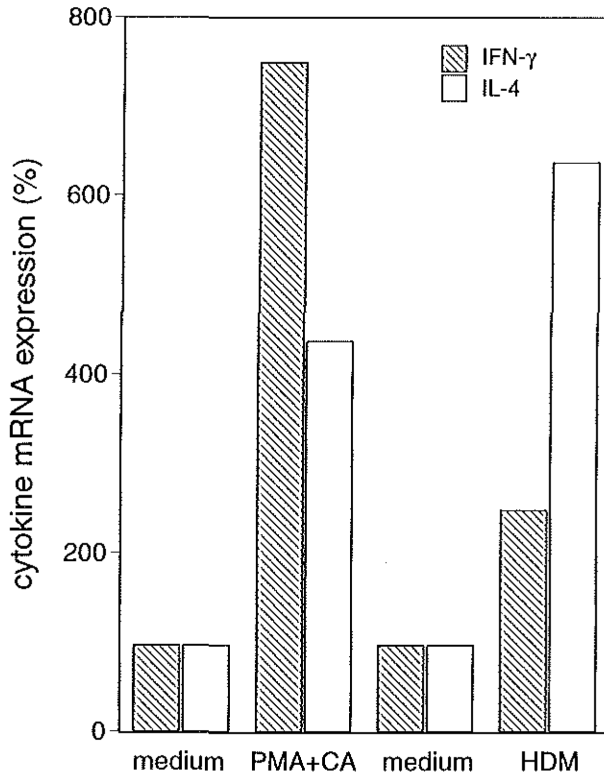

Figure 4. IL-4 and IFN-γ mRNA expression in PBMC cultured in 24-wells plates. PBMC of a *D. pteronyssinus* allergic individual were stimulated for 16-18 hours with PMA + Ca-ionophore or for 4 days with *D. pteronyssinus*-extract (HDM). After stimulation, IL-4 and IFN-γ mRNA expression were determined by RT-PCR analysis. Cytokine expression levels of unstimulated cells were set to 100%. Cytokine expression levels after stimulation were related to the cytokine expression levels of unstimulated cells.

Table 3. Variation of cytokine expression in one patient during a day and between different weeks.

		IL-4 mean ± SD	IFN-γ mean ± SD
Week 1	9.00 am	371.1[1] ± 141.2	709.9 ± 33.6
	2.00 pm	585.9 ± 37.9	648.6 ± 31.6
Week 2	9.00 am	396.3 ± 58.1	640.3 ± 141.9
	2.00 pm	544.1 ± 22.6	746.9 ± 84.7
Week 3	9.00 am	355.0 ± 85.7	858.1 ± 67.2
	2.00 pm	450.8 ± 105.6	805.1 ± 152.0

Results of one representative subject from a series of 3 individuals are shown. The absence of clinical symptoms and the IgE levels remained stable during the tested period of three weeks.
[1] Numbers represent mean scanvalues ± sd after duplicate RT-PCR analysis.

Next, mRNA expression and protein production of IL-4 and IFN-γ were determined in PBMC cultured in different culture plates. Initially, PBMC of a *D. pteronyssinus* allergic subject, cultured in 24-wells plates showed a strong increase of both IL-4 and IFN-γ mRNA expression after stimulation with PMA+Ca-ionophore and after *D. pteronyssinus*-extract stimulation (Fig 4). Subsequently, when more HDM-allergic patients were tested this increase in cytokine mRNA expression could also be found after stimulation in 96-wells plates. No significant differences were found in IL-4 and IFN-γ production after *D. pteronyssinus*-specific or polyclonal stimulation in both types of culture plates.

Furthermore, for determination of the inter-assay variance the reproducibility of cytokine mRNA scanvalues of the T cell clone B21 were compared between 15 different experiments. The inter-assay variance was 24.0% for HPRT mRNA scanvalues, 52.5% for IFN-γ mRNA scanvalues and 39.5% for IL-4 mRNA scanvalues, with a mean coefficient of variance of 38.7%. To determine the variation in cytokine mRNA expression within one individual during the day and between different weeks, blood samples of 3 individuals were taken twice daily at 9.00 am and 2.00 pm, during 3 successive weeks. IL-4 and IFN-γ mRNA expression were determined in duplicate directly after PBMC isolation (Table 3). The variation in cytokine mRNA expression during the day and between weeks was substantial, but consistently smaller than the inter-assay variance.

Taken together, for the analysis of cytokine gene expression and protein production both after polyclonal and allergen-specific stimulation, the largest differences were found using cultures of PBMC in YM in 24-well culture plates.

Influence of blood holding time on cytokine gene expression and protein production

The influence of blood holding time on the subsequent cytokine gene expression and protein production was analyzed in heparinized blood samples

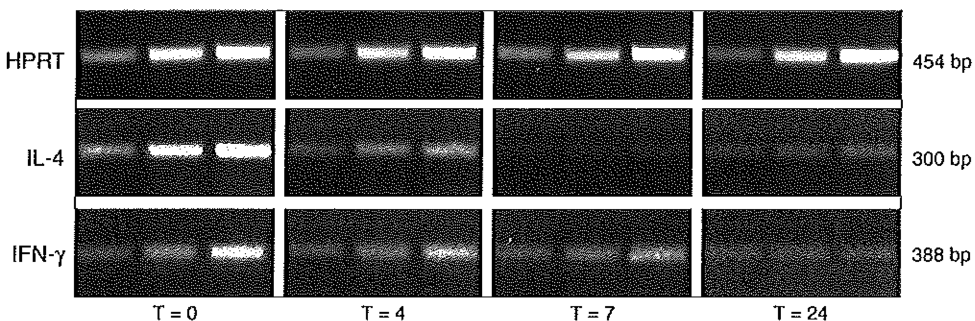

Figure 5. IL-4 and IFN-γ mRNA expression during increasing time-periods. PBMC were isolated after retaining blood for 0, 4, 7 and 24 hours in the heparinized collection tube. In these samples the house-keeping gene HPRT and IL-4 and IFN-γ mRNA levels were determined by RT-PCR analysis.

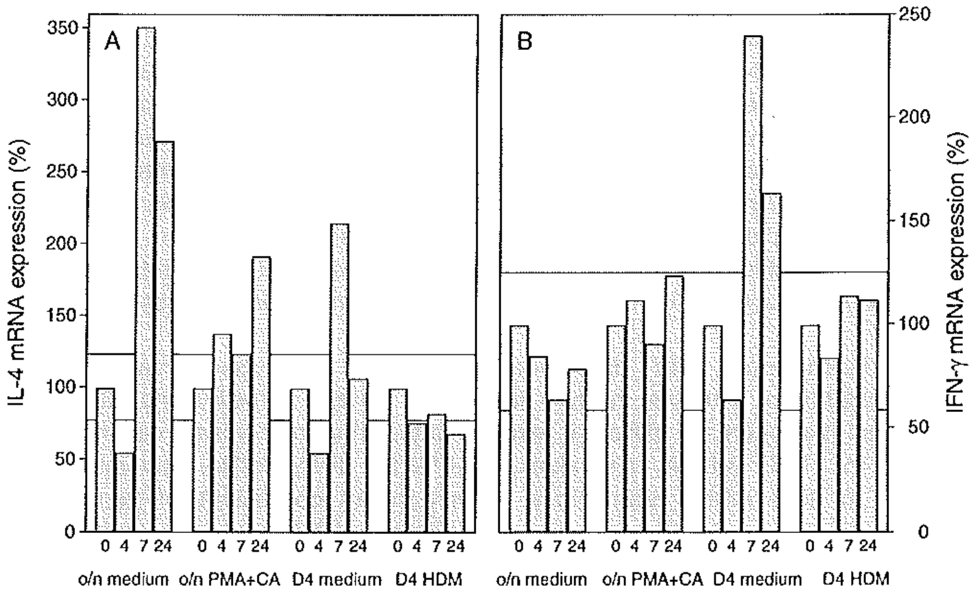

Figure 6. Influence of retaining blood on IL-4 and IFN-γ mRNA expression after stimulation. Blood was retained 0, 4, 7 and 24 hours before isolating PBMC. After stimulation PBMC with PMA + Ca-ionphore or with *D. pteronyssinus*-extract (HDM), IL-4 (a) and IFN-γ (b) mRNA levels were determined by RT-PCR. Data are expressed as percentages relative to scanvalues at T=0 hours (100%).

of 5 subjects. Compared to cytokine expression in PBMC directly after isolation, the mRNA expression of IL-4 and IFN-γ decreased with increasing holding time before further analysis (Fig 5). In all samples IL-4 mRNA expression started to decrease after a storage period of 4 hours after blood collection, while IFN-γ mRNA expression generally started to decrease after 7 hours.

Furthermore, we studied whether induced cytokine mRNA expression levels could return to their initial scanvalues at T=0 hours after a storage period of 4, 7 or 24 hours. In all different culture conditions initial scanvalues at T=0 hours were set to 100%. The cytokine mRNA expression in cultured, non-stimulated PBMC showed a consistent trend of a holding time-dependent increase in cytokine mRNA expression (Fig. 6 A,B). Both after 3 days of PMA+Ca-ionophore and 7 days of D. pteronyssinus-extract stimulation the mRNA expression levels of both IL-4 and IFN-γ returned to the expression levels at T=0 hours (Fig 6. A,B).

Table 4 represents the stimulation index of IL-4 and IFN-γ mRNA expression in stimulated PBMC during the 24 hours observation time period. During prolonged time periods the stimulation index for both stimulation conditions, PMA+Ca-ionophore and *D. pteronyssinus*-extract, fluctuated with a tendency to decrease after a holding time of 4 hours.

After keeping the blood for 4, 7 or 24 hours, the induced cytokine production levels after polyclonal stimulation decreased with longer time periods, both for IL-4 and IFN-γ production (Table 4). After D. pteronyssinus-

Table 4. IL-4 and IFN-γ mRNA expression and protein production during 24 hours.

	IL-4 (%)				IFN-γ (%)			
Hours[1]	PMA + Ca-ionophore[2]		HDM[3]		PMA + Ca-ionophore		HDM	
	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein
0	100[4]	100	100	<16	100	100	100	100
4	251	38	138	<16	132	33	131	130
7	35	36	38	<16	142	19	48	99
24	71	66	64	<16	156	47	68	179

[1] Heparinized blood samples of 5 subjects were kept for 0, 4, 7 or 24 hours at room temperature before isolating the PBMC fraction. Subsequently, cytokine mRNA expression and protein production after stimulation was determined. Results of one representative subject are shown.
[2] PBMC were stimulated for 16-18 hours. Cytokine production was measured in culture supernatants and cytokine mRNA expression was determined by RT-PCR.
[3] PBMC were stimulated for 4 days. Cytokine production was measured in culture supernatant and cytokine mRNA expression was determined by RT-PCR.
[4] Cytokine mRNA scanvalues or protein production levels on T = 0 hour were set to 100%. Other scanvalues and production levels were related to scanvalues and production levels of T = 0 hour.

extract stimulation of PBMC no IL-4 was detectable, while IFN-γ production did not display significant differences (Table 4).

Thus, we conclude that for the optimal analysis of cytokine mRNA expression and protein production, PBMC should be isolated from the blood samples preferably within 4 to 7 hours.

IL-4 and IFN-γ mRNA expression and production in patients

IL-4 and IFN-γ mRNA expression and protein production were determined in PBMC, directly isolated after blood collection of 4 HDM allergic and 3 non-allergic subjects using the optimized culture conditions. After polyclonal and *D. pteronyssinus*-specific stimulation, proliferation assays were performed in 96-wells round bottom plates in RPMI supplemented with 10% HS. Cells for cytokine assays (RT-PCR and ELISA) were cultured in 24-wells plates in YM supplemented with 1% HS.

Proliferative responses to *D. pteronyssinus* were observed only in HDM allergic subjects (mean SI = 5.3 ± 0.75) and not in non-allergic subjects (mean SI = 2.3 ± 0.21). After polyclonal stimulation no significant differences in proliferative responses were found between HDM allergic (mean SI = 96.0 ± 5.8) and non-allergic subjects (mean SI = 94.3 ± 10.9).

After *D. pteronyssinus*-specific stimulation, IL-4 mRNA expression was significantly ($p = 0.03$) increased in HDM allergic subjects compared to non-allergic subjects (Table 5). No significant differences were found in IFN-γ mRNA expression between HDM allergic and non-allergic subjects, both after allergen-specific or polyclonal stimulation (Table 5). After polyclonal stimulation the

Table 5. IL-4 and IFN-γ mRNA expression and protein production after polyclonal and allergen-specific stimulation of PBMC from house dust mite allergic and non-allergic individuals.

		RT-PCR			ELISA		
Cytokine	Stimulation	HDM-all (n = 4)	Non-all (n = 3)	p-value	HDM-all (n = 4)	Non-all (n = 3)	p-value
IFN-γ	PMA + Ca[2]	31.7 ± 15.5[1]	1.0 ± 0.1	0.16	15479.8 ± 10763.8	4460.3 ± 4122.2	0.16
IFN-γ	HDM[3]	70.5 ± 63.9	1.5 ± 0.3	0.12	1965.0 ± 587.7	940 ± 252.9	0.039
IL-4	PMA + Ca[2]	108.8 ± 108.1	1.7 ± 1.6	0.15	182.5 ± 63.5	24.3 ± 21.3	0.0097
IL-4	HDM[3]	7.3 ± 4.0	0.5 ± 0.003	0.034	22.5 ± 6.9	10.0 ± 7.0	0.065

[1] Results are expressed as mean ± sd

[2] IL-4 and IFN-γ were detected after stimulation of PBMC for 16-18 hours with TPA + Ca-ionophore.

[3] IL-4 and IFN-γ were detected after stimulation of PBMC for 4 days with *D. pteronyssinus*-extract.

production of IL-4 of PBMC of HDM allergic subjects was significantly increased (p=0.009) compared to non-allergic subjects, while IFN-γ production was not significantly different between HDM-allergic and non-allergic subjects. Both IFN-γ (p=0.04) and IL-4 (p=0.06) protein were increased after *D. pteronyssinus*-specific stimulation in HDM allergic subjects compared to non-allergic subjects (Table 5).

Taken together, when using optimal culture conditions PBMC from HDM-allergic patients show enhanced proliferation to house dust mite extract associated with increased IL-4 mRNA expression and IL-4 protein production as compared to non-allergic controls.

DISCUSSION

This study shows that culture conditions which allows efficient analysis of proliferative responses are essentially different compared to optimal culture conditions for the proper analysis of cytokine mRNA expression and protein production with respect to medium requirements and type of culture plates. Maximal proliferative responses were obtained when PBMC were cultured in RPMI. To study proliferative responses in polyclonally stimulated PBMC, the type of culture plates used was shown not to be critical. However, higher proliferative responses were observed after allergen-specific stimulation of PBMC in 96-wells round bottom plates. This is most likely due to the dependence on a closer cell-cell contact between antigen-presenting cells and antigen-specific T cells relative to the polyclonal stimulation conditions that are independent of contact with antigen-presenting cells.

Optimal cytokine mRNA expression and protein production resulted after stimulation of PBMC in YM and culturing in 24-wells plates. Analysis of IL-4 protein production has been greatly hampered because the levels following allergen-specific stimulation of PBMC were generally undetectable. In most of the cytokine studies cells were cultured in RPMI [19,21,23, 28] while only rarely [15,20] Yssel's modification of Iscove's medium (YM) was used as culture medium [33]. Our results show that especially for the limited IL-4 mRNA expression and protein production nutrient rich media, like YM are essential. This is all the more necessary when cytokine expression and production is studied after allergen-specific stimulation and therefore, YM is superior to RPMI. Virtually under all conditions tested, PBMC cultured in AIMV resulted in the lowest responses. When culturing polyclonally activated PBMC cultured in AIMV we found that these cells do express IFN-γ mRNA, but do not produce IFN-γ protein. A possible explanation could be that more nutrients provided by the other two media, are necessary to translate mRNA and secrete IFN-γ protein. There are several reasons to explain that after house-dust mite stimulation of PBMC cultured in RPMI no IFN-γ mRNA expression was found, while IFN-γ production was measured. This could be due to different kinetics between cells cultured in RPMI compared to YM. PBMC cultured in RPMI could

start early with IFN-γ synthesis and on day 4 of culture no IFN-γ mRNA remains present. Furthermore, IFN-γ mRNA expression of allergen-specific stimulated PBMC in RPMI could be too low to be detected with our PCR protocol.

Proliferation and cytokine mRNA expression and production by cultured PBMC displayed differential reponsiveness to polyclonal versus allergen-specific stimulation. Different culture requirements were found to be necessary depending on the analysis of these individual stimulation conditions. Only when these optimal culture conditions are used potential functional differences between PBMC of allergic and non-allergic subjects can be detected.

Increasing holding time of heparinized blood before isolating PBMC influenced their proliferative capacity, cytokine mRNA expression and protein production. Generally, after both polyclonal and allergen-specific stimulation, these parameters returned to their initial values. Hence, the blood storage period influenced mostly the cytokine mRNA expression and proliferative capacity of unstimulated cells, possibly due to changes induced in the blood collectión tubes. Most likely, this is due to activation of monocytes by adhesion to the glass wall of the tube or due to endotoxin contaminated heparin. Activated monocytes produce cytokines, like TNF-a, IL-1ß, IL-8 and IL-6 [37,38]. This can lead to a subsequent activation of lymphocytes, resulting in proliferation and cytokine mRNA expression without further stimulation with D. pteronyssinus or PMA + Ca-ionophore. Hence, we consider it crucial to isolate PBMC as soon as possible after collection to enable proper analysis of proliferation and cytokine mRNA expression and protein production potential.

After polyclonal stimulation of PBMC obtained from HDM allergic patients and healthy controls no differences in proliferative responses or SI were observed. However, under these conditions the IL-4 production was increased in HDM allergic patients. We showed before [30]. by studying the intracellular Ki67-antigen expression that PMA + Ca-ionophore stimulation resulted in a preferential proliferation of $CD8^+$ cells. The remaining $CD4^+$ cells could thereby be induced to enhanced cytokine production (e.g. IL-4). This effect, however, is restricted to HDM allergic patients and possibly due to the activated state in which $CD4^+$ cells are present in vivo. Alternatively, comparable to $CD4^+$ lymphocytes, an IL-4 producing subset of $CD8^+$ T cells has been implied [39-41]. These intrinsic differences in the $CD4^+$ and $CD8^+$ subsets in HDM allergic patients only became clear, when PBMC were stimulated under optimal culture conditions and both proliferation and cytokine production were examined.

We showed that after allergen-specific stimulation, 80-100% of the proliferating cells belong to the $CD4^+$ T cell subset [30]. In PBMC of HDM allergic patients and healthy controls we found differences both in proliferative responses and in cytokine production levels after *D. pteronyssinus*-extract stimulation. Proliferative responses to *D. pteronyssinus*-extract were only observed in PBMC of HDM allergic patients. After allergen-specific stimulation of PBMC of HDM allergic patients, both IL-4 and IFN-γ were increased, which is in agreement with the findings of Gauchat et al. [26]. Our findings do not

sustain the widespread belief of selective activation of T_h2-like cells in atopic disease, but suggest activation of both T_h1 and T_h2-like cells besides potentially IFN-γ producing $CD8^+$ T cells. IFN-γ may contribute to the allergic inflammation, by activating macrophages and epithelial and endothelial cells [42,43]. During severe acute exacerbations of allergic asthma, increased serum concentrations of IFN-γ occur, which decrease as the patients improve clinically [44].

In conclusion, we have shown that different culture plates, different culture media and the holding period of blood samples all influenced the cytokine mRNA expression and protein production levels and also the proliferative capacity. To detect the often subtle differences between allergic and non-allergic subjects, it is therefore important to stimulate the cells under optimal culture conditions. Furthermore, analysis of possible functional differences in PBMC between allergic and non-allergic patients, necessitates the stimulation of PBMC both under polyclonal and allergen-specific conditions.

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T CELL RESPONSES DURING THE DEVELOPMENT OF ATOPIC DISEASE

CHAPTER 4

MARKERS FOR EARLY SENSITISATION AND INFLAMMATION IN RELATION TO CLINICAL MANIFESTATIONS OF ATOPIC DISEASE UP TO 2 YEARS OF AGE IN 133 HIGH-RISK CHILDREN

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SUMMARY

Background The combination of genetic susceptibility and environmental factors induce allergic sensitisation and subsequently local inflammation, resulting in atopic manifestations.
Objective To examine whether markers of sensitisation (total and specific IgE levels, allergen-induced proliferative responses and skin tests) and markers of inflammation (plasma sE-selectin and blood eosinophils) are related to the clinical expression of atopy and whether they precede atopic disease in newborns.
Methods The development of these markers during the first 2 years of life was studied prospectively in 133 newborns at high risk to develop atopy.
Results The prevalence of atopy increased from 25% at 12 months to 32% at 24 months of age. The children with food allergy at 12 months, who all had atopic dermatitis (AD), turned out to have asthma-like disease in 40% and AD in 100% at the age of 24 months. Total IgE levels increased with time and from 12 months onward levels started to differ between atopics and non-atopics. Food-specific IgE antibodies were significantly associated with AD (relative risk (RR) = 2.39), food (RR = 1.32) and upper-airway allergy (RR = 1.20), and HDM-specific IgE antibodies with upper-airway allergy (RR = 5.00). A positive skin test was significantly associated with AD (RR = 2.90) and food allergy (RR = 1.36). The inflammation markers investigated, were not related to the clinical expression or preceded atopy at 2 years of age in high-risk children. Parental smoking was a significant, independent risk factor (RR = 2.23) for the development of atopy at 12, but not at 24 months of age.
Conclusion Positive skin tests and specific IgE to food or inhalant allergens were related to the clinical expression of different atopic diseases. The combination of AD and food allergy at 12 months reflected the strongest risk factor in our cohort for the development of asthma-like disease at 24 months of age.

INTRODUCTION

The prevalence of atopic disease is increasing, particularly among children [1]. This creates a necessity to detect children at risk to develop atopy and to initiate preventive measures. During the first year of life, atopic dermatitis (AD) is the most common atopic disease. Sixty percent of the children who will develop AD already have symptoms during the first year of life and 85% during the first 5 years [2]. Approximately 20% of children with AD have allergic reactions to food constituents, mostly cow's milk, egg and peanut [3,4]. The natural course of atopic disease gradually evolves from AD with or without food allergy in the first years of life, to asthma and rhinitis in

the years thereafter. Similarly, in infancy allergic sensitisation occurs predominantly to foods, but in later childhood allergic sensitisation to inhalant allergens, such as house-dust mite (HDM) and pollen, is more frequent [5,6].

The development of atopy is the result of complex interactions between genetic susceptibility and environmental factors. These interactions can induce allergic sensitisation and subsequently lead to local inflammation resulting in clinical symptoms of atopy. The genetic predisposition for the development of atopy in children increases from 10% in children without atopic parents to 30% in the case of one atopic parent. The risk to develop atopy is further modified by several environmental factors, of which passive smoking is the most consistently documented risk factor [7,8].

Early diagnosis of children at high risk for the development of atopic disease will allow measures to be taken to prevent or diminish the clinical expression of atopic diseases. Therefore, several *in vivo* and *in vitro* tests have to be evaluated for detection of early sensitisation and for their role in the early diagnosis of childhood allergy. IgE is highly associated with atopic disease, but only a minority of children who develop atopy are born with increased total IgE levels [9,10]. However, in 80-85% of children who had developed AD the serum total IgE levels were elevated [11,12], and 20% of these children had positive immediate skin tests and serum allergen-specific IgE antibodies to various food or inhalant allergens [3].

Several procedures can be used to test for IgE-mediated food sensitisation or allergy. Skin tests and RAST are widely applied, while oral challenge is still the gold standard test for the diagnosis of food allergy [13-16]. The practical usefulness of skin tests and RAST for the diagnosis of food sensitisation or allergy is still controversial [17-20]. On the other hand, the presence of IgE antibodies specific to hen's egg protein during the first year of life is proposed to precede the development of atopic disease during childhood [21,22].

Increased proliferative responses of peripheral blood mononuclear cells (PBMC) to food allergens were found in children who subsequently developed food-sensitised AD [23,24]. CD4+ T lymphocytes were found to be the predominant responding cells in the PBMC fraction [25]. Moreover, increased proliferative responses were considered to be a more accurate predictor for atopic disease than elevated cord blood IgE levels [23].

The RAST, proliferative responses of T lymphocytes and skin tests are all tests to detect sensitisation to allergens at plasma, cellular and skin level, respectively. Allergic sensitisation could result in inflammation of the target organs and subsequently in clinical expression of atopic disease. We and others have previously described soluble E-selectin (sE-selectin) as a marker of skin inflammation related to the clinical severity of the AD [26,27]. In addition, the degree of the eosinophilia has been shown to correlate with the severity of inflammation and the degree of airway hyperresponsiveness [28].

In this longitudinal study we investigated whether the development of the above mentioned markers is related to the clinical expression of atopy

and whether they precede the atopic manifestation at 24 months of age. To select children with high genetic predisposition for atopy, we included 133 newborns having an atopic mother. We investigated the time course of total- and specific-IgE levels, allergen-induced proliferative responses, skin tests, sE-selectin levels in plasma and blood eosinophil counts in relation to the development of atopic disease up to 2 years of age. Furthermore, in these children parental smoking habits as a factor for the environmental influence, was studied.

MATERIALS AND METHODS

Study population

High-risk children were followed from birth until 24 months of age, in order to monitor the development of atopic disease, such as AD, asthma-like disease, food or upper-airway allergy. All mothers of the newborns were atopic, as established by clinical history and a positive skin test and/or specific IgE to common inhalant and/or food allergens. The Medical Ethical Committees of the participating hospitals approved this study. For the collection of cord blood samples several hospitals participated, while the other visits of the children were all in the Sophia Children's Hospital Rotterdam. Informed consent was obtained from the parents of all children prior to their participation in the study. In total 133 newborns were included. For various reasons, 23 children dropped out the study before one year of age, and 7 children between one and two years of age. The main reasons for drop out were difficulties to come to the hospital and non-acceptance by the parents of repeated venapunctures of their child.

Physical examinations were performed, questionnaires concerning environmental factors and histories of symptoms of atopy were completed and venous blood samples were obtained at ages 0, 6, 12, 18, 24 months. The clinical evaluation during the different visits was made without knowledge of the immunological or skin test results.

Definition of atopy

AD was based on Sampson's criteria: erythema, edema, oozing and excoriation with evidence of itchiness lasting longer than four weeks [2]. The severity of the eczema was measured by the objective SCORAD [29].

Asthma is difficult to define in young children. Therefore, we introduce the term asthma-like disease to classify the complaints that indicate asthmatic symptoms before 2 years of age. This group of asthmatic children include both children with ongoing and temporary respiratory problems after the age of 2 years. Based on criteria of the American Thoracic Society [30] *asthma-like disease* is diagnosed if children either had at least three separate wheezing episodes each lasting three days or more, or had more than two separate

episodes of paroxysmal nocturnal cough with sleep disturbance for three consecutive nights, or required anti-asthma therapy during the last 6 months.

Food allergy was defined as clinical manifestation of erythematous, papular, macrovesicular skin eruptions, diarrhea, vomiting, or respiratory problems in relation to food ingestion. For the definitive diagnosis a positive skin test or specific IgE was necessary. All children suspected for food allergy were asked for an open oral provocation test to confirm the diagnosis.

Upper-airway allergy was defined as seasonal rhinoconjunctivitis, symptoms of sneezing, redness, itching and tearing of the nose or eyes and/or respiratory complaints. Diagnosis was confirmed by the presence of specific IgE in the plasma.

Sensitisation to food or inhalant allergens was defined as a positive skin test or specific IgE without clinical manifestation.

The term *atopy* was used for high-risk children with clinical manifestations of allergy, such as AD, asthma-like disease, food or upper-airway allergy.

Total and specific IgE and eosinophil count

The total IgE concentrations in all cord blood samples were determined with a modified radio-immuno assay to result in a lower detection limit of 0.2 kU/L. At the other time points, in all plasma samples of the children total IgE was measured using the Pharmacia CAP RAST (Kabi Pharmacia, Uppsala, Sweden). Analysis of specific IgE antibodies directed towards the most common inhalant allergens (HDM, grass and birch pollen, cat, dog and moulds) and food allergens (peanut, chicken egg white (EW), cow's milk (CM), codfish, wheat and soy) were performed using the Pharmacia CAP system Phadiatop® RAST and the Pharmacia CAP RAST (Kabi Pharmacia), respectively. Specific IgE was measured in all plasma samples of the children, whether or not having symptoms after food ingestion or after exposure to inhalant allergens at 12 (91 samples) and 24 (88 samples) months of age. Additionally, determination of specific IgE in plasma samples obtained from children of 6 (38 samples) and 18 (32 samples) months of age was considered to be relevant in those children with symptoms of atopic disease, or specific IgE or a positive skin test before 24 months of age.

The percentages of eosinophils were counted in peripheral blood by leucocyte differentiation.

Proliferative responses

From birth up to 24 months of age heparinized blood samples were obtained every 6 months. After diluting 1:1 with PBS, PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia). Lymphocyte proliferation assays were performed as described previously [31]. Briefly, PBMC were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate and 10% heat-

inactivated human serum in 96-wells round bottom microtiter culture plates. PBMC were cultured in quadruplicate at $2x10^5$ cells/well in the presence of D. pteronyssinus extract (HDM, 50 μg/ml, ALK-Benelux, Groningen, The Netherlands) or EW extract (50 μg/ml, ALK) or CM extract (25 μg/ml, ALK) or without additions in a total volume of 200 μl. After 7 days of allergen-specific stimulation, proliferation was measured using $[^3H]$-thymidine incorporation. The results are expressed as stimulation index (SI), equal to the ratio of mean cpm in allergen-specific stimulated cultures over mean cpm in unstimulated cultures. A SI greater than 3 is generally considered a positive proliferative response.

Determination of sE-selectin

After Ficoll centrifugation, plasma samples were obtained and stored at −70°C until analysis. sE-selectin was measured at the different time points in plasma by ELISA as described previously [32].

Skin tests and oral provocation test

Between 9 and 12 months of age two different skin tests, namely the skin application food test (SAFT) and the prick-prick test [19,33], were performed for cow's milk, egg and peanut in 97 children, independent of a history of AD. Only in selected cases other foods were tested in children, on account of a history of complaints to these foods. Children were asked for an oral provocation test, when the skin test was positive.

The SAFT is a reliable and child-friendly skin test, validated for AD in children, more specifically for evaluating (suspected) food allergy in children younger than 4 years [19]. At the Paediatric Dermatology Unit of the Sophia Children's Hospital, the SAFT is usually performed in AD children younger than 3 years to evaluate food allergy. In this cohort, both skin tests were performed in all children, independent of a history of AD. In the SAFT, the food was applied in the same condition as usually consumed on the back of the child, using large Finn chambers. The test was read after 10, 20, and 30 minutes. The results were scored as follows: 0 = no reaction; 1+ = erythema; 2+ = erythema and edema within the area of the Finn chamber; 3+ = eythema and edema up to the outer area of the Finn chamber. Reactions with a score of 2+ or 3+ were regarded as positive.

A microlancet with a 1-mm needle with shoulders (ALK, Benelux) was used in the prick-prick test. The needle was pricked in the food and subsequently vertically into the lower arm. A drop of histamine dihydrochloride solution (10 mg/ml, ALK, Benelux) was used as positive control and a drop of saline as negative control. The test was read after 15 and 30 minutes. The reaction to the food was evaluated by comparing it with the reaction to histamine. The reaction to food was considered positive if the average wheal diameter was at least 3 mm larger than the negative control.

The oral provocation test was performed in a standardised way at the

day-care unit, under appropriate safety conditions. After examination and registration of the blood pressure and pulse rate, a small amount of the food was given to the child. All observed reactions were noted. Positive reactions consisted of an urticarial rash, perioral redness, flare-up of eczema, itching, increased pulse rate, depression of blood pressure, and respiratory or gastro-intestinal complaints. If no reactions were observed after 1 hour, a double amount of the food was given; after 2 hours, the amount was doubled again. After 3 hours, the food was given without restrictions. The total amount given correlated to age-related average 'daily intake'. If a positive reaction was observed, the test was stopped. If no reaction was observed, the child was dismissed after 8 hours. The parents were urged to contact the dermatologist if any late reaction was observed, in order to evaluate this reaction.

Statistics

Statistical analyses were performed using SSPS for Windows, version 8.0. Parameters were evaluated by computing the relative risk factor (RR) with Cornfield's 95% confidence interval (CI) as a measure of association or parameters were tested using an independent sample t-test. P values less than 0.05 were considered significant.

RESULTS

Prevalence of atopic disease

In this group of high-risk newborns, the prevalence of atopy was 16% (18/110 children) at 6 months of age, 25% at 12 months of age and increased gradually to 32% (33/103 children) at 24 months of age (Figure 1). The prevalence of AD increased markedly after birth to 21% at 12 months and 25% at 24 months. The prevalence of asthma-like disease started to increase mainly after the first year of life from 4% at 12 months to 12% at 24 months of age. Five children (5%) had a food allergy at 6 months of age, all had also AD. The prevalence of food allergy did not show marked changes during the first 24 months of life, while the prevalence of upper-airway allergy in the children started to develop after 12 months of life to 4% at 24 months of age.

In the subgroup of 23 children who had AD at 12 months of age, at 24 months 3 children had developed asthma-like disease (13%) and 1 child had upper-airway allergy (4%). Out off the 13 children who had developed asthma-like disease at 24 months, at 12 months 3 of them had AD (23%), 3 had asthma-like disease (23%) while the other 7 children (54%) had no detectable atopic symptoms. Of the 5 children with a food allergy and AD at 12 months, at 24 months 2 children had asthma-like disease (40%) and all 5 children still had AD (100%).

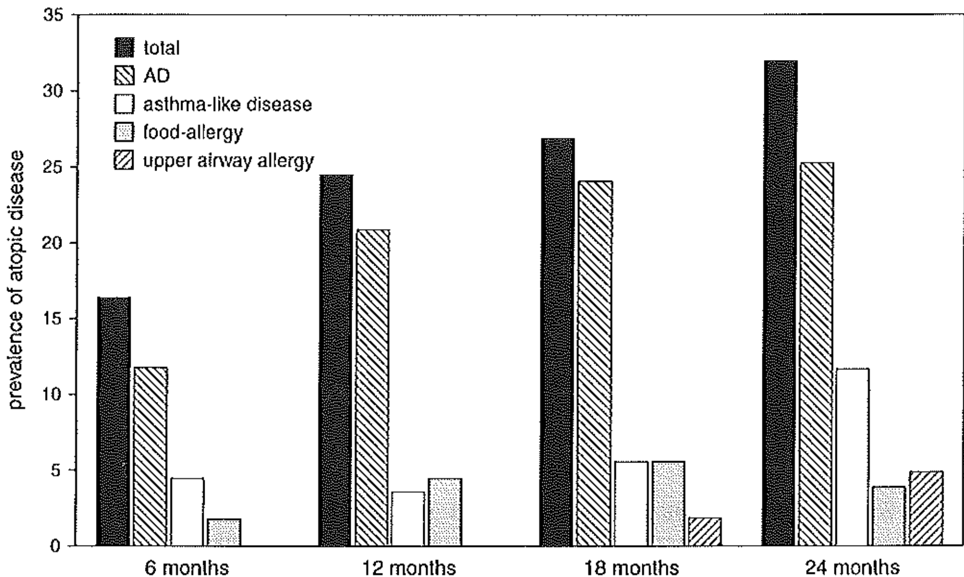

Figure 1. Prevalence of atopic disease at the consecutive time points tested. Children were assessed for atopic symptoms at 6, 12, 18 and 24 months of age. Results were expressed as the total percentage atopic children at the individual time points or the percentage of the children with atopic dermatitis, asthma-like disease, food-allergy and upper-airway-allergy.

Smoking habits were admitted by 34 parents (31%) during the first year of life of the children. In 4 cases the mother, in 14 cases the father and in 16 cases both parents smoked. Smoking by either one of the parents was significantly (RR=2.23, 95% CI=1.20-4.14) associated with the development of atopy at 12 months in the children. A positive, but not significant (RR=1.56, 95% CI=0.91-2.71), association was also observed between smoking of the parents and atopy development at 24 months in the children. No significant associations were found between smoking by the parents and plasma total IgE levels, presence of specific IgE, positive skin test, sE-selectin levels or blood eosinophil count in the children.

Time course of total IgE levels

Figure 2 shows the development of mean total IgE levels between birth and 24 months of age in children who had and had not developed atopy before 24 months of age. The mean total IgE levels measured at the consecutive time points increased gradually over time, whereas this increase was more prominent in children with atopic features at 24 months. At all time points tested, the difference in total IgE levels between atopic and non-atopic children just failed to reach significance.

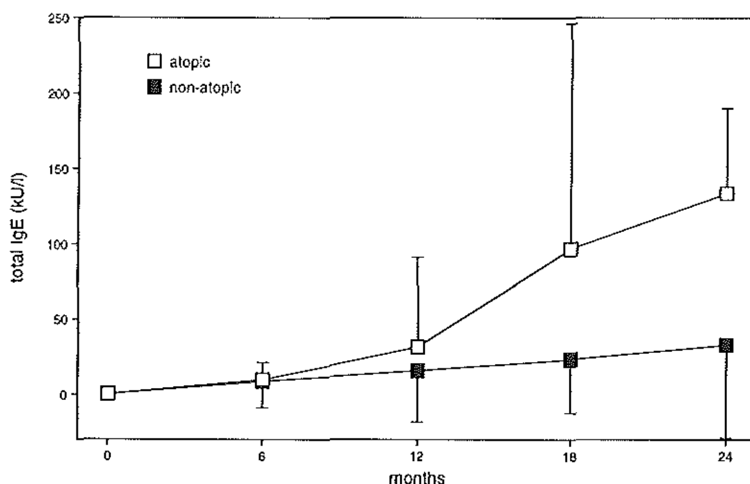

Figure 2. The time course of total plasma IgE levels between birth and 24 months of age in children who had or not had developed atopy at 24 months of age. Results were expressed as mean IgE in kU/l with standard deviation. The number of atopic vs. non-atopic children at the different time points was at birth 29 vs. 54, at 6 months 31 vs. 65, at 12 months 27 vs.62, at 18 months 24 vs. 54 and at 24 months 28 vs. 58, respectively. At 12 months $p=0.07$, at 18 months $p=0.09$ and at 24 months $p=0.06$.

Time course of specific IgE

Specific IgE in plasma of children at 12 months of age was only found against food allergens, while in children at 24 months of age specific IgE against both food and inhalant allergens was observed (Table 1B). Three out of 12 children (25%) with food- specific IgE had allergy to the corresponding food at the age the specific-IgE was found (Table 1A). Out of the 5 children with HDM-specific IgE, four (80%) had an upper-airway allergy. Food and upper-airway allergy was significantly more frequently observed in children with specific IgE to food or inhalant allergens respectively, compared to children without specific IgE (Table 1A).

Food-specific IgE in children before 24 months of age was significantly associated with atopy (RR=2.86), AD (RR=2.39), food allergy (RR=1.32) and upper-airway allergy (RR=1.20) at 24 months of age. Inhalant-specific IgE in children was significantly associated with upper-airway allergy (RR=5.00) at 24 months of age. We could not assess in our cohort that specific IgE measured before 24 months of age preceded the development of atopic disease at 24 months.

Time course of proliferative responses

At the consecutive time points, *in vitro* proliferative responses of PBMC to cow's milk, egg-white (Figure 3) and HDM were determined.

Table 1. Age-related plasma levels of allergen-specific IgE.

A. Overall results

Specific IgE to	Number of children	Food or[†] upper-airway allergy	Atopy at 24 months[‡] AD	FA	A	UA
Food allergens[§]	12	3/12*	8*	3	3	2
Inhalant allergens	5	4/5*	3	1	2	4
None[¶]	84	2/84	17	1	8	0

B. Individual results

Child Study number	Allergic[#] sens. to	Specific-IgE (kU/l) 6 months	12 months	18 months	24 months	clinical** symptoms
28	peanut	0.36	ND	-	-	no
101	peanut	1.1	-	-	-	no
61	EW	-	0.58	-	-	no
70	EW	0.56	0.37	0.55	-	yes
91	EW	1.08	0.40	-	ND	no
126	EW	-	-	0.78	0.83	yes
128	EW	2.31	0.47	0.43	-	no
109	EW	0.36	3.39	4.66	16.6	yes
	wheat	0.42	2.81	2.75	8.96	?
	peanut	-	-	0.69	1.54	?
	HDM	-	-	22.7	93.3	yes
	Soy	-	-	-	0.88	?
39	HDM	-	-	-	4.9	yes
119	HDM	-	-	-	1.04	yes
40	HDM	-	-	-	15.16	no
	CM	-	1.19	ND	3.38	no
57	HDM	-	-	1.7	25.8	yes
	CM	-	-	0.45	1.42	no
113	CM	-	0.79	0.54	-	no
63	CM	-	-	0.46	1.72	no

AD = atopic dermatitis, A = asthma-like disease, FA = food allergy, UA = upper-airway allergy, CM = cow's milk, EW = egg-white, HDM = house-dust mite; - = <0.35kU/l; ND = RAST not done; ? = not clear. *$P<0.05$ relative to children without specific IgE. [†] Number of children with a food or upper-airway allergy corresponding with the specificity of IgE. [‡] Number of children with atopic disease at 24 months of age. [§] Number of children with specific IgE (to food or inhalant allergens) at any of the tested time points. Specific-IgE was measured using the RAST. [¶]At all tested time points no specific-IgE was detectable in these children. [#] Inhalant and food allergens to which IgE was directed. ** Clinical symptoms to the food or inhalant allergen corresponding with the specificity of IgE.

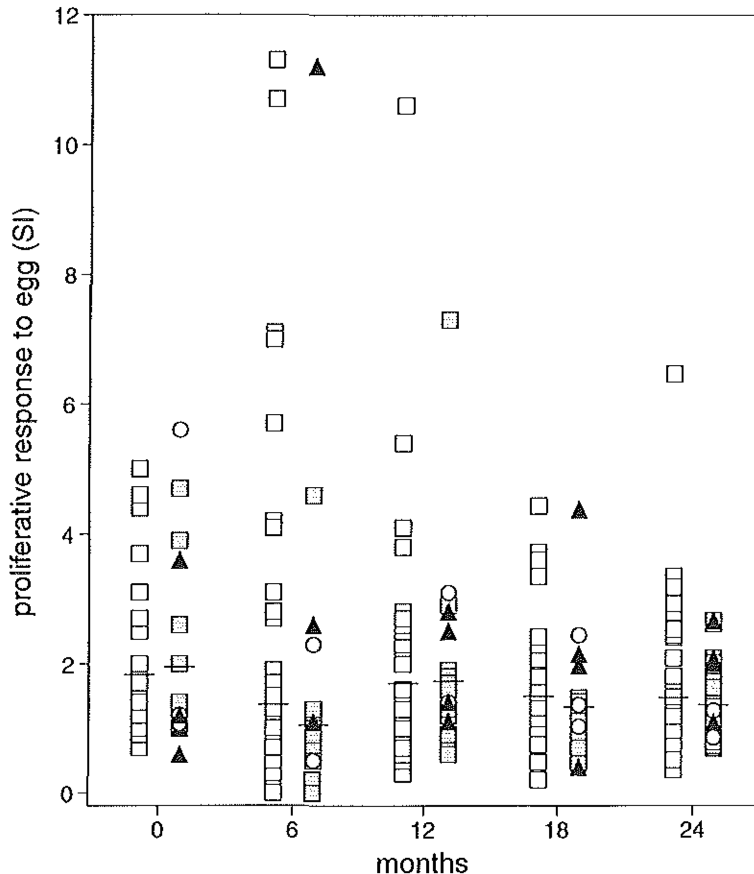

Figure 3. Proliferative responses of PBMC of children who had developed atopy (hatched square) and non-atopic children (open square). Proliferation of PBMC at the different time points was determined by measuring [^{3}H]-thymidine incorporation on day 7 of culture after stimulation with 50 µg/ml egg-white extract. Results are expressed as SI and means of the groups are indicated. Each point represents the mean of a quadriplate from an individual PBMC sample. Atopic children were subdivided in children who had developed clinical symptoms to egg (open circles), children with specific-IgE against egg-white (closed triangle) and children without clinical symptoms or specific IgE to egg-white (hatched square).

Proliferative responses were not significantly different for the various allergens at any time point tested between children who had or had not developed atopy. Atopic children who had developed a food or upper-airway allergy or sensitisation at any of the different time points, did not differ in their proliferative responses from the other atopic children without a food or upper-airway allergy or sensitisation (children with clinical symptoms to egg or with specific IgE to egg-white are marked in figure 3). Relationships

between allergen-specific proliferative responses and the development of atopic disease at 24 months of age were not observed.

Skin tests and oral provocation test

In 11 out of 97 children tested (11%), a positive skin test for food was observed (Table 2A). The children with a positive skin test had significantly more AD at 12 months (8/11) compared to skin test negative children (15/86). Also significantly more children with a positive skin test had food allergy (4/11) compared to children with a negative skin test (1/86). A positive skin test at 12 months was significantly associated with atopy (RR = 2.86), AD (RR = 2.90) and food allergy (RR = 1.36) at 24 months.

In 5% of the children a positive skin test for egg-white or cow's milk was observed. Out of the 14 positive skin test children, only 5 (36%) were related to the diagnosis of food allergy (Table 2B). This discrepancy between a positive skin test and a food allergy was mainly due to false positive peanut prick-prick tests. Eight positive prick-prick tests for peanut were observed, while no symptoms to this food product were mentioned in these children.

Table 2. Skin test results and atopic symptoms at 12 and 24 months of age.

A. Overall results

Skin test[†]	Number of children	Food allergy[‡]	Atopy at 12 months[§]				Atopy at 24 months[§]			
			AD	FA	A	UA	AD	FA	A	UA
Positive	11/97	4/11	8*	4*	0	0	8*	3	2	0
Negative	86/97	1/86	15	1	4	0	18	1	10	4

B. Individual results

Skin test[†]	Positive test[¶]		Food allergy[‡]
Pr-Pr	EW	3	3/3
	Peanut	8	0/8
SAFT	CM	1	1/1
Pr-Pr plus SAFT	EW	1	0/1
	Banana	1	1/1

AD = atopic dermatitis, A = asthma-like disease, FA = food allergy, UA = upper-airway allergy; Pr-Pr = prick-prick test, SAFT = skin application food test, CM = cow's milk, EW = egg-white. *$P<0.05$. [†] Skin tests for cow's milk, egg and peanut were performed at the age between 9 and 12 months in all children, independent of having a history of AD. (A) represents the total number of children with a positive and negative skin test. (B) represents the number of positive Pr-Pr and/or SAFT tests. [‡] Children with a food allergy as defined in Materials and Methods section, for the corresponding food as positive in the skin test. [§] Number of children with atopic disease at 12 or 24 months of age. [¶] Number of food positive skin tests. Two children had more than one positive test: one had a positive prick-prick tests for egg and peanut and another had a positive prick-prick tests for egg, peanut and banana in combination with a positive SAFT for banana.

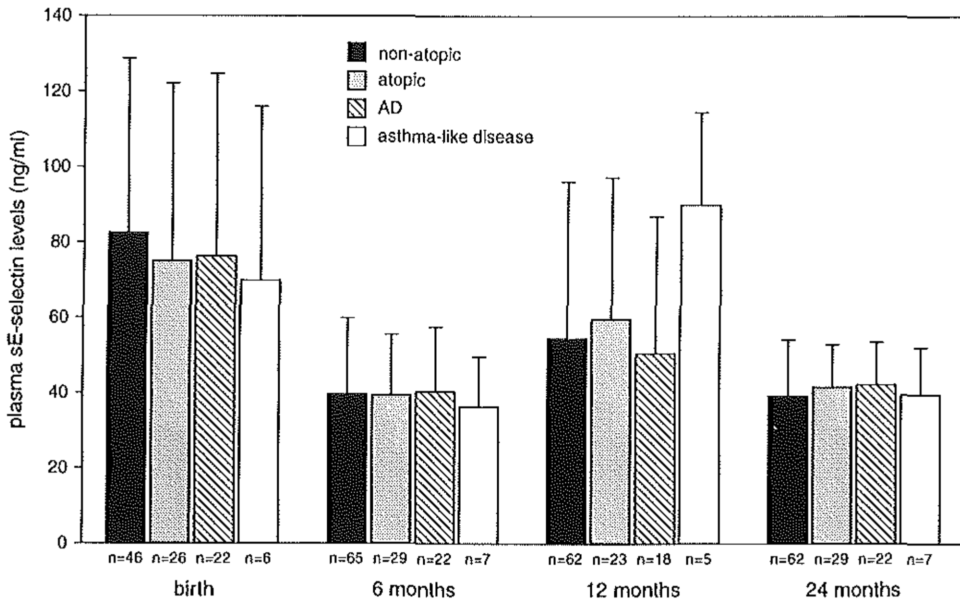

Figure 4. sE-selectin levels in plasma at the consecutive time points. Results are expressed as mean sE-selectin levels in ng/ml with SD measured in plasma of non-atopic children, children with atopy, atopic dermatitis and asthma-like disease as defined at 24 months of age. No significant differences exist between the patient groups and the different time points.

Oral provocation tests were performed in 7 children to evaluate the clinical impact of the skin tests. These provocation tests were negative in all cases related to peanut, while in 1 child with a history of symptoms to egg, peanut and banana, the egg provocation test was positive. Provocation to banana was not performed in this child, because of a strong flare-up of the eczema during the skin test with banana.

Time course of plasma sE-selectin levels and blood eosinophil count

At the consecutive time points, sE-selectin levels were not significantly different between children diagnosed as non-atopic, atopic, AD or asthma-like disease (Figure 4). In all patient groups the sE-selectin levels were not significantly higher at birth than at later time points. Between the sE-selectin levels and the development of atopic disease at 24 months of age no associations were observed.

The percentage eosinophils in peripheral blood did not increase with consecutive time points and no significant differences were found between the different patient groups at the consecutive time points (Figure 5).

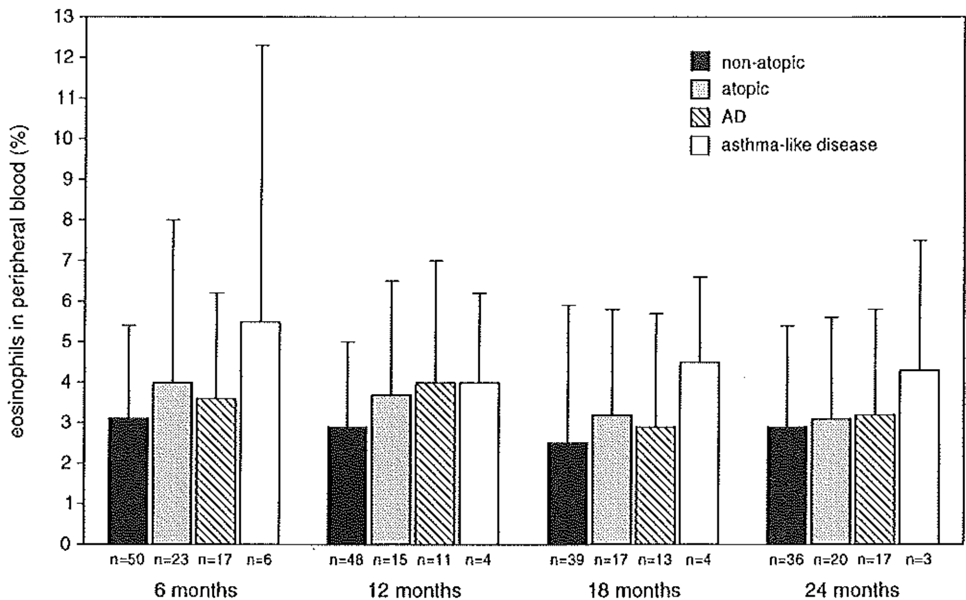

Figure 5. Percentage of eosinophils in peripheral blood at the consecutive time points. Results are expressed as mean percentage eosinophils with SD measured by leucocyte differentiation of blood samples of non-atopic children, children with atopy, atopic dermatitis and asthma-like disease as defined at 24 months of age. No significant differences exist between the patient groups and the different time points.

DISCUSSION

Detection of children at risk for atopy is important for initiating preventive measures. In 133 newborns at high risk for the development of atopy, we studied the development of several markers for allergic sensitisation and inflammation at consecutive time points up to two years of age. An important difference in the conduct between this and most other studies is that we did not select patient groups retrospectively. This study underscored the relevance of sensitization, but not of inflammatory markers in relation to the clincial expression of atopy and whether they preceded atopic manifestations up to 24 months of age.

The prevalence of atopic manifestations (AD, asthma-like disease, food and upper-airway allergy) rose from 16% at 6 months to 25% and 12 months and to 32% at 24 months, which is in agreement with the expected genetic predisposition of 30% in the case of an atopic mother [34]. The prevalence for AD at one (21%) and two years (25%) of age is in agreement with other studies [35,36]. Most children, who had AD at 24 months of age, had their initial symptoms within their first 12 months. The prevalence for asthma-like disease in our cohort at one (4%) and two (12%) years was

lower than that in the 'Isle of Man study' being 19% and 27%, respectively [35]. This could be due to differences in the selection criteria for high-risk children or the severity of the AD. In our cohort, the severity of AD, considered as a risk factor for the development of asthma [37], was mostly classified as mild (objective SCORAD <15).

AD within the first months of life, mostly the initial expression of atopy, is considered to be a risk factor for the development of asthma at later ages [37]. In our study, the percentage of children with asthma or upper-airway allergy at two years was not higher in the group of children who had AD at one year compared to the whole cohort. Also Sporik et al. could not confirm AD as a risk factor for asthma [38]. The sample size of children who had a food allergy was too small for definite recommendations, but our results suggest that the combination of AD and food allergy at 12 months of age turned out to be a better risk factor for asthma-like disease at 24 months. Out of the 5 children with AD and food allergy, 2 developed asthma-like symptoms at 24 months of age.

Several studies have demonstrated that early sensitisation to food and inhalant allergens must be regarded as a risk factor for the development of atopic disease [22,25,37]. Allergic sensitisation is frequently asymptomatic, but sensitisation is generally considered the first step in developing atopic disease [6,39]. Allergic sensitisation was studied at the plasma (total and specific IgE), cellular (proliferative responses of T lymphocytes) and skin (skin tests) level. Plasma sE-selectin levels and eosinophil counts were both evaluated as a reflection of the intensity of inflammation in the target organs [26-28].

Between 12 and 24 months the IgE levels in atopic children increased markedly and in the non-atopic children gradually, resulting in an increasing difference between the total IgE levels of atopic and non-atopic children.

The clinical heterogeneity in both groups of children, atopic and non-atopic, may influence the impact of these total IgE levels as well as of other markers. The non-atopic group of children can be divided in children who never developed atopy and children who were not yet atopic, but will develop an atopy after 2 years of age. The heterogeneity in the atopic group of children is related to the observation that before the age of 2 years it is difficult to distinguish between temporary and persistent atopic symptoms, especially for asthmatic features [40]. Most of the children who wheezed before their second birthday did not persist in wheezing [38]. This implies that in the wheezing group a part of the children will appear to be free of such symptoms and mostly non-atopic some years later. Clinical implications of total IgE levels and the other markers as risk factors for the development of atopy can become more obvious after longer follow-up.

In agreement with the MAS-90 study, specific IgE antibodies in children before the age of 2 years were predominantly observed against food allergens [41]. In the group of children with food-specific IgE antibodies significantly more children with a food allergy were observed compared to

children without such antibodies. Out of the children with food-specific IgE antibodies, however, only 25% had a food allergy. This percentage is in agreement with previous studies, showing that only 30% to 40% of patients with food-specific IgE experienced clinical symptoms after ingestion of the food-at-risk [12,18]. The determination of specific IgE at the different time points revealed that there was a significant association between the presence of food-specific IgE in children before the age of 2 years and the clinical expression of AD, food and upper-airway allergy at 2 years. HDM-specific IgE antibodies measured at 24 months of age was in 80% of these children related to the presence of an upper-airway allergy at this age.

We could not assess that specific IgE preceded the clinical expressions of atopy in our cohort, as most children with atopy at 24 months had that atopic manifestation already before 24 months. This is in contrast with the findings of Lilja et al. [18] showing that three out of four children with detectable specific IgE to food at 6 months of age, developed clinical symptoms of atopic disease before 18 months of age. These authors concluded that although the presence of circulating IgE antibodies as detected by RAST precede the development of atopic disease during childhood, the usefulness of the test is limited by its low sensitivity, as also confirmed by our data for food-specific IgE (We found a sensitivity of 18% for food-specific IgE before 12 months and atopy at 24 months).

In 5% of the children a positive skin test was observed for egg or milk, as was also found by others [8,35]. Despite the frequently false positive prick-prick test for peanut, a food allergy was significantly more often observed in children with a positive skin test (2/3 SAFT and 4/13 prick-prick tests) compared to a negative skin test. Moreover, a positive skin test was significantly more frequent observed in children with AD. Even without excluding the possibility of a false positive prick-prick test for peanut, the skin test was related to the clinical expression of AD and food allergy. A positive skin test at 12 months was significantly associated with AD and food allergy at 24 months in these children. As also described for specific IgE, we could not detect that a positive skin test preceded the clinical expressions of atopy. Skin sensitivity and IgE antibodies to egg in the first year of life have been reported to be associated with upper-airway allergy at 7 years of age [8,21]. This predictive effect over prolonged periods is important and underscores the further follow up of our cohort.

We found that determination of the markers related to inflammation, plasma sE-selectin levels and blood eosinophil counts, were not related to or would preceede the clinical expression of atopy in children before two years of age. This could imply that the development of the inflammatory process has not (yet) resulted in the presence of these markers in the circulation. This may be a consequence of level of symptoms, since the determinations were performed in children without exacerbations of their atopic disease at the moment of examination.

The influence of smoking habits of the parents on the development of atopy in their high-risk children was studied as an example for the environmental influence. Passive smoking, which may potentially influence sensitisation, is the most consistently documented environmental risk factor for the development of atopic disease [7,8]. In our study smoking habits of the parents were also found to be a risk factor for atopy at 12 months of age, but not at 24 months of age. This suggests that the influence of passive smoking is the most pronounced in the first year of high-risk children.

In conclusion, in these genetically predisposed children the total prevalence of atopy at 2 years of age was 32%. The prevalence of AD increased markedly during the first 12 months, while asthma-like disease started to increase mainly after 12 months of age. Markers for atopy were a positive skin test in relation to the clinical expression of AD and food allergy. Food-specific IgE antibodies were associated with AD, food and upper-airway allergy, and HDM-specific IgE antibodies with upper-airway allergy. Moreover, the combination of AD and food allergy at 12 months is the strongest risk factor in our cohort for the development of asthma-like disease at 24 months of age. The effect of passive smoking as an independent risk factor for developing atopy in high-risk children, was the most pronounced in the first year of life.

None of the markers for allergic sensitisation or inflammation was found to precede the clinical expression of atopy, especially asthma or upper-airway allergy at 24 months, which may be the case in follow-up after 2 years of age. In most other studies where markers such as skin tests or specific IgE were claimed to be useful in predicting atopy, the subjects studied were older than 2 years [5,6,8,39].

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CHAPTER 5

A TH0 CYTOKINE PROFILE PRODUCED BY STIMULATED CORD BLOOD CELLS OF CHILDREN WHO DEVELOPED ATOPY. ANALYSIS OF ENVIRONMENTAL AND GENETIC RISK FACTORS AND IMMUNOLOGICAL RESPONSES IN A FOLLOW-UP STUDY OF 133 NEWBORNS

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ABSTRACT

Understanding the immunological processes in early life and the influence of environmental and genetic factors is necessary to develop a strategy to reverse the trend of increasing atopic disease in children. We selected 133 newborns at high risk for the development of atopy. Retrospectively, these children were divided in those who did and did not develop atopy at 12 months of age. The aim was to find out whether environmental or genetic risk factors or immunological responses at birth contribute to the development of atopic disease at 12 months of age. At that time 27/110 children had developed an atopic disease, confirming the role of parental atopy. Two atopic parents, parental smoking and atopic dermatitis of at least one of the parents was significantly related to the presence of atopy in their children at 12 months of age. In cord blood of newborns who developed atopy, an increased percentage of $CD4^{+}CD45RO^{+}$, $CD25^{-}$ cells was observed. In both groups of newborns a significant correlation was observed between IFN-γ and IL-4 protein production by cord blood mononuclear cells (CBMC) after polyclonal stimulation, suggesting the presence of a previously activated Th0 population. In the children who developed atopy, a reduction of the IL-4 and IFN-γ production in combination with high IL-10 production after house-dust mite (HDM) stimulation of CBMC was observed. This suggests a role for the recently described regulatory Tr1 cells during the allergic sensitization of children.

INTRODUCTION

In the Western world the prevalence of atopic disease is increasing, particularly among children (1). To develop a strategy to reverse this trend, a clear understanding is necessary of the immunological processes in early life which potentially promote Th2-polarized atopic sensitization. Data have become available suggesting that the initial sensitization to allergens could already start in utero (2,3). The T cell memory that develops against allergens perinatally is currently believed to be the result of complex interactions between genetic susceptibility and environmental factors. Factors such as parental smoking, infections, feeding methods, birthweight, elevated total IgE levels in cord blood, early exposure to household pets and inhalant allergens were assumed to increase the risk for developing atopic disease (4-7).

Accumulating evidence suggests that the immune system of newborns is relatively immature compared to that of adults, which may lead to an increased susceptibility to develop atopy. For example in newborns, the capacity of cord blood T cells to produce cytokines such as IL-4 and IFN-γ is diminished (8,9), the secretion of immunoglobulins by B cells is strongly decreased (10) and antigen-presenting cells are functionally immature

compared to adults (11). Moreover, the percentage of cord blood $CD45RO^+$ T cells, a marker for activated or memory T cells, is lower than in pheripheral blood of adults (12,13).

Recently, some studies described the phenotypic expression of cell surface markers and functional analysis of cytokines and proliferative responses of cord blood mononuclear cells (CBMC) of children who did and did not develop atopy at later ages (3,14-17). From the results of these studies, it has been proposed that at birth, a weak Th2 skewing is present in all children (18). In susceptible children, the relatively immature T cell compartment being skewed towards a Th2 activity could lead to a failure of tolerance induction or immune deviation towards a profound Th1 response. Therefore, in susceptible children, with atopic parents, allergen exposure may well result in the development of long-term Th2 skewed allergen-specific immunological memory and atopic disease.

Recent findings relating to the postnatal development of immune function have suggested that children who are to develop atopy have a delayed postnatal maturation of cellular immune function, in particular a reduced production of IFN-γ (19,20). In these newborns also a reduced capacity to mount a Th2 response was suggested (21). However, the above hypotheses are based on relatively small patient numbers and insufficient distinction was made between allergen-specific responses of children with or without clinical manifestations of upper-airway or food allergy.

For the longitudinal study presented here, we selected 133 newborns at high risk for the development of atopy for a prospective study. From these newborns at least the mother had an atopic disease. In order to get more insight into the process of allergic sensitization, we examined whether environmental and genetic risk factors and immunological responses at birth predisposed and possibly contributed to the development of clinical symptoms of atopic disease. In newborns who did develop atopy at 12 months of age, we observed the presence of a potential Th0 cytokine profile produced by cord blood cells, which had previously been activated. Decreased IL-4 mRNA, IL-5 protein and IFN-γ protein in combination with a relatively high IL-10 protein production was observed after house-dust mite (HDM)-specific stimulation in newborns who developed atopy. This cytokine profile suggests a role for Tr1 cells in allergic sensitization of children.

MATERIALS AND METHODS

Study population

Cord blood samples were obtained from 133 newborns at high risk for developing atopy. At least the mother of the newborns was atopic, as established by clinical history and a positive skin-prick test (SPT) and/or specific IgE to common inhalant and/or food allergens. After permission from the

pregnant women, informed consent was obtained from the parents of all children prior to their participation in the study. The Medical Ethical Committees of the participating hospitals approved this study.

All children were followed for at least two years in order to monitor the development of atopic disease such as atopic dermatitis (AD), asthma-like disease and food or upper-airway allergy. At the ages 6, 12, 18 and 24 months, physical examinations were performed, questionnaires on environmental factors and histories of symptoms of atopy were filled out and venous blood samples were obtained. The environmental factors were the presence of pets, strategies for HDM avoidance, parental smoking and feeding method. The study populations included in this study comprised 110 children who did or did not suffer from atopy at 12 months of age.

Definition of atopy

At the age of 12 months, a clinical judgement was made without knowledge of the immunological results. Atopic children were defined as children with AD, asthma-like disease, food allergy, upper-airway allergy or a combination of these diseases.

AD was based on Sampson's criteria of (22). These were erythema, edema, oozing and excoriation with evidence of itchiness lasting for longer than four weeks. The severity of the AD was measured by the objective SCORAD. *Asthma* or asthma-like disease was diagnosed if children either had more than two separate wheezing episodes each lasting three days or more, or had more than two separate episodes of paroxysmal nocturnal cough with sleep disturbance for three consecutive nights, or required anti-asthma therapy. *Food allergy* was defined as a clinical manifestation of erythematous, papular, macrovesicular skin eruptions, diarrhea, vomiting, or respiratory problems in relation to food ingestion. For the definitive diagnosis a positive prick-prick test or a positive skin application food test (SAFT) (23) or specific IgE was necessary. Specific IgE was measured using Phadebas CAP radio-allergo-sorbent test (RAST) (Pharmacia, Uppsala, Sweden) (24). *Upper-airway allergy* was defined as seasonal rhinoconjunctivitis, symptoms of sneezing, redness and itching and tearing of the eyes and/or respiratory complaints. Diagnosis was confirmed by the presence of specific IgE in the plasma.

Isolation of CBMC

At birth, heparinized umbilical cord blood samples were obtained from 85 newborns. After diluting 1:1 with PBS, CBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia). In order to control for maternal blood contamination, IgA content was determined in cord blood samples of all newborns whose IgE values were detectable. Two cord blood samples with an IgA content higher than 10 mg/l were therefore excluded from this study.

Determination of IgE and IgA

The total IgE concentrations in cord blood were determined using a modified radio-immuno assay which resulted in a lower detection limit of 0.2 kU/L. At the age of 12 months a sample of venous blood was obtained to determine specific IgE levels directed towards the most common inhalation allergens (house-dust mite, grass and birch pollen, cat, dog and moulds) and food allergens (peanut, chicken egg white, cow's milk, codfish, wheat and soy). Measurements were performed using the Pharmacia CAP system Phadiatop® RAST and the Pharmacia CAP RAST (Kabi Pharmacia, Uppsala, Sweden). IgA was determined by a nephelometric method with a detection limit of 10 mg/l.

Proliferative responses

Lymphocyte proliferation assays were performed as described previously (25). Briefly, CBMC were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate and 10% heat-inactivated (HI) human serum (HuS) in 96-well round-bottom microtiter culture plates. CBMC were cultured in quadriplate at $2x10^5$ cells/well in the presence of PMA (2 ng/ml; Sigma, St.Louis, MO) and Ca-ionophore (1 μg/ml; A23187, Sigma) or D. pteronyssinus extract (HDM, 50 μg/ml, ALK-Benelux, Groningen, The Netherlands) or egg-white extract (egg, 50 μg/ml, ALK) or cow's milk extract (CM, 25 μg/ml, ALK) or without additions in a total volume of 200 μl. After 3 days of polyclonal or 7 days of allergen-specific stimulation, proliferation was measured using $[^3H]$-thymidine incorporation.

Detection of cytokine mRNA expression in CBMC cultures

CBMC were cultured in 24-well plates at $1x10^6$ cells/well in Yssel's medium (YM) containing 1% HI-HuS. CBMC were cultured with or without the addition of 2 ng/ml PMA and 1 μg/ml Ca-ionophore for 16-18 h, or in the presence of HDM (50 μg/ml), egg (50 μg/ml) or CM (25 μg/ml) for 4 days in a total volume of 1 ml. After we harvested the cells, supernatants were stored at -80°C. Total RNA was isolated from the cells, followed by cDNA synthesis, amplification by semi-quantitative RT-PCR using primers specific for either the housekeeping gene HPRT (hypoxanthine phosphoribosyl transferase) or IL-4 and densitometric analysis as described previously (26).

Detection of cytokine protein production in CBMC cultures

The levels of IFN-γ, IL-4, IL-10 and IL-13 in the culture supernatants and in plasma were determined using a Pelikan Compact™ human cytokine kit (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands) according to the manufacturers' instructions.

IL-5 was determined using an ELISA based on TRFK5 (a kind gift of Dr. R.L. Coffman (DNAX Research Institute, Palo Alto, CA)) as a capture mAb and

biotin-labeled rat anti-human IL-5 (JES1-5A10, 0.25 μg/ml, Pharmingen, cat. 18522D) as detection mAb. 96-well plates (flat-bottom, Falcon 3912, Becton Dickinson, San Jose, CA) were coated overnight at 4°C with anti-IL-5 mAb TRFK5, in an optimal coating concentration of 0.25 μg/ml at 100 μl/well in 0.1 M carbonate buffer pH 8.2. After each incubation step plates were washed with PBS/0.05% Tween. After washing the plates three times, non-specific binding was prevented by blocking the plates for 2 hours at room temperature (RT) with 200 μl/well PBS/10%FCS-HI (BioWhittaker, Verviers, Belgium). Plates were washed three times after which standard IL-5 (1000-15.6 pg/ml; Rec. human IL-5, Pharmingen) diluted in YM 1% HuS and samples were added in duplicate (100 μl/well). After overnight incubation at 4°C, and washing the plates 4 times, they were incubated for 45 minutes at RT with anti-IL-5 biotin (0.25 μg/ml) diluted in PBS/10%FCS-HI (100 μl/well, BioWhittaker). After washing 6 times, poly-streptavidine-peroxidase (CLB) diluted in PBS/10%FCS-HI (BioWhittaker) was added. After 30 minutes incubation at RT and additional 6 times washing, peroxidase activity was revealed by the addition of TMB (3,3',5,5'-tetramethylbenzidine) substrate (Kirkegaard & Perry Lab., Gaithersburg, MD). The reaction was stopped with 0.1 M H_3PO_4 and read at 450 nm. The lower detection limits were 2 pg/ml for IFN-γ, 1 pg/ml for IL-4, 2.5 pg/ml for IL-5, 2 pg/ml for IL-10 and 0.5 pg/ml for IL-13.

Phenotypic analyses of CBMC

One to five x10^5 CBMC were stained (10 min, RT) with optimally titered mAb specific for B cells (Leu12-PE (CD19), Coulter Cytometry, Hialeah, FL), T cells (Leu4-FITC (CD3), Becton Dickinson), monocytes (My4-FITC (CD14), Coulter Cytometry) and NK cells (Leu11-PE (CD16) and Leu19-PE (CD56), Becton Dickinson). For the analysis of CD4/CD8 ratios, CBMC were double stained with Leu4-FITC (CD3) and Leu3-PE (CD4, Becton Dickinson) or with Leu4-FITC and Leu2-PE (CD8, Becton Dickinson). For the analysis of CD45RA and CD45RO expression, CBMC were double stained with Leu3-PE or Leu2-PE and 2H4-PE (CD45RA, Coulter Cytometry) or with Leu3-PE or Leu2-PE and UCHL1-PE (CD45RO, DAKO, Glostrup, Denmark). After staining, the normoblasts were lysed with NH_4Cl, and the cells were washed twice and resuspended in 100 μl FACSflow solution (Becton Dickinson). Flowcytometry was performed on a FACScan (Becton Dickinson).

Statistical analyses

Statistical analyses were performed using SSPS for Windows, version 8.0. Environmental and genetic factors were evaluated by computing the relative risk (RR) with Cornfield's 95% confidence intervals (CI) as a measure of association or by the Chi-square linear association test. Immunological parameters were tested using an independent sample t-test or the Mann-Whitney test. Correlation was assessed using Spearman's rank correlation coefficient. P-values less than 0.05 were considered significant.

RESULTS

Prevalence of atopic disease at 12 months

For this study, a group of 133 high-risk newborns were selected, the mothers of whom all had an atopic disease, defined as described in the materials and methods section. At the age of 12 months, 110 of the 133 included children (83%) were assessed for the presence of clinical symptoms of atopic disease defined as AD, asthma-like disease and food or upper-airway allergy. At this time point 27 of these children (25%, Table I) had developed an atopic disease, almost exclusively AD, which is in agreement with the expected genetic predisposition of 30% in the case of an atopic mother (27). The median severity of the AD, determined by the objective SCORAD, was classified as mild AD. Of the atopic children, 5 children had a food allergy. Of these children, one child had a food allergy against cow's milk and four children against egg. Four children had developed asthma-like disease, whereas none of the children had developed upper-airway allergy at 12 months of age. The prevalence of atopy was similar in boys and girls. Cord blood IgE above the detection limit of 0.2 kU/l, was detected in 41% (35/85) of the children, of which 23% (8/35) had developed atopy at 12 months of age.

Table I. Characteristics and prevalence of atopic diseases in children who did and did not develop atopy at 12 months of age.

			Clinical signs of atopy		
	Atopic[1]	Non-atopic[1]	AD	AD and food allergy	Asthma-like disease
n	83	27	23	5	4
%	75.5	24.5	85.2[4]	18.5[4]	14.8[4]
sex M/F	35/47	13/14	11/12	2/3	2/2
total IgE[2]	27/63	8/22	8/20	1/4	0/2
birthweight[3] gr.	3190 (1670-4750)	3355 (2350-4580)	3360 (2710-4210)	3150 (2350-3440)	3785 (3070-4580)
SCORAD			7 (2-35)	2 (2-11)	

M = male, F = female. [1]Children were divided in two groups: those who did (atopic) and who did not (non-atopic) develop clinical features of atopy at 12 months of age. [2]Data represent number of newborns with detectable IgE levels in cord blood. Total IgE levels were determined by IgE CAP RAST with a lower detection limit of 0.2 kU/L. [3]Data represents the median value (range) in grams. [4]Calculation of percentage of children relative to atopic children.

Risk factors for the development of atopy

Several genetic (atopy of both parents, asthma or AD of the parents and male sex) and environmental (presence of pets, strategies for HDM avoidance, parental smoking and feeding method) risk factors were studied for the development of atopy at 12 months of age. Smoking habits were admitted by 34 parents (31%), of which in four cases only the mother, in 14 cases only the father and in 16 cases both parents smoked. 14/28 children (50%) who had developed atopy at the age of 12 months had smoking parents. Of nine mothers who also smoked during pregnancy, three children (33%) had developed atopy.

Of 9/28 children (33%) who did, and of 11/82 (13%) children who did not develop atopy, both parents had an atopic disease. In total 26 parents (24%) had AD of which 11 children had developed atopy at 12 months of age. The RR of these and other factors were calculated for all atopic children (Table II). Three risk factors were significantly related to the presence of atopy in the children at 12 months of age: smoking by at least one of the parents, AD of at least one of the parents, and atopy in both parents.

For the analysis of cord blood IgE level as a risk factor for the development of atopy, different cut-off values for total IgE levels were used, i.e. 0.5 and 0.9 kU/l (28,29). The RR for both cut-off values did not differ significantly from 1. The analysis of the feeding method revealed that 5 children received a restricted diet of casein hydrolysate. Moreover, 23 children were breast-fed for more than 6 months, 24 children for more than 3 months but less than 6 months, and 26 children for less than 3 months. Formula feeding was received by 32 children. The chi-square linear association test did not reveal a significant association between feeding method and atopy development at 12 months of age (data not shown).

Table II. Risk factors for the development of atopy in high-risk children.

Risk factor	RR	95% CI
Atopy both parents	2.13	1.14-3.99*
Asthma of parents	1.51	0.75-3.02
AD of parents	2.04	1.10-3.78*
Male sex	1.17	0.62-2.21
Cord IgE >0.5 kU/l	1.64	0.26-10.16
Cord IgE>0.9 kU/l	1.35	0.23-8.07
Parental smoking[1]	2.23	1.20-4.14*
Pets	1.06	0.55-2.03
HDM avoidance	0.84	0.40-1.80

RR= relative risk, CI= confidence interval, * $P < 0.05$. The contribution of different possible risk factors for the development of atopy in high-risk children was determined. RR was calculated for all atopic children relative to children without atopic disease at 12 months of age. [1] Smoking of either one of the parents.

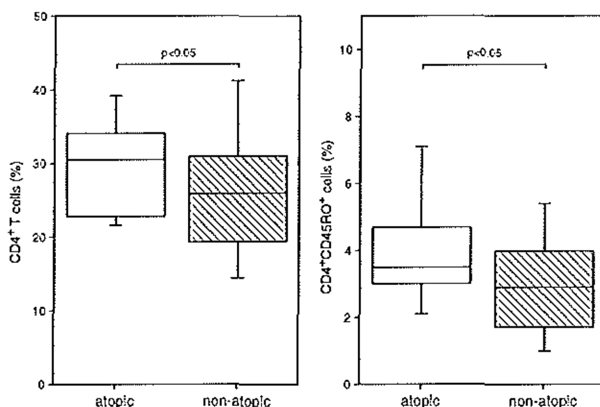

Figure 1. T cell subpopulations in CBMC of children who did and did not develop atopy within the first year of life. CBMC were double stained with monoclonal antibodies specific for $CD4^+$ T cells, and with antibodies specific for $CD4^+CD45RO^+$ cells after which flowcytometry was performed. Boxplots of mean percentages of $CD4^+$ T cells (left), and mean percentages of $CD4^+CD45RO^+$ T cells (right) are shown from newborns who did (atopic) and did not (non-atopic) develop atopy at 12 months of age.

Phenotypic analyses of CBMC

In order to elucidate possible lymphocyte differences in newborns who developed atopy compared to those who did not, we investigated T cell subpopulations and activation markers at birth. Flowcytometric analyses revealed that the percentage of $CD4^+$ T cells was significantly ($P<0.05$) increased in the CBMC of those children who developed atopy at 12 months of age (Fig. 1). The determination of the percentage of cells, which were double positive for either CD4 or CD8 and CD45RO (activated or memory cells) or CD45RA (naive cells) revealed an increased percentage of $CD4^+CD45RO^+$ cells in those children who later developed atopy ($P<0.05$, Fig. 1). No differences were found for $CD25^+$ T cells and other markers analyzed (Table III).

Proliferative capacity and cytokine profiles after polyclonal stimulation of CBMC

Proliferative responses of CBMC of both groups were studied after in vitro stimulation with PMA and Ca-ionophore. The baseline proliferation of CBMC of children who had developed atopy at 12 months (median = 2750 cpm (646-13249) was significantly increased compared to those who did not (median = 1674 cpm (152-8386); $P<0.05$). Also proliferative responses after polyclonal stimulation of the CBMC were significantly increased in the group of children who had developed atopy at 12 months (median = 47589 cpm (744-107374) compared to those who did not (median = 37631 cpm (3599-103391); $P<0.05$). These results, together with the increased

Table III. Phenotypic analyses of CBMC.

	Atopic	Non-Atopic
T cells	42.7 (15.1-61.9)	36.4 (16.2-65.2)
B cells	10.2 (4.1-26.9)	10.3 (4.6-24.5)
Monocytes	22.2 (7.5-41.9)	21.4 (4.3-52.4)
NK cells	13.2 (4.4-29.9)	16.8 (6.4-43.3)
$CD4^+$ T cells	30.7 (13.4-49.2)	25.9 (8.7-49.2)*
$CD8^+$ T cells	11.2 (3.1-20.2)	11.7 (3.6-18.8)
Ratio CD4/CD8	2.7 (1.2-10)	2.3 (1.1-6.5)
$CD25^+$ T cells	3.1 (0.6-6.4)	2.8 (1.0-6.2)
$CD4^+CD45RO^+$ cells	3.2 (1.7-7.6)	2.9 (1.0-9.6)*
$CD4^+CD45RA^+$ cells	23.5 (6.9-36.8)	20.5 (2.9-44.2)
$CD8^+CD45RO^+$ cells	2.9 (0.7-14.9)	1.9 (0.4-10.5)
$CD8^+CD45RA^+$ cells	11.3 (3.7-29.4)	10.2 (2.7-25.8)

CBMC were stained with monoclonal antibodies specific for T cells, B cells, monocytes, NK cells, $CD4^+$ and $CD8^+$ T cells, CD25, CD45RO and CD45RA after which flowcytometry was performed. Data represent median percentages of cell populations identified by the indicated cell surface molecules, present in the CBMC fraction (range). $^*P<0.05$.

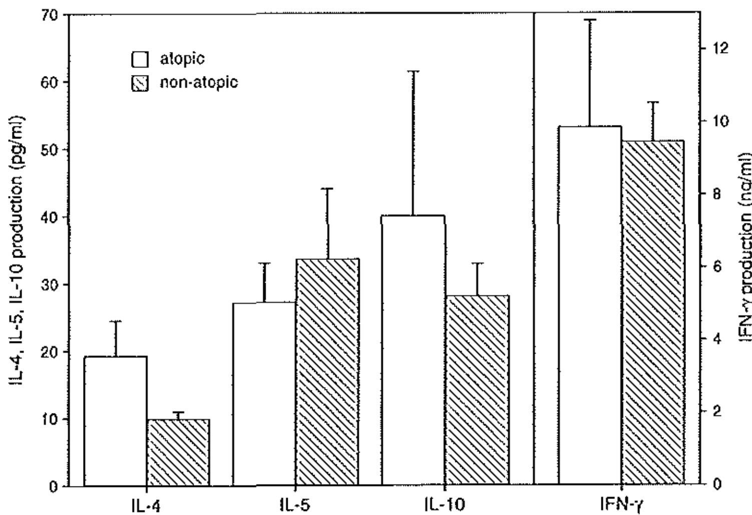

Figure 2. Cytokine protein production by CBMC of children who did and did not develop atopy at 12 months of age. CBMC were polyclonally stimulated with PMA and Ca-ionophore for 16-18 hours. In the culture supernatants IL-4, IL-5, IL-10 and IFN-γ were measured by ELISA. Group means and standard deviations are shown. No significant differences were observed for the various cytokine levels between the patient groups.

percentage of $CD4^+CD45RO^+$ cells but not $CD25^+$ cells, indicate that newborns who had developed atopy at 12 months of age, had a pool of apparently previously activated cells at birth, which had the capacity to respond more vigorously to non-specific stimuli.

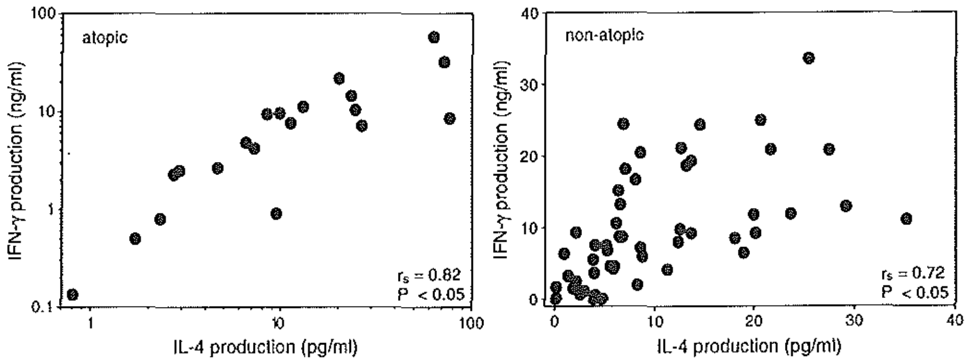

Figure 3. Correlation between IFN-γ and IL-4 protein production of CBMC of children who did (left) and did not (right) develop atopy at 12 months of age. CBMC were polyclonally stimulated with PMA and Ca-ionophore for 16-18 hours after which IFN-γ and IL-4 production levels were determined by ELISA. Measurements were performed in duplicates.

To determine the cytokine protein production profile of the CBMC, we measured IL-4, IL-5, IL-10, IL-13 and IFN-γ levels in the supernatants of CBMC cultured with or without a polyclonal stimulator. Without stimulation, only IL-5, IL-10 and IFN-γ were detectable (data not shown), while after polyclonal stimulation all measured cytokines were detectable. However, no significant differences were observed between both groups of newborns (Fig. 2). Only a trend of increased IL-4 and IL-10 production levels occurred in those children who later developed atopy. IL-13 production was not different between children who did and did not develop atopy at 12 months (data not shown). A significant correlation between IFN-γ and IL-4 protein production by CMBC was observed for atopic (r_s=0.82, P<0.05, Fig. 3A) and non-atopic children (r_s=0.72, P<0.05, Fig. 3B) at 12 months of age. The concomitant production of these two cytokines after polyclonal stimulation supports the existence of a Th0 population of cells at birth.

Proliferative responses and cytokine profiles after allergen-specific stimulation of CBMC

In order to get more insight in the occurrence of allergen-specific proliferative responses at birth, CBMC were stimulated with different allergens e.g. cow's milk, egg and house-dust mite (HDM). No differences were observed in the proliferative responses between CBMC of children who did and did not develop atopy at the age of 12 months in response to allergen-specific stimulation (Fig. 4). Children who had developed a food allergy at the age of 12 months (marked in figure 4) did not distinguish themselves at birth with regard to their proliferative responses, compared to the other atopic children without a food allergy.

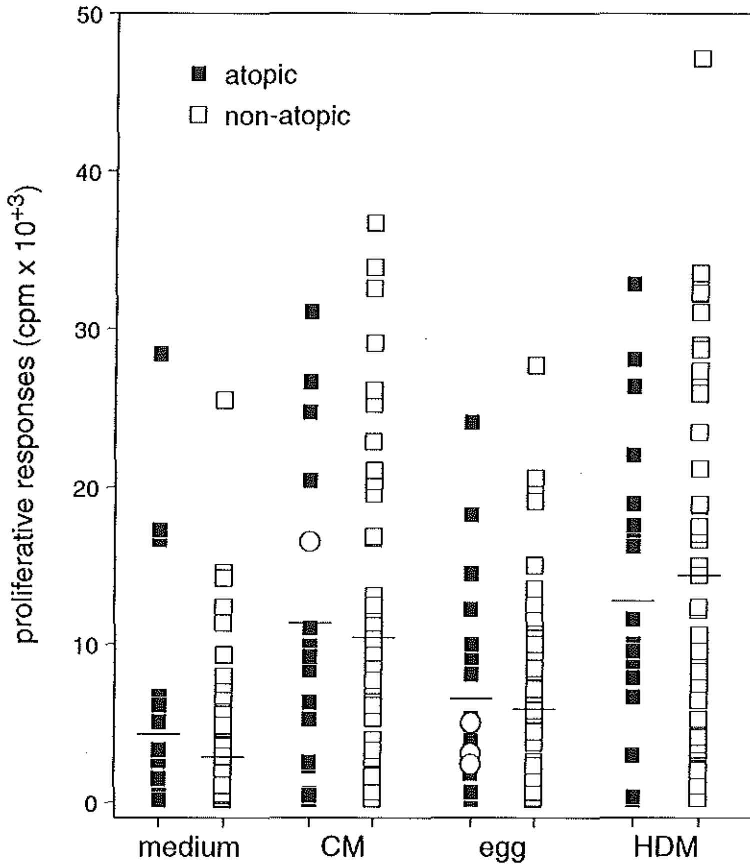

Figure 4. Proliferative responses of CBMC of children who did and did not develop atopy at 12 months of age. Proliferation of CBMC was determined by measuring [^{3}H]-thymidine incorporation on day 7 of culture after stimulation with various allergens (CM = cow's milk; egg; and HDM = house-dust mite) or cultured in medium only. Results are expressed as total cpm. Each point represents the mean of quadruplicate measurements from an individual CBMC sample. Open circles represent newborns who developed clinical symptoms of food allergy to either cow's milk or egg within 12 months of age.

Cytokine profiles induced by allergen-stimulated CBMC were studied for the food allergen egg and the inhalant allergen HDM. These two representative allergens were used to study the occurrence of allergen-specific memory cells at birth, despite the fact that virtually no children had developed clinical symptoms to these allergens at 12 months of age. The levels of IL-4 protein produced in these allergen-stimulated cultures were below the detection limit of the sensitive ELISA method employed, necessitating detection of IL-4 mRNA expression by a semi-quantitative RT-

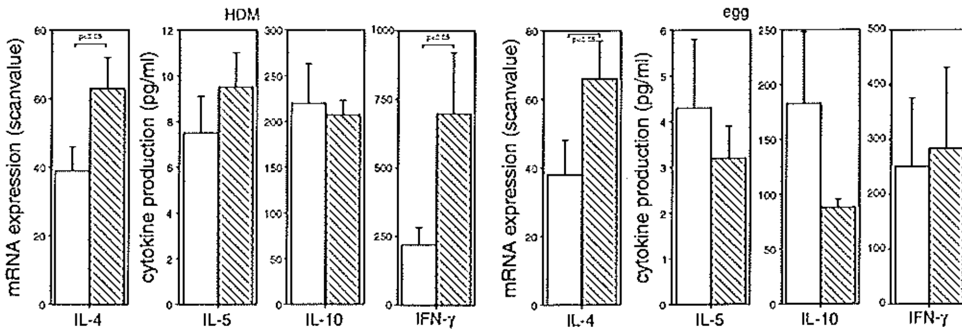

Figure 5. Cytokine mRNA expression and protein production by CBMC of children who did and did not develop atopy at 12 months of age. CBMC were stimulated allergen-specifically for 4 days with house-dust mite (left) and egg-extracts (right). After stimulation, RNA was isolated, semi-quantitative RT-PCR was performed for IL-4, while IL-5, IL-10 and IFN-γ protein production was measured in the culture supernatants by ELISA. Group means and standard deviations are shown. Open bar = atopic, hatched bar = non-atopic.

PCR method. IL-4 mRNA and IFN-γ protein were significantly reduced after HDM-specific stimulation of CBMC of children who had developed atopy at the age of 12 months (Fig. 5). The IL-10 production was not different between both groups of newborns, but the ratio of IL-10 to other cytokines was relatively increased in the newborns who later developed atopy. The cytokine profile characterized by relatively high IL-10 levels and low IL-4 levels is suggestive for the presence of Tr1 cells. This was further substantiated by the reduced IFN-γ production after HDM-specific stimulation of CBMC, which is one of the characteristics of Tr1 cells.

In agreement with the correlation between the IL-4 and IFN-γ production capacity after polyclonal stimulation of CBMC, after HDM-specific stimulation of CBMC a significant correlation between IL-4 and IFN-γ production was also observed in the atopic group ($r_s=0.62$, $P<0.05$, data not shown), again indicating the existence of a substantial Th0 population of cells at birth.

The cytokine profile observed after stimulation with a food allergen was essentially different from that found after stimulation with an inhalant allergen. Only IL-4 mRNA expression was significantly reduced after stimulation of CBMC with egg in children who had developed atopy, whereas a trend for increased IL-10 production existed (Fig. 5). IL-5 and IFN-γ production were not different between atopic and non-atopic children. IL-13 protein levels produced after allergen-specific stimulation were below the detection limit of the ELISA.

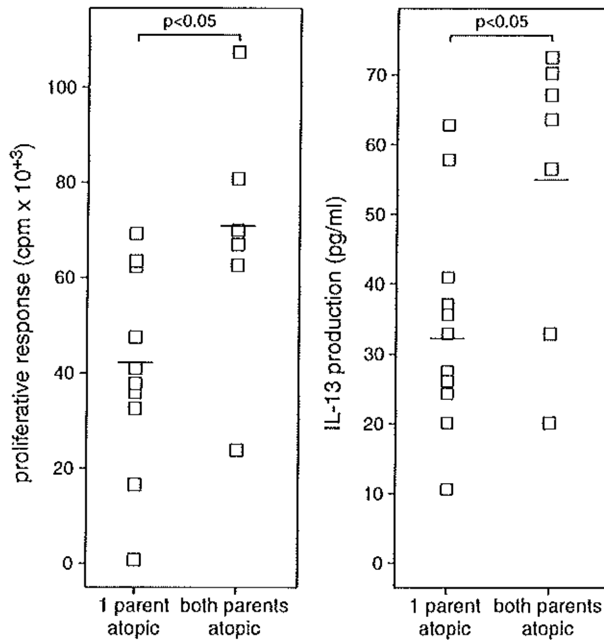

Figure 6. Association between immunological responses and a genetic factor in atopic children. Atopic children were divided in two groups: those with an atopic mother and those with two atopic parents. Between both groups of atopic children the proliferative capacity of CBMC (left) and the IL-13 production capacity of CBMC (right) were compared. To assess the proliferative capacity of CBMC, the cells were stimulated with PMA and Ca-ionophore for 3 days after which proliferation was measured by [^{3}H]-thymidine incorporation. IL-13 production was measured after stimulating the CBMC for 16-18 hours with PMA and Ca-ionophore. Each point respresents the mean of a quadruplicate measurement from an atopic child in case of proliferative responses and of a duplicate measurement in case of IL-13 production. The lines indicate the group means.

Associations between immunological and environmental or genetic risk factors

Associations were studied between immunological responses and environmental or genetic risk factors in the children who had developed atopy at 12 months of age. Significantly higher proliferative responses after polyclonal stimulation of CBMC were observed in those newborns with two atopic parents compared to those with one atopic parent ($P<0.05$, Fig. 6). Next to the increased proliferative capacity in this group of children, the IL-13 protein production by CBMC after polyclonal stimulation was also significantly elevated ($P<0.05$, Fig. 6).

DISCUSSION

A cohort of 133 newborns, at high risk for the development of atopy, was followed from birth to 12 months of age. At this age, 25% of the children had developed atopic disease, mainly AD. We analyzed whether environmental and genetic risk factors and immunological responses at birth contribute to the development of clinical symptoms of atopy within the first year of life. Parental smoking, two atopic parents and AD of at least one of the parents were significantly related to the presence of atopy at 12 months of age in their children. An increased percentage of $CD4^+$ and $CD4^+CD45RO^+$ cells and an increased proliferative capacity were observed in CBMC of those children who had developed atopy at 12 months of age. A strong correlation between IL-4 and IFN-γ production after polyclonal stimulation of CBMC was found in both groups. After allergen-specific stimulation with HDM, the CBMC of newborns who did develop atopy, showed a decrease in IL-4 mRNA, IL-5 and IFN-γ production, but not in IL-10 production. Moreover, a correlation between IL-4 and IFN-γ production was observed in this group of children after HDM stimulation of CBMC. These results are indicative for the presence of a Th0 cytokine profile produced by stimulated cells in cord blood, which had presumably been activated in utero.

The generation of allergen-specific responses as a result of the interaction with environmental and genetic factors in the risk of developing an atopic disease is still largely unknown. Because of mutual influences, associations between immunological responses and environmental risk factors were difficult to interpret. For genetic risk factors the situation is somewhat less complicated. In the children who had developed atopy at 12 months of age, we observed associations between immunological responses and a genetic risk factor. Atopic children who had two atopic parents showed an increased proliferative capacity and an increased IL-13 production by CBMC compared to atopic children with one atopic parent, clearly indicating genetic influences on the development of atopy. More data on the nature of these and other associations between immunological responses of CBMC and genetic risk factors are needed to further identify possible predictors for the development of atopy.

Passive smoking is the most consistently documented environmental risk factor for the development of atopic disease in children (4-7,27). Also the presence of atopy in the family is a well-known risk factor for atopy (4-6). Indeed, also in this study both risk factors had a strong association with the development of atopy in the first year of life. Several studies have shown elevated IgE levels in umbilical cord blood to be a good predictive test for atopy, but more recent studies described the IgE level as a poor predictor (7,28). The present study confirms that the cord blood IgE level is a poor predictor of atopic disease. Other risk factors analyzed, e.g. the presence of pets, strategies for house-dust mite avoidance, breast-feeding and male sex,

were not associated with the presence of atopic disease at 12 months of age. These can still be risk factors for other atopic diseases (e.g. allergic asthma) after the first year of life (6).

The increased T cell proliferative capacity observed in CBMC of children who developed atopy is in agreement with findings of others (3,30). In contrast, in another study from our group (31), comparing proliferative responses after polyclonal stimulation of PBMC from children with established AD and from healthy children, no differences were detected. The disappearance of the increased proliferative capacity during the establishment of the atopy suggests that this increased proliferative capacity of CBMC is due to perinatal T cell activation or to an altered intrinsic capacity to proliferate to polyclonal stimulation during the developmental phase of an atopic disorder.

Phenotypic studies of T cells have shown lower percentages of $CD3^+$, $CD4^+$, $CD8^+$ and $CD45RO^+$ cells in cord blood than in adult peripheral blood samples (12,13). In accordance with the data of Miles et al. (16) we did not find a difference in the percentage of $CD25^+$ (activated) T cells between newborns that did and did not develop atopy within the first year after birth. However, a significantly increased percentage of $CD4^+CD45RO^+$ cells did occur in the cord blood of those children, who later developed atopic symptoms. This finding supports the hypothesis that at the time of birth, T helper cells of children who later developed atopy had been activated in utero. This is in agreement with recent data suggesting that the initial allergenic sensitization could already start in utero (3).

The existence of a previously activated Th population at birth is further substantiated by the observation of a higher baseline proliferative response of CBMC in the same patient group. This finding corroborates the hypothesis of priming of the fetal immune system (17,32). In contrast to our findings, Miles et al. found a significantly decreased percentage of $CD4^+CD45RO^+$ cells in cord blood comparing a small group of high-risk children who did and did not suffer from eczema within the first year of life (16). However, in cross-sectional studies comparing atopic with healthy children, increased percentages of $CD4^+CD45RO^+$ cells were also described in the peripheral blood (33,34).

Our cytokine data after polyclonal stimulation indicate that at the time of birth a potential Th0 cytokine production profile is present, rather than a weak Th2 skewing (18). In contrast to other studies (3,20,29), we did not find a decreased IFN-γ production after polyclonal stimulation of CBMC of children who later developed atopy. Moreover, our results after polyclonal stimulation did not show an intrinsic T cell defect for cytokine production in CBMC of newborns who developed atopy.

The increased percentage of $CD4^+CD45RO^+$ cells showed that the major T cell subpopulation considered to respond to allergen-specific stimulation is present in increased frequencies in atopics. The increased

percentage of $CD4^+CD45RO^+$ cells in newborns who later developed atopy did not result in a higher production of cytokines to inhalant stimulation compared to newborns who did not develop atopy. The failure to detect an increased cytokine production in atopics could be partially explained by a defect or immaturity of the antigen-presenting cell compartment (35-37). The most likely explanation is that the percentage of $CD4^+CD45RO^+$ cells in newborns who later developed atopy is still low compared to the percentages found in adults (12,13).

Allergen-induced lymphoproliferative responses were present at birth in newborns who did and did not develop atopy at 12 months, suggesting that in utero allergen priming had occurred in both groups. In spite of the increased percentage of memory cells in newborns who developed atopy, CBMC of these newborns did not display a stronger proliferative response to allergen stimulation. Newborns who developed food allergy did not distinguish themselves from the other atopic children in the level of proliferative response to food stimulation. It remains to be established to what degree expression of food allergic symptoms during the first year of life is related to the probability of developing upper-airway allergy at ages above three years (38-40).

More apparent than proliferative responses were the differences in the cytokine responses of these allergen-reactive mononuclear cells. We observed differential cytokine responses induced either by HDM or egg stimulation. An interesting question is whether this difference also has consequences for atopic sensitization after the first year of life.

After HDM stimulation the cytokine profile, the decreased release of IL-4, IL-5, IFN-γ and the relationship between IL-4 and IFN-γ production were consistent with a Th0 profile. This corroborates the finding of a Th0 cytokine profile as observed after polyclonal stimulation. In spite of the Th2 state in pregnancy (41,42) our results indicate that the immune response of newborns is not Th2-skewed, but polarized to a Th0 cytokine response. This is in contrast with the postulation of a Th2 profile by others (18,43), based on reduced (3,19,20) or even undetectable (21) IFN-γ production in atopics after allergen-specific stimulation.

Holt et al. (21) postulated that cord blood cells of children who develop atopy, compared to those who do not, display reduced house-dust mite induced Th1 and Th2 cytokine responses. Indeed, our findings of reduced IL-4 mRNA expression and IFN-γ production observed after HDM stimulation confirmed such reduced Th1 and Th2 cytokine responses of newborns who developed atopy. This reduced response of Th1 and Th2 cells concerns only inhalant-induced but not food-induced cytokine responses. After egg-specific stimulation only a decreased IL-4 mRNA level was observed, while no differences were found in IFN-γ production. These allergen-related differences illustrate that a distinction should be made between food and inhalant allergen-induced cytokine responses. In future

research, we will study whether children with food or inhalant allergen-induced cytokine responses will develop different clinical disorders, e.g. AD vs. asthma at later ages.

The process of allergic sensitization is currently only discussed in the light of the Th1-Th2 dogma. It is, however, of interest to note the observed relatively high IL-10 production in the presence of reduced IL-4 expression in atopics after allergen-specific stimulation with either egg or HDM. As we showed previously, allergen-specific stimulation results in a predominant activation of $CD4^+$ T cells (31), suggesting that these T cells are the major source of IL-10 production. Our results of high IL-10 production in combination with reduced IL-4 expression point to the newly described regulatory T cell subset, called Tr1 cells. These cells are able to produce high amounts of IL-10, but low IL-4 (44). It is conceivable that sensitization in utero creates a milieu of high IL-10 release, inducing both a general inhibition of cytokine release by Th1 as well as Th2 cells, but more profoundly inhibiting the IFN-γ production (45-47). In addition, the high IL-10 release may result in a preferential development of Tr1 cells. Thus, we here propose a role for Tr1 cells in atopic sensitization of children.

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CHAPTER 6

THE SELECTIVE DEVELOPMENT OF A TH2 CYTOKINE PROFILE IS INDEPENDENT OF THE PRODUCTION OF IFN-γ IN CHILDREN WHO DEVELOP ATOPY

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ABSTRACT

Established atopic disease in patients is characterized by a Th2 cytokine profile. We investigated in a longitudinal study whether the intrinsic capacity of T cells to develop Th1 or Th2 cytokine profiles was different between high-risk newborns who did and did not develop atopy. We therefore performed quantitative analysis of mRNA and protein production of IL-4, IL-5, IL-10, IL-13 and IFN-γ in isolated PBMC on consecutive time points starting at birth. Stimulation of cord blood mononuclear cells (CBMC) indicated that all children had the capacity to induce a Th0 cytokine profile at birth. However, this Th0 profile shifted gradually to a Th2 profile at 6 months of age in children who developed atopy at this time. A Th2 cytokine profile was also observed at 6 months in children who developed their first atopic symptoms between 6 and 12 months of age. No significant differences were observed for IFN-γ production between children who did and did not develop atopy. We conclude that the first 6 months of life represents a critical time window for the initiation of immunological changes reflecting the allergic sensitization that becomes clinically detectable at 12 months. Furthermore, the selective development of a Th2 cytokine profile in atopic children is independent of the production of IFN-γ.

INTRODUCTION

Recently, it was proposed that children who develop atopy have a delayed postnatal maturation of cellular immune functions, especially a reduction in the production of IFN-γ (1-3). A change in the balance of T-helper cell subsets (Th1 vs. Th2) towards a polarized Th2 population is generally accepted to occur in established atopic disease. In adults and children with established atopic disease, increased levels of IL-4, IL-5 and IL-13 protein in combination with a decreased IFN-γ protein production have been described both after polyclonal and allergen-specific stimulation of PBMC or purified T cells (4-7).

However, the described Th2 cytokine profile was less pronounced after stimulation of PBMC at very young age as compared to the strongly polarized Th2 population observed in patients with established atopy (8). A weak Th2 skewing of stimulated cord blood mononuclear cells (CBMC) has been proposed to be present in all children at time of birth (9). In addition, a decreased IFN-γ production was found after polyclonal stimulation of CBMC in those children who developed atopy compared to children who did not develop atopy before 2 years of age (3,8).

Many immune functions in newborns are less mature compared to adults (10-13). Recent studies suggest that these maturational deficiencies may be more marked in newborns who develop atopy (8). In susceptible newborns, a relatively immature T cell compartment that is skewed towards a

Th2 phenotype could lead to failure of tolerance induction or lack of immune deviation towards a Th1 response. As a result, such children develop a long term Th2-skewed allergen-specific immunological memory and atopic disease upon allergen exposure. It was proposed repeatedly that the failure to develop a profound Th1 response after stimulation of CBMC of atopic children was especially due to a reduced IFN-γ production (3,8,14-16).

The proposed allergen-induced weak Th2 priming at birth in all children has made it challenging to investigate whether the intrinsic capacity of T cells to give rise to Th1 or Th2 cytokine profiles is different between atopy-prone children and children who do not develop atopy within their first year of life. We have therefore started a prospective study on a group of 133 high-risk newborns and followed them from birth onwards for the development of clinical signs of atopic disease together with a detailed analysis of their cytokine profiles. PBMC of these children were stimulated polyclonally after which intrinsic differences in T cell cytokine profiles were analyzed by ELISA and by a quantitative "real time" PCR using TaqMan technology, at consecutive follow-up time points. Our results provide evidence for the expression of a Th2 cytokine profile within the first 6 months of life in the presence of an undisturbed production of IFN-γ in those children developing atopic disease in their first year of life.

MATERIALS AND METHODS

Study population

High-risk children (n = 133) were followed from birth until 24 months of age to monitor the development of asthma-like disease, atopic dermatitis (AD), food and upper-airway allergy. The mothers of all newborns were atopic, as established by a clinical history and a positive skin test and/or specific IgE to common inhalant and/or food allergens. This study was approved by the Ethical Committees of the participating hospitals and informed consent was obtained from the parents prior to participation.

The populations included in this study comprised children who did (atopic) and did not (non-atopic) develop atopy before 12 months of age. At the age of 6 and 12 months, questionnaires were filled out, physical examinations were performed and a clinical evaluation was made without knowledge of the immunological results. Both groups of children, atopic and non atopic, had received the appropriate routine vaccinations for their age.

AD was based on Sampson's criteria (17). These were erythema, edema, oozing and excoriation with evidence for itchiness lasting for longer than four weeks. *Asthma* or asthma-like disease was diagnosed if children either had more than two separate wheezing episodes each lasting three days or more, or had more than two separate episodes of paroxysmal nocturnal cough with sleep disturbance for three consecutive nights, or required anti-asthma therapy. *Food allergy* was defined as clinical manifestation of

erythematous, papular, macrovesicular skin eruptions, diarrhea, vomiting, or respiratory problems in relation to food ingestion. For the definitive diagnosis a positive skin test or specific IgE was necessary. Mostly the diagnosis was confirmed by an open oral challenge test. Specific IgE was measured using RAST Phadebas CAP system (Pharmacia, Uppsala, Sweden). *Upper-airway allergy* was defined as seasonal rhinoconjunctivitis, symptoms of sneezing, redness, itching and tearing of the eyes and/or respiratory complaints. Diagnosis was confirmed by the presence of specific IgE in plasma.

Analysis of cytokine mRNA expression by TaqMan technology

Heparinized blood samples were obtained at birth, 6 and 12 months of age. After diluting 1:1 with PBS, PBMC/CBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia). PBMC/CBMC were cultured for 16-18 h in 24-well culture plates at $1x10^6$ cells/well in Yssel's medium (YM) containing 1% heat-inactivated human serum (HuS-HI) with or without the addition of 2 ng/ml PMA (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1 µg/ml 4-bromo-calcium-ionophore (Sigma-Aldrich) in a total volume of 1 ml (18). After harvesting the cells, supernatants were stored at -80°C. RNA was isolated from the cells by the RNAzol B method (5) (Campro Scientific, Veenendaal, The Netherlands). Briefly, per 10^6 pelleted cells, 200 µl RNAzol B was added as well as chloroform (10 µl per 100 µl RNAzol B). After vigorous shaking and centrifugation, the aqueous phase was collected and an equal volume of acid phenol:chloroform (1:1) was added. After mixing and centrifugation the aqueous phase was collected and an equal volume of chloroform was added. Following centrifugation, an equal volume of isopropanol was added to the aqueous phase and 20 µg glycogen (Boehringer, Mannheim, Germany) was added as a carrier. After incubation at 4°C for at least 30 minutes, the RNA was pelleted by centrifugation and washed with 50 µl 75% ethanol per 10^6 cells. The RNA was resuspended in 10 µl DEPC treated water and the OD260/OD280 ratio was determined by spectrophotometry.

cDNA synthesis was performed starting with 1 µg total RNA, after heating for 10 min at 65°C. The reaction mixture contained 2 µl of 10x AMV-RT buffer (0.5 M Tris-HCl containing 0.1 M $MgCl_2$, 0.5 M DTT, 10 mM EDTA and 100 µg/ml BSA; pH 8.3), 0.25 mM of each deoxynucleotide triphosphate (dNTP, Boehringer), 1 mM spermine HCl (Sigma-Aldrich), 40 units of RNAguard (Pharmacia), 2.5 OD $(dN)_6$ (Pharmacia), 0.2 µg oligo$(dT)_{15}$ (Boehringer) and 5 units reverse transcriptase (AMV-RTase, Promega, Leiden, The Netherlands). The total reaction volume was 20 µl. Incubation was performed for 1 hour at 41°C. Afterwards the cDNA was diluted to 200 µl with DEPC water and stored at -70°C.

Quantitative "real time" PCR was performed using a 7700 Sequence Detector ABI PRISM on 25 ng cDNA. The PCR mixture consisted of TaqMan^R Universal PCR Master Mix (PE Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands), 900 nM of sense- and anti-sense primer, 250 nM FAM Dye labeled probe, 50 nM of sense- and anti-sense 18S rRNA primer and 50 nM 18S

rRNA VIC Dye labeled probe (PE Applied Biosystems). PCR was performed in a 96 well optical reaction plate (PE Applied Biosystems). Primers and probes for IL-4, IL-5, IL-10, IL-13 and IFN-γ (Predeveloped TaqMan Assay Reagents, PE Applied Biosystems) were specific and did not amplify genomic sequences. Distilled water and 25 ng genomic DNA were used as negative controls. The Thermal cycler conditions were set at 2 min 50°C, 10 min 95°C and subsequently 40 cycles of 15 sec 95°C and 1 min 60°C. The amount of PCR product was calculated by the sequence detection system computerprogram (PE Applied Biosystems) using the C_T Value (Cycle of Threshold) as read-out and quantitated against a standardcurve (100 pg to 0.001 pg) of a 10 fold dilution of plasmid DNA, containing the full-length cytokine cDNA of interest as insert. The input of cDNA was adjusted according to the amount of 18S rRNA.

Detection of cytokine protein production in PBMC/CBMC cultures

The levels of IFN-γ, IL-4, IL-10 and IL-13 in the culture supernatants were determined by Pelikan Compact™ human cytokine kits (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands) according to the manufacturers' instructions.

IL-5 was determined using an ELISA based on TRFK5 as a capture mAb in a concentration of 0.25 μg/ml in 0.1M carbonate buffer pH 8.2. Biotin-labeled rat anti-human IL-5 (0.25 μg/ml, JES1-5A10, Pharmingen, San Diego, CA) was used as detection mAb in combination with poly-streptavidine-peroxidase (CLB) diluted in PBS/10% FCS-HI. Standard IL-5 ranging from 1000 pg/ml to 15.6 pg/ml (recombinant human IL-5, Pharmingen) and samples were diluted in YM/1% HuS and run in duplicate. After each incubation step, plates were washed with PBS/0.05% Tween. Peroxidase activity was revealed by the addition of TMB (3,3',5,5'-tetramethylbenzidine) substrate (Kirkegaard & Perry Lab., Gaithersburg, MD). The reaction was stopped with 0.1 M H_3PO_4 and read at 450 nm. The lower detection limits were 2 pg/ml for IFN-γ, 1 pg/ml for IL-4, 2.5 pg/ml for IL-5, 2 pg/ml for IL-10 and 0.5 pg/ml for IL-13.

We used two different criteria to define Th phenotypes. The first one was based on differences in the levels of Th2 cytokines (IL-4, IL-5 and IL-13) and IFN-γ between children who developed atopy relative to those that did not. The second criterion was based on the proportional production of IL-4 to that of IFN-γ. A Th2 cytokine profile was defined as less than 5000 pg/ml IFN-γ and more than 5 pg/ml IL-4 protein production.

Statistical analyses

Statistical analyses were performed using SSPS for Windows, version 8.0. Parameters were tested using an independent sample t-test, paired sample t-test or the Mann-Whitney test. Correlation was assessed using Spearman's rank correlation coefficient. P-values less than 0.05 were considered significant.

RESULTS

Prevalence of atopic disease at 12 months of age

In order to facilitate the detection of potential differences in cytokine production profiles, a group of 133 high-risk newborns were selected. At the age of 12 months, the children were assessed for the presence of clinical symptoms of atopic disease defined as AD, asthma-like disease and food- or upper-airway allergy. At this time point 27/110 of these children (25%) had developed atopic disease, of which 23 children had AD (85%) and four children (15%) had asthma-like disease. Of the children with AD, 5 children had developed food allergy, whereas none of the children had developed upper-airway allergy at 12 months of age. These results confirm the reported atopy prevalence of 30% in children with an atopic mother (19).

Time course of cytokine production profiles

In order to assess the cytokine production capacity, IL-4, IL-5, IL-10, IL-13 and IFN-γ were determined in PBMC of cord blood and blood obtained at 6 and 12 months of age following polyclonal stimulation. Children were divided in two groups: those that did (atopic) and did not (non-atopic) develop atopy within the first 12 months of life.

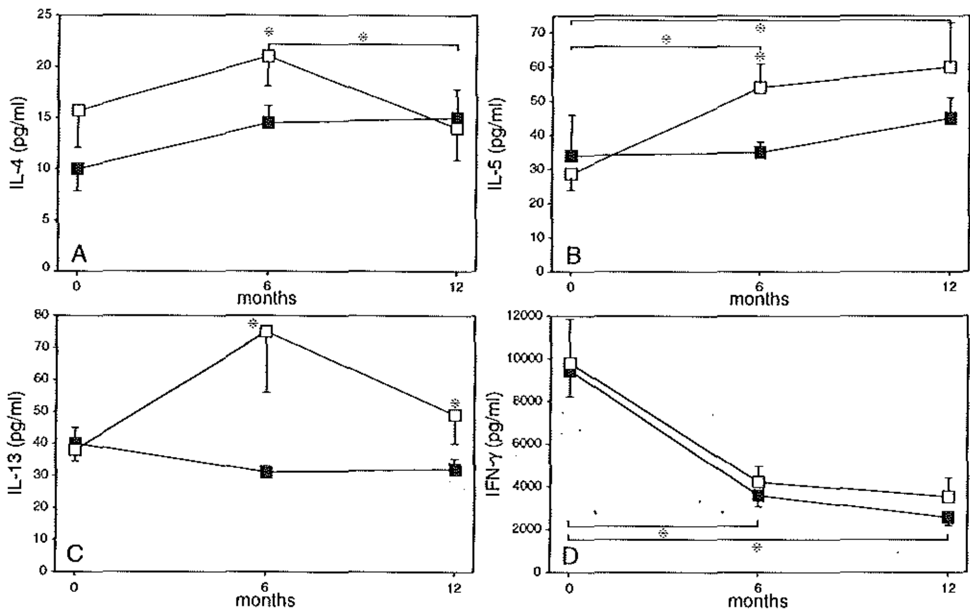

Figure 1. Time course of cytokine protein production by PBMC of children who did (atopic, open square) and did not (non-atopic, closed square) develop atopy at the age of 12 months. PBMC were isolated from blood samples obtained at birth, and 6 and 12 months of age. PBMC were polyclonally stimulated with PMA and Ca-ionophore for 16-18 hours. In the culture supernatants IL-4 (A), IL-5 (B), IL-13 (C) and IFN-γ (D) production were measured in duplicate by ELISA. Group means and standard deviations are shown. *$P<0.05$.

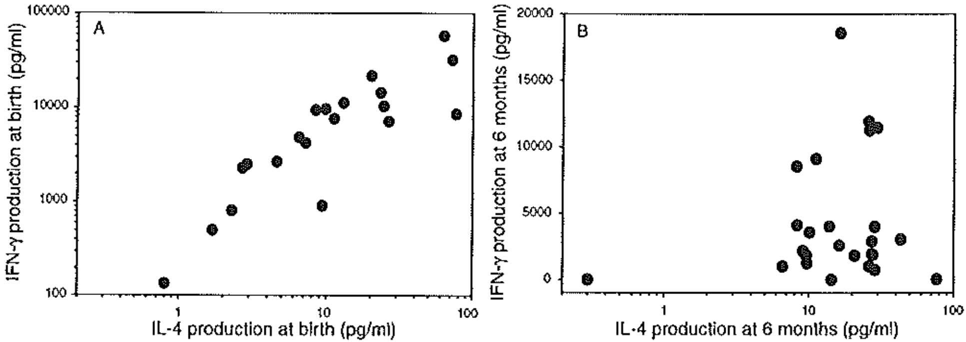

Figure 2. Concomitant production of IFN-γ and IL-4 protein in atopic children by PBMC isolated from cord blood (A) or blood obtained at 6 months of age (B). PBMC were polyclonally stimulated with PMA and Ca-ionophore for 16-18 hours after which IFN-γ and IL-4 production levels were determined by ELISA. Measurements were performed in duplicate.

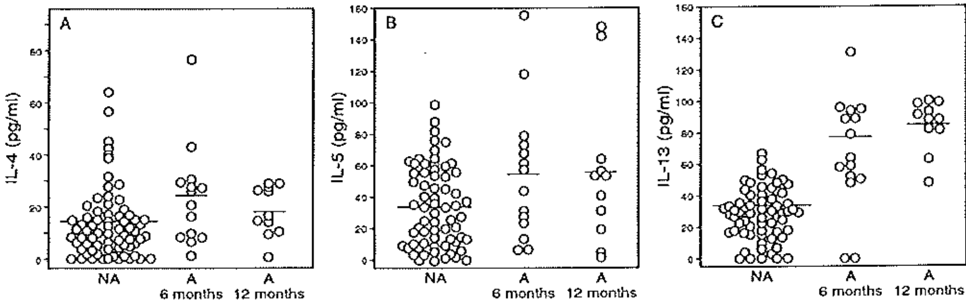

Figure 3. Cytokine production by PBMC of non-atopic children (NA), children who developed atopy within 6 months (A 6 months) and children who developed atopy between 6 and 12 months of age (A 12 months). PBMC were isolated from blood samples obtained at 6 months of age. PBMC were polyclonally stimulated with PMA and Ca-ionophore for 16-18 hours. In the culture supernatants IL-4 (A), IL-5 (B), and IL-13 (C) production were measured in duplicate by ELISA. Group means are indicated. *$P<0.05$.

None of the measured cytokine levels, including IFN-γ, which were present in supernatants of stimulated cord blood cells, were significantly different between atopic and non-atopic children (Fig. 1). In cord blood of all children, whether or not they developed atopy within 12 months, a Th0 profile was found, based on the concomitant production of both IL-4 and IFN-γ by the PBMC of the individual children (Fig. 2).

In supernatants of stimulated PBMC isolated from blood at 6 months of age, IL-4 (Fig. 1A), IL-5 (Fig. 1B) and IL-13 (Fig. 1C) were significantly increased in the atopic children compared to the non-atopic children. IFN-γ (Fig. 1D) and IL-10 (data not shown) levels were not different between both groups of children. Furthermore, at 6 months of age, 18 of the 25 atopic children showed a profound Th2 profile, while 7 of the 25 atopic children remained embedded in a Th0 profile as shown by the concomitant production of both IL-4 and IFN-γ by the individual children (Fig 2).

Of all children who had developed atopic manifestations at 12 months of age, about 50% had developed an atopic disease within 6 months of age, while the remainder developed their first atopic symptoms between 6 and 12 months of age. Analysis of cytokine production at 6 months of age in both groups of children revealed increased IL-4, IL-5 and IL-13 production for both subgroups compared to non-atopic children (Figs. 1 and 3).

At 12 months of age, IL-5 production was increased but not significantly, while IL-13 was significantly increased in the atopic children compared to the non-atopic children (Fig. 1). IL-4, IL-10 and IFN-γ were not significantly different between both groups of children at 12 months of age.

Kinetic analysis revealed a significant decrease in the production of IL-4 between the 6 and 12 months period for the atopic children (Fig. 1A). Moreover, in the atopic children the production of IL-5 increased significantly between birth and 6 months of age and between birth and 12 months of age (Fig. 1B). IL-13 production was significantly increased at 6 and 12 months of age between atopic and non-atopic children, but did not further increase between 6 months of age and 12 months of age. For IL-10 production no significant kinetic differences were found between atopic and non-atopic children. Interestingly, the capacity for IFN-γ production decreased significantly after birth till 6 months and then normalized, both for the atopic and non-atopic children (Fig. 1D).

The kinetic analyses in the atopic children revealed that after birth IL-4, IL-5 ($p<0.05$) and IL-13 production increased, while IFN-γ production decreased. This indicated a transition from a Th0 cytokine profile at birth to a Th2 cytokine profile at 6 months of age.

Time course of quantitative cytokine mRNA expression

In order to further validate and study the differences in cytokine protein levels between the different groups, we assessed cytokine mRNA expression by the sensitive and quantitative TaqMan PCR technology, in two random selected subgroups containing 15 atopic and 15 non-atopic children. All non-atopic children did not show signs of atopic disease until at least 24 months of age. The mRNA expression levels of IL-5, IL-10, IL-13 ($p<0.05$) and IFN-γ, except IL-4, were consistently increased in stimulated cord blood cells of atopic children compared to non-atopic children (Fig. 4). At 6 months of age the mRNA expression levels for IL-4 were still lower in atopics than in non-atopics. The mRNA expression levels for IL-5 (Fig. 4B) and IL-13 (Fig. 4C) were significantly increased in the atopic children compared to the non-atopic children at this time point. The mRNA expression levels of the other measured cytokines were not significantly different between both groups of children. These quantitative cytokine mRNA expression levels further support the transition from a Th0 profile at birth to a Th2 cytokine profile present at 6 months of age, selectively expressed in the atopic children. At 12 months of

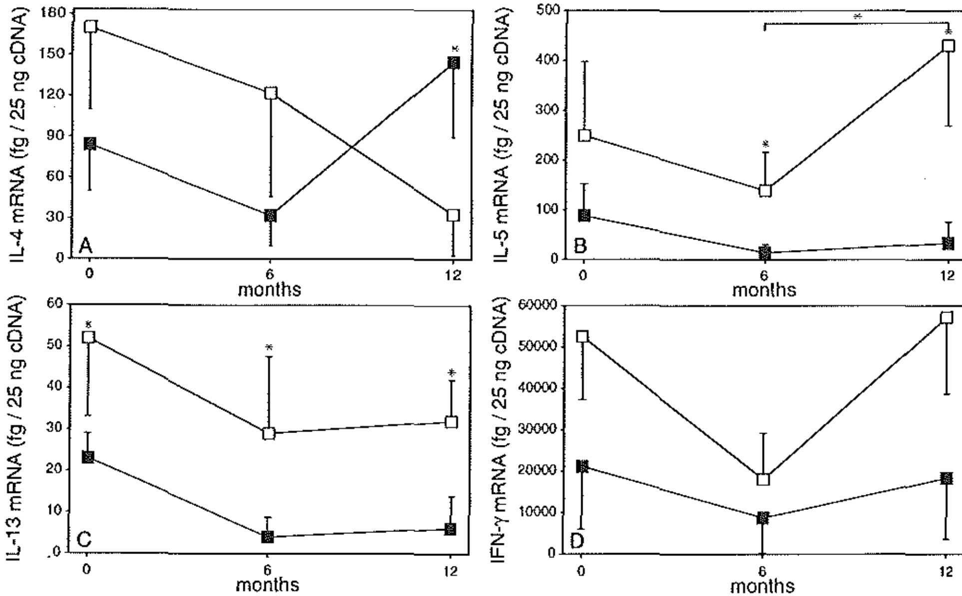

Figure 4. Time course of cytokine mRNA expression levels by PBMC of 15 atopic (open square) and 15 non-atopic (closed square) children at 12 months of age. PBMC were isolated from blood samples obtained at birth, and 6 and 12 months of age. PBMC were polyclonally stimulated with PMA and Ca-ionophore for 16-18 hours. RNA was isolated followed by cDNA synthesis and quantitative "real time" PCR by TaqMan technology. IL-4 (A), IL-5 (B), IL-13 (C), and IFN-γ (D) mRNA levels were determined. Group means and standard deviations are shown. *$P<0.05$.

age, IL-4 (Fig. 4A), IL-5 (Fig. 4B) and IL-13 (Fig. 4C) were significantly increased in atopic children, while IFN-γ (Fig. 4D) and IL-10 (data not shown) were increased, but not significantly.

In atopic children, IL-5 and IL-13 showed a quantitatively increased mRNA expression at all time points evaluated. In particular for IL-5 a further rise between 6 and 12 months was observed (Fig. 3A).

DISCUSSION

The proposed weak Th2 priming at birth in all children, independent of developing atopic disease has made it challenging to investigate whether the intrinsic capacity of T cells to give rise to Th1 or Th2 cytokine production profiles is different between atopy-prone children and children who do not develop atopy within their first year of life. Analysis of a cohort of children born to atopic mothers who have an assumed prevalence of 30% to develop atopy within 12 months, provides an unique oppotunity to examine such intrinsic capacity of T cells. In this study, we provide evidence for (a) a Th0 cytokine profile at birth, (b) a transition from a Th0 cytokine profile at birth to

a Th2 cytokine profile within 6 months of age in atopic children , (c) the absence of an intrinsic defect in the capacity of T cells of atopic children to produce IFN-γ and (d) the presence of increased Th2 cytokines already at 6 months of age in children who subsequently developed their first clinical symptoms of atopic disease between 6 and 12 months of age.

In newborns many immune functions are less mature compared to adults. The capacity of cord blood T cells to produce cytokines, such as IL-4 and IFN-γ was found to be diminished (10,11,20), the secretion of immunoglobulins by cord blood B cells is strongly decreased (12) and cord blood antigen-presenting cells are functionally immature (13). For the process of allergic sensitization, this relative immaturity of the immune system of newborns could create a window starting in utero and up to 12 months of age with increased susceptibility to develop atopy.

Here, we showed that at birth CBMC of all newborns whether or not they developed atopy before 12 months of age, had the capacity to induce a Th0 cytokine profile. This Th0 profile was based on the proportional production of IL-4 and IFN-γ in the culture supernatants, and is in contrast with the postulated (allergen-induced) Th2 profile (9). This Th2 profile was primarily based on reduced IFN-γ production (both after polyclonal and allergen-specific stimulation of CBMC) and the assumption that the Th2 milieu present in the placenta results in a Th2 milieu in the fetus (14,21-24). Another factor possibly influencing the cytokine profile is the delivery process. In our cohort, most children (>90%) were born by vaginal delivery. The vaginal delivery process of a child takes hours, and during this delivery-related stress-period for a fetus its immune system may become non-specifically activated *in vivo* (25,26). This is supported by the observed higher baseline proliferative response of CBMC of all newborns compared to the baseline proliferative response at 6 months of age (unpublished observation). In addition, all cytokine mRNA expression levels, as determined by quantitative "real time" PCR, were increased at birth compared to 6 months of age.

Our data are most consistent with a transition from a Th0 cytokine profile at birth to a Th2 cytokine profile at 6 months in atopics. This Th2 profile is defined as increased production of Th2 cytokines IL-4, IL-5 and IL-13 in atopics relative to non-atopics after polyclonal stimulation of PBMC, and was detectable at the mRNA expression level (IL-5, IL-13) but also at the protein production level (IL-4, IL-5, IL-13). We could not confirm that decreased IFN-γ production, as described in other studies, was responsible for the profound Th2 skewing present in early life (1-3,8,14). These studies described reduced mitogen induced IFN-γ production in children either at risk for atopy or in children who later developed atopy (1-3,14,15,21). Our results showed no intrinsic defect in the capacity of T cells to express or produce IFN-γ between children who did and did not develop atopy during the first 12 months of life.

An explanation for the discrepancy between our and other published reports concerning IFN-γ production could be a difference in patient groups that were studied. In this study, only high-risk (HR) newborns were included and divided in atopic and non-atopic at 1 year of age. Other studies compared HR with low-risk (LR) newborns and did not follow them with respect to the development of atopy (14,22) or compared HR children who developed atopy with LR children who did not develop atopy (8,15).

Additional factors that can lead to a discrepancy in the results between these studies and ours are the different definitions for atopy and the follow-up period of the newborns (between 1 and 3 years of age) as wells as protein or mRNA detection methods, culture conditions and stimulation protocols (1-3,8,14,15,21,22). For example, important differences between these studies and ours were the culture medium (RPMI, serum-free medium and YM), culture period (ranging between 18 hours and 72 hours) and the mitogenic stimulus (PHA, anti-CD3, PMA and Ca-ionophore) (1-3,8,14).

Optimal culture conditions and the combined analysis of mRNA expression levels and protein production are important to quantitate the production of cytokines (27). Especially, the analysis of IFN-γ is complicated by the rapid degradation of IFN-γ in culture supernatants. Here, the mRNA expression levels were determined by a quantitative "real time" PCR using TaqMan technology, which is a very sensitive method to detect small differences in expression levels between different patients samples and patient groups (28).

We used the stimulus PMA plus Ca-ionophore to study intrinsic differences in cytokine profiles. This stimulus does not involve triggering of the T cell receptor (TCR)/CD3 complex, and results in maximal T cell stimulation and high production levels of both Th1 and Th2 cytokines (18,29). Therefore, possible differences in IFN-γ production levels must be a consequence of a difference in regulatory mechanisms between atopic and non-atopic children, also suggested by others (30,31).

Using these defined methods and conditions we could exclude an intrinsic T cell defect for IFN-γ production. However, for the *in vivo* situation we can not exclude a difference in IFN-γ production between atopic and non-atopic children, as stimulation conditions, APC and other factors, including genetic predisposition can not be duplicated *in vitro*.

We found evidence for a significantly increased IL-4 production in atopic children at 6 months of age compared to non-atopics. This finding reveals a window of time in which the active induction of a Th2 cell population may occur. This short-lived increase in IL-4 production, concomitant with an active development of Th2 cells correlated in time with the first signs of clinical symptoms characteristic of an atopic disease.

Analysis of cytokine mRNA and protein production revealed that children who developed their first atopic symptoms between 6 and 12 months of age, already displayed a Th2 cytokine profile at 6 months of age. These children were predisposed to express an 'atopic' Th2 cytokine

production profile before the onset of clinical symptoms. Therefore, a Th2 cytokine profile could possibly be used as a predictor for the onset of atopy development. Further research is necessary to investigate this predictive factor in more detail.

Our study demonstrates that the first 6 months of life is the crucial period for the initiation of immunological changes reflecting the (previously started) allergic sensitization, which is clinically detectable at 12 months. At birth all children expressed a Th0 cytokine profile, which gradually shifted to a Th2 cytokine profile in children who became atopic at 6 or 12 months of age. During this window of time a strong and active induction of a Th2 cytokine profile occurred, resulting in the presence of IL-4, IL-5 and IL-13, rather than an inhibition of the production of IFN-γ protein. This indicates that T cells of atopics have no intrinsic defect in their capacity to produce IFN-γ protein. We therefore conclude that the selective development of a Th2 skewing in atopics is independent of the production of IFN-γ.

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T CELL RESPONSES AND SOLUBLE ADHESION MOLECULES IN CHILDREN WITH ESTABLISHED ATOPY

CHAPTER 7

PEANUT-ALLERGEN-SPECIFIC STIMULATION OF PBMC IN CHILDREN WITH ATOPIC DERMATITIS

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SUMMARY

To study the reactivity of peripheral blood mononuclear cells (PBMC) in peanut-allergic (PA) children, a peanut-allergen specific proliferation assay was developed. Peak proliferative responses occurred at 500 µg/ml peanut-extract on day 7. proliferation to peanut-allergen stimulation was present in PA, but not in peanut-non-allergic (PNA) children. After stimulation IFN-γ mRNA expression measured by RT-PCR was optimal on day 4 of culture. IFN-γ mRNA expression and production decreased in PBMC of PA patients in contrast to PNA children after peanut-allergen stimulation.

INTRODUCTION

Approximately 60% of children with severe atopic dermatitis have allergic reactions to food constituents: mostly cow's milk, egg, peanut and soy. Peanuts are considered to be one of the most allergenic foods and multiple cases of fatal or near-fatal anaphylaxis have been reported [1].

We studied the role of T cells and cytokines in food allergy in children, and peanut allergy was used as a model. A peanut-allergen specific proliferation assay was developed. Furthermore, the optimal conditions were examined for in vitro IFN-γ mRNA expression and production after peanut-allergen specific stimulation of PBMC of young children. Analysis of proliferative and cytokine responses to peanut-allergen are relevant for understanding of the immune responses to peanut-allergen and is essential for the development of an effective more casual therapy.

MATERIAL AND METHODS

Subjects

Three PA patients (mean age 3.9 years) and three PNA controls (mean age 2.9 years) were selected from the Sophia Children's Hospital in Rotterdam. All PA patients had a positive immediate skin prick test reaction, a positive radio-allergo-sorbent test (RAST) to peanut and a history of mild to severe eczema according to the criteria of Hanifin and Rajka [2]. All PNA patients were healthy controls without eczema or food-allergy.

Lymphocyte proliferation assay

PBMC from heparinized venous blood were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). PBMC were washed three times and resuspended at 2.0×10^6 cells/ml in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1 mM pyruvate and 10% heat-inactivated human serum. Proliferation assays were performed in quadruplicate in 96-wells round-

bottomed plates with $2x10^5$ cells/well in the presence or absence of different concentrations of peanut-extract (ALK, Groningen, The Netherlands). Tetanus toxoid (2 If/ml; RIVM, Bilthoven, The Netherlands) was used as positive control. Culture times varied from 5 to 8 days as indicated in the results. The last 8 hours before harvesting, the cells were cultured in the presence of 0.5 µCi/well methyl-[^{3}H]-thymidine. The results are expressed as stimulation index (SI), equal to the ratio of mean cpm in antigen-stimulated cultures to mean cpm in unstimulated cultures.

Analysis of IFN-γ mRNA transcripts and production in PBMC cultures

PBMC were cultured in 24-wells plates, $1x10^6$ cells/well, for various time points in Yssels medium [3] containing 1% human serum with and without the addition of peanut-extract at 37°C, 5% CO_2. After harvesting the cells, supernatants were stored at -80°C and RNA was isolated from the cells by the RNAzol B method [3]. 1 µg RNA was used for cDNA synthesis and amplification by semi-quantitative reverse-transcriptive polymerase chain reaction (RT-PCR) using primers specific for either HPRT (housekeeping gene) or IFN-γ [3]. Production of IFN-γ was determined by ELISA (Eurogenetics, Tessenderlo, Belgium).

RESULTS AND CONCLUSIONS

Optimization of the proliferative response of PBMC to peanut-extract

Peanut-induced proliferation assays were performed using PBMC of PA and PNA children. To determine the optimal concentration of peanut-extract for proliferation, dose-response studies were performed with concentrations ranging from 50 to 1000 µg/ml. Maximum proliferation was observed with 500 µg/ml peanut-extract (SI = 11; Fig.1A).

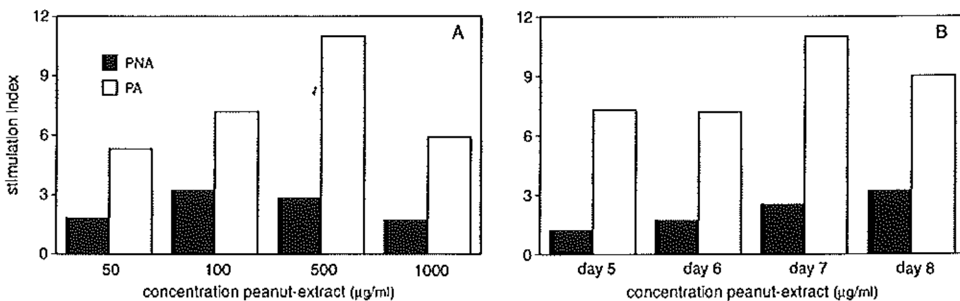

Figure 1. Proliferative responses of PBMC of PNA (■) and PA (□) children after stimulation with peanut-extract. The data of a representive experiment are shown. (A) dose-dilution curves with different concentrations of peanut-extract (50 to 1000 µg/ml). (B) time-response curves with various culture times (5 to 8 days).

The optimal incubation period was determined by kinetic studies of the proliferative response (Fig.1B). On day 7 after peanut-allergen stimulation the SI was maximal (SI = 11). In contrast to PA patients, the PBMC of PNA children did not react to peanut-allergen. Dorion et al.[4] did not observe a difference in peanut-allergen specific proliferation between adult PA patients and normal controls. This discrepancy most likely results from differences in the (patho)physiology of the immune system of children and adults [5].

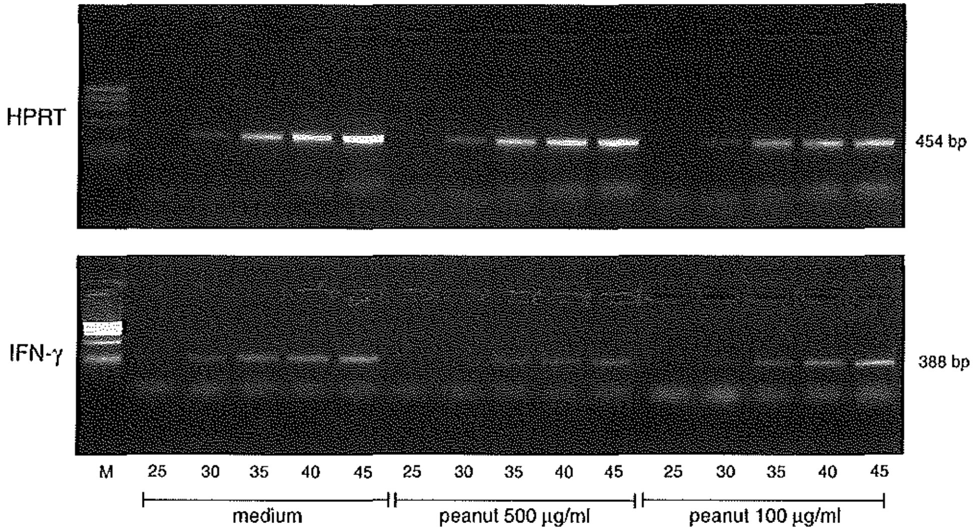

Figure 2. HPRT and IFN-γ mRNA expression in a PA child. PBMC were stimulated with or without peanut-extract. Cells were harvested on day 4 and PCR was performed for HPRT and IFN-γ. Samples were collected from the reaction tube after different numbers of cycles. The Hae 111 digest of Ph1X174 was used as a molecular weight marker (M).

IFN-γ mRNA expression in response to peanut-allergen stimulation

PBMC of PA and PNA children were stimulated with 100 μg/ml and 500 μg/ml peanut-extract. Cells were harvested on days 3 to 6.

Day 4 was optimal to observe IFN-γ mRNA expression (data not shown). When comparing between PA and PNA children IFN-γ mRNA expression decreased after peanut-allergen specific stimulation (500 μg/ml) in PBMC of PA patients (Fig. 2), but not in PNA patients (data not shown). The lower peanut concentration (100 μg/ml) did not decrease the IFN-γ mRNA expression.

After culturing PBMC in the presence or absence of peanut-extract no difference in expression level of the HPRT house-keeping gene was found.

IFN-γ production after peanut-allergen stimulation

After peanut-allergen (500 μg/ml) stimulation of PBMC of both PA and

PNA patients, supernatants were collected on day 4 for the measurement of IFN-γ levels by ELISA. Supernatants of PBMC of PNA children contained 23.0 U/ml and 16.0 U/ml IFN-γ. In supernatants of PA patients lower IFN-γ levels (4.4 U/ml and 9.9 U/ml) were detected, corresponding to the decreased levels of IFN-γ mRNA in these patients.

This study shows we have developed a reliable peanut-allergen proliferation assay, using 500 µg/ml peanut-allergen with a maximal incubation period of 7 days. IFN-γ mRNA expression and production could be detected after peanut-allergen peanut-allergen stimulation and was optimal on day 4. Further analysis is in progress to increase understanding of T cell and cytokine regulation in relation to peanut-allergy.

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CHAPTER 8

CD4^{+} CELLS PROLIFERATE AFTER PEANUT-EXTRACT-SPECIFIC AND CD8^{+} CELLS PROLIFERATE AFTER POLYCLONAL STIMULATION OF PBMC OF CHILDREN WITH ATOPIC DERMATITIS

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SUMMARY

Background Few studies describe *in vitro* food-allergen induced proliferative responses and cytokine production of PBMC of children with atopic dermatitis. This is especially true for peanut-allergen.

Objective To analyze the specificity of the T cell in proliferative responses, in children with atopic dermatitis with or without peanut allergy and healthy age-matched children.

Methods Proliferative responses were measured by [^{3}H]-thymidine incorporation and by expression of the intracellular Ki67-antigen using flow cytometry after antigen-specific stimulation of PBMC with peanut-extract (day 7) or polyclonal stimulation with Phorbol-12-myristate-13-acetate and Ca-ionophore (day 3). Cytokine mRNA (Interferon-γ (IFN-γ), IL-4) was detected by semi-quantitative RT-PCR. Cytokine production (IL-4, IFN-γ) was measured by ELISA.

Results Peanut-extract induced proliferative responses of PBMC from children with atopic dermatitis and peanut allergy (AD^+PA^+) were significantly higher as compared to the other studied groups. Ki67-antigen double staining revealed that 80-100% of the proliferating cells were $CD4^+$. These proliferative responses correlated significantly with the increase in IL-4 mRNA expression after peanut-extract specific stimulation. After polyclonal stimulation, however, $CD8^+$ cells preferentially proliferated. The degree of proliferation after polyclonal stimulation correlated inversely with the ratio of IL-4/IFN-γ production.

Conclusion The principal responding population of T cells in proliferative responses is different after peanut-extract specific and polyclonal stimulation of PBMC from AD^+PA^+ patients. Furthermore, we found indirect evidence that the PBMC fraction of AD^+PA^+ children contains increased frequencies of peanut-specific T helper-2 cells.

INTRODUCTION

Atopic dermatitis (AD) is an intensely pruritic inflammatory skin disorder, especially common in infants [1]. Food allergens and aeroallergens besides genetic determinants, are considered to play an important role in the pathogenesis of AD [2]. Approximately 60% of children with severe AD have allergic reactions to food constituents: mostly cow's milk, egg, peanut and soy [3,4]. Allergic reactions and allergies represent the combination of a positive skin test and clinical symptoms. The prevalence of allergies to cow's milk and egg tend to decrease with increasing age, while allergy to peanut persists with increasing age [5]. Peanuts are the subject of increasing concern based on an increasing number of reports of fatal or near-fatal anaphylaxis and local allergic reactions [6].

Profound immunological dysregulation with various immune alterations has been described in children with AD [7,8]. Several findings suggest a role for IgE-mediated food hypersensitivity in the pathogenesis of AD. It has been shown that two thirds of AD children have positive family histories for atopic disease [9]. About 50% to 80% of children with AD develop allergic rhinitis or asthma [10]. Moreover, serum total IgE concentrations were elevated in about 80% of children with AD [11] and most of these children had positive immediate skin tests and serum allergen-specific IgE antibodies to various dietary allergens [12].

Furthermore, it appears that some forms of cell-mediated immunity may be involved in the pathogenesis of AD, as observed from the cellular infiltrate in eczematous skin lesions [13]. Most cells in eczematous skin lesions are $CD4^+$ helper T (Th) cells, many of which are considered to be allergen-specific Th2 cells [14,15].

Activated Th2 cells secrete a variety of cytokines, including IL-4, IL-5, IL-10 and IL-13 [16], which play a role in the down-regulation of Th1 activity, mainly IFN-γ production [17]. It has been demonstrated that IL-4 acts as an isotype switch inducing factor that commits B cells to the IgE pathway [18-20]. In contrast, IFN-γ inhibits *in vivo* and *in vitro* IgE synthesis [21-23]. The allergenic specificity of infiltrating Th2 cells generally correlates with the presence of allergen-specific IgE antibodies.

Few studies, all using adult patients, describe *in vitro* food-allergen induced proliferative responses of isolated PBMC [24]. Such studies are especially lacking for the peanut-allergen [25]. Kondo et al. [26] described that in AD patients who were sensitive to hen's egg, the $CD4^+$ T lymphocytes proliferate predominantly after *in vitro* ovalbumin stimulation. It is well established that T cells proliferate *in vitro* to soluble antigens such as tetanus toxoid presented by antigen-presenting cells. However, the evidence that this can also occur in the case of food-antigens, requires confirmation.

Ki67-antigen is a nuclear antigen that is expressed in actively cycling cells, but not in resting G_0 cells. Ki67-antigen levels increase during the S phase with a maximum in the G_2/M phase of the cell cyclus [27]. By a double staining method using Ki67-antigen and lymphocyte cell surface markers it is possible to determine which cell population is proliferating [28].

We studied the T cell subsets, $CD4^+$ and $CD8^+$ cells, of proliferative responses by Ki67-antigen expression after peanut-extract specific and polyclonal stimulation of PBMC from children with AD with or without peanut-allergy and from healthy children. Furthermore, we studied in these children relationships between proliferative responses, cytokine gene expression and production.

Table 1. Characteristics of the patient groups.

	n	Age (months)	Sex distr.	Total IgE (kU/l)	T cells (%)	B cells (%)	$CD4^+$T cells (%)	$CD8^+$T cells (%)	CD4/CD8 ratio	NK cells (%)	monocytes (%)
Atopic dermatitis											
PA^+	13	51 (17-78)	9♂ 4♀	$605^{p=0.02}$ (8-2747)	62.4±6.0	18.7±1.2	39.3±3.2	23.3±3.3	1.8±0.2	5.6±1.5	11.6±0.9
PA^-	8	46 (13-95)	2♂ 6♀	$808^{p=0.005}$ (58-1990)	53.2±5.5	22.9±5.1	32.8±4.2	19.8±3.1	1.7±0.2	8.0±2.8	12.0±2.5
Healthy children	10	45 (19-86)	6♂ 4♀	18 (0-137)	59.8±4.1	18.0±3.1	37.9±4.9	20.1±2.9	2.0±0.4	9.2±2.6	8.1±4.0

Ages are expressed in months (range). Total IgE levels were determined by IgE CAP RAST and are expressed as kU/l.
PBMC were stained with monoclonal antibodies specific for T cells, B cells, $CD4^+$ and $CD8^+$ T cells, NK cells and monocytes after which flow cytometry was performed. Data represent mean percentages of a cell population from the PBMC fraction ± SEM. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

MATERIAL AND METHODS

Patients

We studied children suffering from atopic dermatitis and peanut allergy (AD^+PA^+), children suffering from AD without peanut allergy (AD^+PA^-) and healthy children (HC) (Table 1). The Medical Ethical Committee of the University Hospital Rotterdam approved this study. Informed consent was obtained from the parents of the children prior to participation in the study.

AD patients were selected on a history of mild to severe eczema according to the diagnostic criteria of Hanifin & Rajka [29]. All AD^+PA^+ children had a positive scratch test or skin application food test (SAFT) [30] specific for peanut and a positive RAST [31] specific for peanut (Pharmacia, Uppsala, Sweden) in plasma. None of the AD^+PA^+ patients had a history of systemic anaphylaxis to peanuts. AD^+PA^- children had a positive scratch test or SAFT specific for milk, egg, wheat, soja or codfish, but not peanut, and also a positive RAST for inhalant allergens (Phadiatop cap RAST, Pharmacia) or food mix (Pharmacia) in plasma, but not for peanut. The healthy controls had no history of dermatitis or allergy and a negative family history as judged by a questionnaire. All healthy children had no specific IgE for inhalation and/or food allergens in plasma as measured by RAST (Phadiatop and food mix).

Lymphocyte proliferation assay

Five ml of heparinized venous blood was collected and PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia) [32]. PBMC were washed two times and resuspended in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1 mM pyruvate and 10% heat-inactivated human serum pooled from healthy donors. This serum pool was previously shown not to induce non-specific stimulation.

Proliferation assays were performed in quadruplicate in 96-wells round-bottomed plates (Falcon, Becton Dickinson & Company, Mountain View, CA) at 2×10^5 cells/well in the presence of PMA (2 ng/ml; phorbol-12-myristate-13-acetate; Sigma, St.Louis, MO) and 4-bromo-calcium-ionophore (1 µg/ml; A23187, Sigma) or peanut-extract (500 µg/ml; ALK-Benelux, Groningen, The Netherlands) or tetanus toxoid (2 lf/ml; RIVM, Bilthoven, The Netherlands) or without additions in a total volume of 200 µl. The amount of endotoxin in the peanut-extract was measured using the Limulus amebocyte lysate micromethod [33] and was below 12.5 EU/mg. At 3 days of polyclonal stimulation (PMA+ Ca-ionophore) or 7 days of allergen-specific stimulation, proliferation was measured using tritium-($[^3H]$)-thymidine incorporation. Optimized doses and kinetic conditions were used as described previously [34]. After addition of $[^3H]$-thymidine (0.5 µCi/well; Amersham, Aylesbury, UK) the plates were incubated for 8 hours before harvesting. $[^3H]$-thymidine incorporation was measured with a BetaPlate (Pharmacia LKB, Turku, Finland).

The results are expressed as SI, equal to the ratio of mean cpm in polyclonal- or antigen-stimulated cultures over mean cpm in unstimulated cultures.

Flow cytometric determination of proliferation by Ki67-antigen expression

Polyclonal and peanut-extract specific proliferation of the PBMC fraction of 4 AD^+PA^+ patients was also studied by intracellular expression of the nuclear Ki67-antigen by flow cytometric analysis [28].

Cultured cells were collected at day 3 after polyclonal stimulation and at day 7 after peanut-extract specific stimulation and stained for immunophenotyping. The cells were washed twice with wash solution (PBS containing 0.5% BSA and 20 mM sodium azide). After spinning down the cells for 5 min 400 g at 4°C, the cells were resuspended in 50 µl PBS containing 0.5% BSA and 20 mM sodium azide. The following monoclonal antibodies specific for cell-surface antigens were used for the staining of the cells: B cells by 50 µl B4-PE (4 µg/ml; Coulter Cytometry, Hialeah, FL), T cells by 50 µl Leu4-PE (2.5 µg/ml; Becton Dickinson), monocytes by 50 µl My4-PE (2.5 µg/ml; Coulter Cytometry), NK cells by a combination of 50 µl CD16-PE and 50 µl CD56-PE (both 2.5 µg/ml; Becton Dickinson), $CD4^+$ cells by 50 µl Leu3-PE (10 µg/ml; Becton Dickinson), $CD8^+$ cells by 50 µl Leu2-PE (12.5 µg/ml; Becton Dickinson) and as an isotype control 50 µl IgG1-PE (2.5 µg/ml; Becton Dickinson) and 50 µl IgG2-FITC (2.5 µg/ml; Becton Dickinson). The cells were incubated at room temperature for 10 min, after which they were washed twice with wash solution. For staining of Ki67-antigen [28], the cells were resuspended in 1 ml freshly prepared FACS Lysing solution (Becton Dickinson) and after vortexing thoroughly kept at room temperature for 10 min. After washing twice, FITC-conjugated anti-Ki67 antibody (Dako, Glostrup, Denmark) or a control IgG1-FITC antibody (Becton Dickinson) was added. The cells were subsequently incubated for 10 min at room temperature. After intracellular staining, cells were washed twice and resuspended in 100 µl isotonic FACSflow solution (Becton Dickinson). Flow cytometric analysis was performed on a FACScan (Becton Dickinson).

Analysis of IL-4 and IFN-γ mRNA expression and production in PBMC cultures

PBMC were cultured in 24-wells plates (Falcon), at 1×10^6 cells/well in Yssel's medium (YM) [35] containing 1% heat-inactivated human serum either with the addition of 2 ng/ml PMA and 1 µg/ml Ca-ionophore or the addition of 500 µg/ml peanut-extract in a total volume of 1 ml at 37°C, 5% CO_2. PBMC stimulated with PMA + Ca-ionophore were cultured for 16-18 hr, and PBMC stimulated with peanut-extract were cultured for 4 days [34]. After harvesting the cells, supernatants were stored at -80°C and RNA was isolated from the cells by the RNAzol B method [36] (Cinna-Biotecx Laboratories Inc., Houston, TX). As described previously [32], 1 µg RNA was used for cDNA synthesis and

amplification by semi-quantitative RT-PCR using primers specific for either the housekeeping gene HPRT (hypoxanthine phosphoribosyl transferase), IL-4 or IFN-γ [32]. After the PCR amplification, the 5 samples, taken after 5 different cycle numbers, were loaded on a 1.2% agarose gel (Sea Kem Leagarose, FMC Bio Products, Rockland, ME), stained with 75 μg/500 ml ethidium bromide (Boehringer Mannheim, Germany). 0.5 μl PhiX174 (HaeIII digest, 0.5 μg/μl, New England Biolabs, Beverly, MA) was used as marker. The bands of the PCR products on the gel were visualized with UV light. A life-size photo was taken and used for scanning. Collected scan values were analyzed as follows: the mean scan values were calculated at two different cycle numbers (30-35 cycles for HPRT and IFN-γ, 35-40 cycles for IL-4), in the linear phase of the PCR reaction. In order to correct for inter-experimental variation a batch of cDNA of B21 (a Th0 clone, Paliard [37]) was used for standardization of HPRT, IFN-γ and IL-4 expression. The scanvalues obtained were further corrected for differences in the quality of isolated RNA and efficacy of the cDNA reaction by dividing the corrected scan values of IFN-γ and IL-4 by corrected HPRT values of the same sample. In the Results section some data are presented as ΔIL-4 mRNA or ΔIFN-γ mRNA. This refers to the IL-4 or IFN-γ mRNA scanvalue after peanut-extract specific stimulation divided by the scanvalue of cultured but unstimulated PBMC of the same patient.

The levels of IFN-γ and IL-4 in the culture supernatants were determined by ELISA (IFN-γ Eurogenetics, Tessenderloo, Belgium; IL-4 CLB, Amsterdam, The Netherlands) according to the manufacturers instructions. The lower detection limit was 5 U/ml for IFN-γ and 3 pg/ml for IL-4.

Statistical analyses

Statistical analyses were performed with STATA™ (Computing Resource Center, Los Angeles, CA). Normally distributed parameters were compared using the Student's t-test and correlation coefficients were calculated by linear regression. Non-parametric parameters were tested using the Mann-Whitney test and correlation coefficients (r_s) were calculated by the Spearman sign-rank correlation. In either test, P values smaller than 0.05 were considered significant.

RESULTS

Proliferative responses of PBMC

Table 2 shows the proliferative responses of PBMC to peanut-extract, tetanus toxoid and PMA + Ca-ionophore in the three patient groups, AD^+PA^+, AD^+PA^- and HC. The peanut-extract induced proliferative responses of PBMC in AD^+PA^+ patients (mean SI = 7.3) were significantly increased compared to the proliferative responses of PBMC of AD^+PA^- (mean SI = 2.7; p = 0.041) and

Table 2. Proliferative responses of PBMC from children with atopic dermatitis with and without peanut-allergy and from healthy children.

	Atopic dermatitis		Healthy children
	PA+ (n=13)	PA- (n=8)	(n=10)
Peanut-extract			
Baseline cpm	1721.6±1683.1*	2840.7±2397.7	1472.8±1097.3
Total cpm	9283.7±8753.8	7040.1±7376.6	2283.6±2045.1
SI (range)	7.3 (1.6-21.0)	2.7**(1.0-5.9)	1.3***(1.0-3.2)
Tetanus toxoid			
Baseline cpm	1721.6±1683.1	2840.7±2397.7	1472.8±1097.3
Total cpm	17766.6±1308.1	24186.1±17312.2	15241.2±9810.2
SI (range)	20.0 (4.4-79.4)	22.6 (3.4-82.6)	10.9 (3.1-34.2)
PMA + Ca-ionophore			
Baseline cpm	409.8±148.8	543.5±242.9	551.4±327.9
Total cpm	18181.7±14562.9	26740.4±2397.7	24275.5±10904.6
SI (range)	49.2 (10.1-105)	46.2 (23.5-73.2)	55.7 (16.8-151.7)

PA+ = peanut allergic AD children; PA- = AD children without peanut-allergy. PBMC were incubated for 3 days with PMA (2 ng/ml) + Ca-ionophore (1 µg/ml) or 7 days with peanut-extract (500 µg/ml) or tetanus toxoid (2 lf/ml) and then assessed for proliferation by [^{3}H]thymidine incorporation. Results are expressed as * mean cpm ± SD or median SI (range). ** Indicates p=0.041 as compared to AD+PA+; *** Indicates p = 0.0003 as compared to AD+PA+.

HC (mean SI=1.3; p=0.0003). In the children with AD (both AD+PA+ and AD+PA-), tetanus toxoid induced proliferative responses, expressed as mean SI, were consistently higher, but not significantly (p=0.16), as compared to healthy controls. The baseline proliferation after 7 days of culture were higher than after 3 day cultures. The polyclonal proliferative responses to PMA + Ca-ionophore were not significantly different between the three patient groups.

Relationship between proliferative responses and cytokine expression and production

After PMA + Ca-ionophore stimulation no significant correlation was observed between the degree of proliferation and the production of either IFN-γ (p=0.91) or IL-4 (p=0.83). The PMA + Ca-ionophore induced proliferative responses and the ratio of IL-4/IFN-γ production showed a significant inverse correlation (r_s= -0.81, p=0.049; Fig. 1).

After peanut-extract induced stimulation, IL-4 production could not be measured in any of the culture supernatants and only in 2 out of 8 AD+PA+ patients we were able to detect IFN-γ production. Therefore, we studied the relationship between ΔIL-4 or ΔIFN-γ mRNA expression after peanut-extract specific stimulation and the peanut-extract induced proliferative responses. The ΔIL-4 or ΔIFN-γ refers to the IL-4 or IFN-γ mRNA scanvalue after peanut-extract

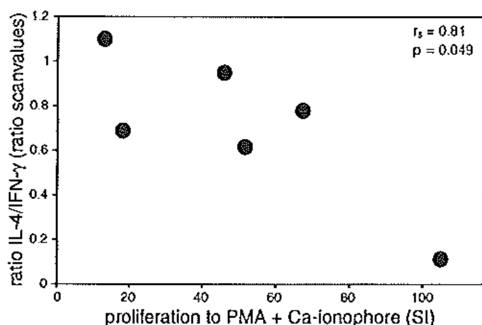

Figure 1. Correlation between proliferative responses to polyclonal stimulation and the ratio of IL-4/IFN-γ production in the culture supernatants of PBMC from AD^+PA^+ patients (r_s=-0.81, p=0.049). Proliferative responses (SI) were measured on day 3 of culture. IL-4 and IFN-γ production, measured after 16-18 hours of culture with PMA + Ca-ionophore, was expressed as ratio of levels of IL-4 over levels of IFN-γ.

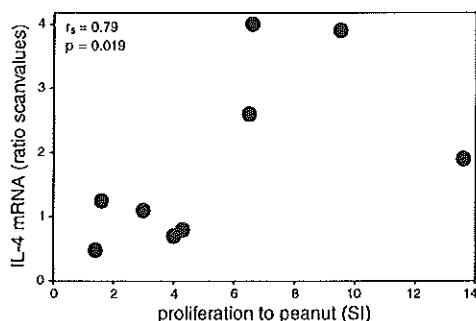

Figure 2. Correlation between the proliferative responses to peanut (expressed as SI) and the ratio of IL-4 mRNA (expressed as ratio of scanvalue after peanut-extract specific stimulation over scanvalue of no addition) with a correlation coefficient of r_s=0.79 and p=0.019.

specific stimulation divided by the scanvalue of cultured but unstimulated PBMC of the same patient. In cultures of PBMC of AD^+PA^+ patients a significant positive correlation (r_s=0.79, p=0.019) was observed between the degree of proliferative responses and the ΔIL-4 mRNA after peanut-extract specific stimulation (Fig. 2). No significant relationship could be detected between ΔIFN-γ mRNA and the proliferative responses to peanut-extract.

Secrist et al. [37] described that the optimal allergen doses for proliferation and cytokine production were different. Therefore, dose-response curves for cytokine production were made for different concentrations of peanut-extract, ranging from 500 µg/ml to 500 ng/ml. No differences were observed in cytokine production levels after stimulation of PBMC with different concentrations of peanut-extract (data not shown).

Phenotype of stimulated cells

FACScan analyses were performed to determine the cellular distribution in the PBMC fraction. No significant differences were found in the percentages of $CD4^+$, $CD8^+$, and $CD3^+$ T cells, B cells, monocytes and NK cells present in the PBMC fractions from AD^+PA^+, AD^+PA^- or HC children (Table 2).

A proliferative response to peanut-extract was observed in PBMC of AD^+PA^+ patients only. The degree of the proliferative responses to PMA + Ca-ionophore was not different between the three patient groups. Therefore, the phenotype distribution after stimulation was only determined in PBMC of

Table 3. Surface marker phenotype of cultured PBMC AD⁺PA⁺ children stimulated with peanut-extract.

AD^+PA^+	$CD3^+$lymphocytes (%)[a]			$CD4^+$lymphocytes (%)			$CD8^+$lymphocytes (%)			B-lymphocytes (%)		
Patients	medium[b]	peanut[c]	p-m[d]	medium	peanut	p-m	medium	peanut	p-m	medium	peanut	p-m
1	68.4	66.3	-2.1	30.6	33.2	+2.6	28.9	29.2	+0.3	22.9	20.8	-2.1
2	68.7	68.2	-0.5	34.4	43.8	+9.4	23.4	18.1	-5.3	25.7	20.7	-5
3	56.5	59.2	+2.7	39.5	44.0	+4.5	14.8	11.7	-3.1	33.8	27.1	-6.7
4	67.1	63.3	-3.8	44.0	47.6	+3.6	15.4	15.0	-0.4	25.6	28.6	+3
			-0.93 ± 1.3[e]			$+5.0 \pm 1.5^{p=0.029}$			-2.1 ± 1.2			-2.7 ± 2.1

[a] Percentage positive cells in 7 days cultured PBMC. [b] Cultures without addition. [c] Cells stimulated with peanut-extract (500 µg/ml). [d] Subtracting the percentage in 'b' from the percentage in 'c'[26]. [e] Mean ± SEM

Table 4. Surface marker phenotype of cultured PBMC from AD⁺PA⁺ children stimulated with PMA + Ca-ionophore.

AD^+PA^+	$CD3^+$lymphocytes (%)a			$CD4^+$lymphocytes (%)			$CD8^+$lymphocytes (%)			B-lymphocytes (%)		
Patients	medium[b]	PMA+Ca[c]	t-m[d]	medium	PMA+Ca	t-m	medium	PMA+Ca	t-m	medium	PMA+Ca	t-m
1	59.9	52.7	-7.2	45.4	29.1	-6.3	22.6	33.6	+11	25.1	35.3	+10.2
2	60.5	71.8	+11.3	27.7	31.7	+4.0	22.7	46.8	+24.1	28.5	30.9	+2.4
3	60.9	53.1	-7.8	38.4	36.1	-2.3	24.9	42.3	+17.4	16.8	24.0	+7.2
4	69.5	67.8	-1.7	46.3	50.5	+4.2	25.5	38.5	+13.0	17.1	27.0	+9.9
			-1.35 ± 4.4[e]			-2.6 ± 4.8			$16.4 \pm 2.9^{p=0.029}$			$7.4 \pm 1.8^{p=0.029}$

[a] Percentages in 3 days cultured PBMC. [b] Cultures without addition. [c] Cells stimulated with PMA (2 ng/ml) + Ca-ionophore (1 µg/ml). [d] Subtracting

AD^+PA^+ patients. After *in vitro* stimulation of PBMC from AD^+PA^+ patients with peanut-extract for 7 days, the proportion of $CD4^+$ T-lymphocytes was significantly increased (p=0.029) compared to medium (Table 3). The percentages of $CD3^+$ cells, $CD8^+$ T cells and B cells did not change significantly compared to medium. The percentages of NK-cells and monocytes were both below 5%, in unstimulated and stimulated cultures.

After polyclonal stimulation of PBMC from AD^+PA^+ patients (for 3 days) with PMA + Ca-ionophore, the percentages of $CD3^+$ cells and $CD4^+$ T cells did not change significantly, but the percentage of $CD8^+$ T cells increased significantly (p=0.029) compared to medium, as was the case for the percentage of B cells (p=0.029) (Table 4).

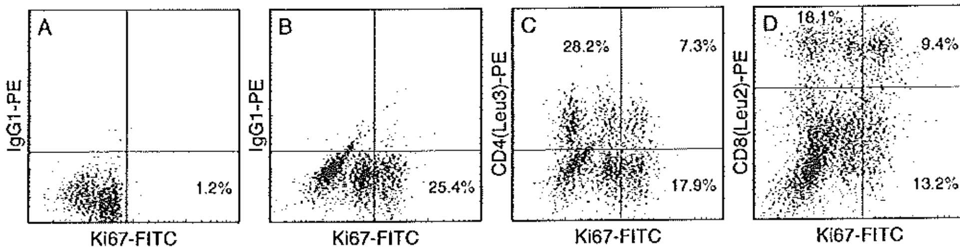

Figure 3. Ki67-antigen expression after polyclonal stimulation of PBMC for 3 days with PMA + Ca-ionophore. (A) 1.2% of unstimulated PBMC expressed the Ki67-antigen. (B) 25.4% of polyclonally stimulated PBMC expressed the Ki67-antigen. (C) 20.6% of $CD4^+$ cells expressed the Ki67-antigen after polyclonal stimulation. (D) 34.2% of $CD8^+$ cells expressed the Ki67-antigen after polyclonal stimulation.

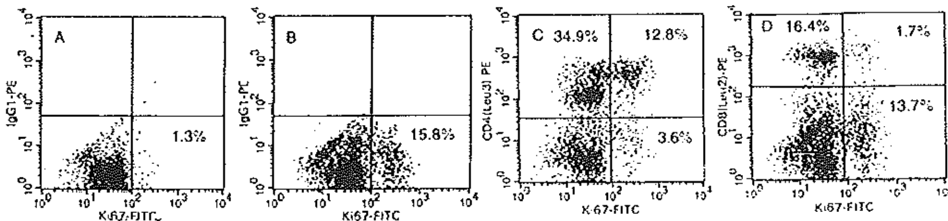

Figure 4. Ki67-antigen expression after specific stimulation of PBMC for 7 days with peanut-extract. (A) 1.3% of unstimulated PBMC expressed the Ki67-antigen. (B) 15.8% of peanut-extract specific stimulated PBMC expressed the Ki67-antigen. (C) 26.8% of $CD4^+$ cells expressed the Ki67-antigen after peanut-extract specific stimulation. (D) 9.4% of $CD8^+$ cells expressed the Ki67-antigen after peanut-extract specific stimulation.

Phenotype distribution of proliferating cells after stimulation

In addition to the phenotype of cultured cells, we studied which of the cell populations preferentially proliferated upon stimulation *in vitro*, using combined detection of Ki67-antigen and membrane markers. In this way it was possible to determine which cell population responded preferentially to

polyclonal (PMA + Ca-ionophore) or peanut-extract specific stimulation. Unstimulated cells expressed no (<5%) Ki67-antigen (Fig 3A, Fig 4A). Figure 3B showed the expression of the Ki67-antigen at day 3 after polyclonal stimulation indicating that 20-70% of the cells in the PBMC fraction expressed the Ki67-antigen. Both $CD4^+$ (20-60%, Fig 3C) and $CD8^+$ (30-75%, Fig 3D) cells expressed the Ki67-antigen after polyclonal stimulation, as did 40-70% of the B cells. Of those cells that did express the Ki67-antigen, 25-40% were $CD4^+$, 35-55% were $CD8^+$ and 6-20% were B cells. At 7 days after peanut-extract specific stimulation, 6-16% of the cells in the PBMC fraction expressed the Ki67-antigen (Fig. 4B). 15-27% of $CD4^+$ (Fig 4C) and 2-10% of $CD8^+$ cells (Fig 4D) were Ki67-antigen positive. Under these conditions, all Ki67-antigen positive cells were $CD3^+$ T-lymphocytes and of those 80-100% were $CD4^+$ cells and 0-20% $CD8^+$ cells. B-lymphocytes did not express the Ki67-antigen after peanut-extract specific stimulation *in vitro*.

DISCUSSION

We have shown that $CD4^+$ and $CD8^+$ subsets from AD^+PA^+ children respond differentially to peanut-extract specific and to polyclonal stimulation. Furthermore, peanut-extract induced proliferative responses of PBMC of AD^+PA^+ children were significantly increased as compared to controls. Upon peanut-extract specific stimulation, 80-100% of the proliferating cells were $CD4^+$ cells, and increased levels of IL-4 mRNA in stimulated cells as compared to medium were observed in these cultures.

Most of the studies of proliferative responses to specific antigens concern inhalant allergens [39-41]. Few studies have been published on food antigen-induced lymphocyte responses. Kondo et al. [24] showed that food-specific (ovalbumin and bovine serum albumin) proliferative responses were only found in children with AD who were sensitive to hen's egg or cow's milk. In contrast, Dorion et al. [25], who studied proliferative responses after peanut-allergen stimulation, observed no differences in proliferative responses between peanut-allergic and control subjects. A difference between their study and ours is that we studied PBMC of children and we used a total peanut-extract, whereas Dorion et al. [25] studied PBMC of adults and used only the major peanut-allergen, Ara hII. The proliferative responses to tetanus toxoid showed a trend to be higher in AD children compared to healthy children. An explanation could be that many T cells of AD children are already activated and therefore respond easier to antigenic stimulation than T cells of healthy children. After polyclonal stimulation no differences could be observed in the levels of proliferative responses between PBMC of atopic and non-atopic children. This indicates that there are no intrinsic differences in PBMC of atopic patients versus healthy control children in their capacity to respond to aspecific triggers.

To investigate the food-induced cell-mediated immunity in more detail, we examined the relationship between proliferative responses to peanut-extract and cytokine mRNA expression. Our results in AD^+PA^+ patients indicate that after peanut-extract specific stimulation, a significant positive correlation existed between proliferative responses and increases in IL-4 mRNA expression as compared to medium. Secrist et al. [38] described that the two processes, proliferation and IL-4 production, have optima at different doses of antigen, but we could not find any such difference.

Dorion et al. [25] could only detect IL-4 mRNA in PBMC after stimulation with peanut (Ara hII) and not in unstimulated PBMC. No differences in IL-4 mRNA expression between healthy controls and peanut-allergic patients were reported so far. In our study, a peanut-extract specific proliferative response could only be detected in AD^+PA^+ patients. Based on the intracellular expression of Ki67-antigen, we were able to demonstrate that $CD4^+$ cells proliferate preferentially after peanut-extract specific stimulation. This was correlated to an increase of IL-4 mRNA expression. No correlation was observed between proliferation and IFN-γ mRNA expression. Therefore, we provide indirect evidence that PBMC of peanut allergic children contain increased frequencies of peanut-specific Th2 cells, but not Th1 cells, in their peripheral blood. Studies on determination of precursor frequencies of peanut-specific Th2 cells can further elucidate this.

This study shows clearly that the preferentially responding subset of T cells is different for peanut-extract specific and polyclonal induced proliferation. After allergen-specific stimulation, the allergen is processed by antigen-presenting cells and presented in the context of MHC-class-II molecules. These will be principally recognized by the T cell receptor of $CD4^+$ T cells, thereby outgrowing. As demonstrated by analyzing the Ki67-antigen expression, virtually only the $CD4^+$ cells proliferate after peanut-extract specific stimulation. Polyclonal stimulation of PBMC with PMA + Ca-ionophore resulted in proliferation of primarily $CD8^+$ cells and to a lesser extent $CD4^+$ cells.

$CD8^+$ cells are considered potent IFN-γ producers [44,45]. This is consistent with our finding of an significant inverse correlation between the proliferative response after polyclonal stimulation and the ratio of IL-4/IFN-γ production. Furthermore, after polyclonal stimulation apart from both subsets of T cells, also B cells proliferate, probably as a result of shared second messenger systems activated by this stimulus.

PMA + Ca-ionophore stimulation is highly efficient in induction of IL-2 production. IL-2 induces primarily $CD8^+$ cells to proliferate [43]. Antigen-specific stimulation activate cells to produce several cytokines, including T cell growth factors specific for $CD4^+$ cells, e.g. IL-4. Due to this cytokine release the $CD4^+$ population is the mean proliferating subset.

Thus, when analyzing the cytokine expression and production of PBMC between patient groups and healthy controls, it is important to realize that

allergen-specific stimulation activates predominantly $CD4^+$ T cells, while polyclonal stimulation increases especially the percentage of $CD8^+$ cells.

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CHAPTER 9

PEANUT-SPECIFIC IgG4 ANTIBODIES AND T CELL CYTOKINE PRODUCTION PROFILES IN CHILDREN WITH ATOPIC DERMATITIS SUFFERING FROM PEANUT SENSITIZATION

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SUMMARY

Background Peanuts are the subject of increasing concern based on the number of reports of fatal or near-fatal anaphylaxis and local allergic reactions.
Objective To investigate peanut-induced allergic responses, that are correlated with the pathogenesis of peanut sensitization in children suffering from atopic dermatitis (AD).
Methods Peanut-specific IgE and IgG4 antibodies in plasma and the cytokine production profiles (mRNA expression and protein production) after polyclonal and peanut-specific stimulation of peripheral blood mononuclear cells (PBMC) were measured in children suffering from AD with (AD^+PA^+) or without (AD^+PA^-) peanut sensitization and in healthy children (HC).
Results Significantly increased peanut-specific IgG4 antibodies and decreased IL-10 levels were observed in children with AD compared to HC, independent of the presence of a peanut sensitization. In general, peanut-specific IgG4 antibodies of AD^+PA^+ recognized peanut proteins with lower molecular weights than peanut-specific IgG4 antibodies of AD^+PA^- and HC. In AD^+PA^-, no Th2 cytokine production profile was observed after peanut-specific or polyclonal stimulation, while in AD^+PA^+ children increased IL-5 mRNA and IL-13 mRNA and protein and decreased IFN-γ protein production were observed after stimulation.
Conclusion These results show that the T cell cytokine profiles after polyclonal stimulation of PBMC from AD^+PA^+ are significantly different from those in AD^+PA^-, indicating that peanut sensitization does not constitute a representative model for food-allergen sensitization. Moreover, we propose that the observed decreased IL-10 production in AD children is responsible for the increase in peanut-specific IgG4 antibodies in these children.

INTRODUCTION

Several food allergens induce IgE-mediated disease in humans. Peanuts, milk and eggs account for approximately 80% of adverse reactions to foods in patients with atopic dermatitis (AD) [1]. Peanut sensitization starts early in life, is usually severe and does not resolve. An increasing number of reports describe fatal or near-fatal anaphylaxis and local allergic reactions [2]. Peanut is an oil-legume containing about 44-56% oil and 22-30% protein [3]. The peanut protein can be divided into albumins, the globulins arachin and conarachin and other proteins such as peanut lectins [4]. One of the first peanut allergens identified was peanut-1, with 2 major bands at 20 and 30 kD [5]. Barnett et al. [6] subsequently demonstrated IgE binding to a Concanavalin A-reactive glycoprotein of 65 kD. Burks et al. [7,8] identified two major allergens, Ara hI and Ara hII, with molecular weights of 63.5 and 17 kD, respectively. Ara hIII was later identified as a 14 kD protein [9]. Other peanut allergens with molecular weights of ≈ 44, 40, 33, 21, 20 and 18 kD have been proposed [10].

In AD, the serum total and allergen-specific IgE levels have been reported to be elevated [11,12], while some studies also described increased serum total and allergen-specific IgG4 levels [12-14]. The presence of IgG4 antibodies is often considered to be the result of chronic exposure to allergen [14,15]. The role of IgG4 in atopic disease is still debated. A possible protective effect of IgG4 antibodies in the allergic response might be due to an effective interference with the allergen-induced triggering of IgE positive cells [16]. On the other hand, small amounts of IgG4 have been shown to sensitize mast cells and basophils to release histamine [17,18], although others were not able to confirm this [19].

A disturbed balance between Th cell subsets (Th1 vs. Th2) is generally thought to play a role in the pathogenesis of AD and to result in the accompanying presence of allergen-specific IgG4 and IgE. It has been suggested that allergens preferentially activate Th2 cells, leading to the production of cytokines (IL-4, IL-5 and IL-13) that are crucial factors in the induction and maintenance of atopic disease [20,21]. Increased IL-4 and IL-5 mRNA levels have been described after polyclonal stimulation of both peripheral blood mononuclear cells (PBMC) and purified T cells from patients with AD [22-24]. In addition, in AD patients reduced production of IFN-γ has been reported [25], possibly due to enhanced release of IL-10 protein [26]. Recent findings indicate that IL-10 protein can also prevent the allergic inflammation by inhibiting production of Th2 cytokines, such as IL-5, and by suppressing eosinophil and mast cell function [27]. These data suggest that IL-10 could have opposing effects in AD by inhibiting both Th1 and Th2 type cytokines. Hence, we addressed the question on the role of IL-10 in the cytokine production by T cells and in the immunoglobulin production by B cells in AD and healthy children (HC).

Other Th2 cytokines, such as IL-4 and IL-13, play a crucial role in the regulation of IgG4 and IgE synthesis [28-30]. IL-13 is two- to five-fold less potent than IL-4 in inducing IgE synthesis [31], but is produced earlier and for longer time periods than IL-4 [32]. Recently, increased levels of IL-13 mRNA have been described in PBMC of adult AD patients [33], while in children a positive correlation between IL-13 protein production and the severity of AD was observed [23]. Moreover, several reports suggest an important role of IL-13 in enhanced IgE synthesis in AD patients [32-34]. However, the precise function of IL-4 and IL-13 in the induction and maintenance of the allergic sensitization in children remains to be established.

The immunological analysis of peanut-specific immune responses has been limited so far. Some investigators [35,36] found increased peanut-specific proliferative responses of PBMC from peanut-allergic subjects compared to non-allergic subjects, but others did not [37,38]. Moreover, in peanut-allergic subjects decreased production of IFN-γ protein was described after peanut-specific stimulation of PBMC [35] or peanut-specific T cell clones [37,38]. Increased IL-4 protein production could only be detected upon stimulation of the

T cell clones [37,38]. The role of additional Th2 cytokines, such as IL-5, IL-10 and IL-13 in the pathogenesis of peanut sensitization has not yet been described.

In order to investigate food allergic responses, in particular to peanuts, we studied peanut-specific IgE and IgG4 plasma antibodies and the Th1 and Th2 cytokine profiles in the pathogenesis of peanut sensitization in children suffering from AD.

MATERIALS AND METHODS

Patients

We studied 24 AD^+PA^+ children, 11 AD^+PA^- children and 21 HC. The Medical Ethical Committee of the University Hospital Rotterdam approved this study. Informed consent was obtained from the children's parents prior to their participation in the study.

AD patients were selected based on a history of mild to severe eczema according to the diagnostic criteria of Hanifin & Rajka [39]. All AD^+PA^+ children had a positive skin test specific for peanut, and had peanut-specific IgE in their plasma. None of the peanut-sensitized AD^+PA^+ patients had a history of systemic anaphylaxis to peanuts. AD^+PA^- children had a positive skin test specific for milk, egg, wheat, soy or codfish, but not for peanut. They also had specific IgE in their plasma to inhalation allergens (Phadiatop®) and/or food allergens, but not for peanut. The severity of the AD was determined by the objective SCORAD [40]. Further details of these patients are listed in *Table 2*.
The HC had no history of eczema or atopy and a negative family history as judged by a validated questionnaire [23,24]. None of the HC had specific IgE in their plasma to the most common inhalation allergens (house-dust mite, grass and birch pollen, cat, dog and moulds) or food allergens (peanut, chicken egg white, cow's milk, codfish, wheat and soy).

Analysis of immunoglobulin levels in plasma

Determination of the total serum IgE levels was performed using the Pharmacia CAP RAST (Kabi Pharmacia, Uppsala, Sweden). Analyses of the specific IgE antibodies directed towards inhalation allergens and food allergens were performed using the Pharmacia CAP system Phadiatop® RAST and the Pharmacia CAP RAST, respectively. Peanut-specific IgG4 antibodies were measured using a RAST system [41]. The peanut-specific IgE and IgG4 binding patterns were determined by Alablot systems (Diagnostic Products Corporation (DPC), Apeldoorn, the Netherlands) according to the manufacturers' instructions.

Lymphocyte proliferation assay

Five ml of heparinized venous blood was collected and after diluting the blood 1:1 with phosphate-buffered saline (PBS), PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia). Phenotypic analysis of several surface markers by flowcytometry [42] revealed no differences in cell populations between the different patient groups. Lymphocyte proliferation assays were performed as described previously [42]. Briefly, PBMC were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate and 10% heat-inactivated (HI) human serum (HuS) in 96-well round bottom microtiter culture plates, which were previously shown to be optimal conditions [43]. PBMC were cultured at $2x10^5$ cells/well in the presence or absence of an optimally titered dose of peanut extract (500 μg/ml; ALK-Benelux, Groningen, The Netherlands) in a total volume of 200 μl [42]. After 7 days of allergen-specific stimulation, proliferation was measured using [^{3}H]-thymidine incorporation. The results were expressed as stimulation index (SI), defined as the ratio of mean cpm in cultures stimulated with peanut extract over mean cpm in unstimulated cultures. A SI greater than 3 was considered to represent a positive proliferative response.

Analysis of cytokine mRNA expression in PBMC cultures

PBMC were cultured in 24-well culture plates at $1x10^6$ cells/well in Yssel's medium (YM) containing 1% HI-HuS either with the addition of 2 ng/ml phorbol myristate acetate (PMA) (Sigma, St.Louis, MO) and 1 μg/ml Ca-ionophore (A23187, Sigma) or the addition of 500 μg/ml peanut extract in a total volume of 1 ml. PBMC stimulated with PMA and Ca-ionophore were cultured for 16-18 hr, while PBMC stimulated with peanut extract were cultured for 4 days [42]. After harvesting the cells, the supernatants were stored at -80°C. Total RNA was isolated from the cells followed by cDNA synthesis, amplification by semi-quantitative reverse transcriptase (RT)-PCR using primers specific for either the housekeeping gene HPRT (hypoxanthine phosphoribosyl transferase), IL-4, IL-5, IL-10, IL-13 or IFN-γ, as described previously [23,24]. For the analysis of HPRT, IL-4 and IFN-γ mRNA expression, a life-size photograph of the gel was taken and used for densitometric analysis. IL-5, IL-10 and IL-13 mRNA expression were determined by densitometric scanning of the film after gel electrophoresis, Southern blotting and subsequent hybridization with a radioactive cytokine-specific probe [23].

Analysis of cytokine protein production in PBMC cultures

The levels of IFN-γ, IL-4, IL-10 and IL-13 in the culture supernatants and in plasma were determined by a Pelikan Compact™ human cytokine kit (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands) according to the manufacturers instructions.

IL-5 was determined using an ELISA based on TRFK5 (a kind gift of Dr. R.L. Coffman; DNAX Research Institute, Palo Alto, CA) as a capture mAb and

biotin-labeled rat anti-human IL-5 (JES1-5A10, 0.25 μg/ml; Pharmingen, San Diego, CA) as detection mAb. Briefly, 96-well plates (flat-bottom, Falcon 3912, Becton Dickinson, San Jose, CA) were coated overnight at 4°C with anti-IL-5 mAb TRFK5, with an optimal coating concentration of 0.25 μg/ml with 100 μl/well in 0.1 M carbonate buffer pH 8.2. After each incubation step the plates were washed with PBS/0.05% Tween-20 (Sigma). After washing the plates three times, non-specific binding was prevented by blocking the plates 2 hours at room temperature (RT) with 200 μl/well PBS/10% fetal calf serum (FCS)-HI (BioWhittaker, Verviers, Belgium). Plates were washed three times after which standard IL-5 (15.6-1000 pg/ml; Rec human IL-5, Pharmingen) diluted in YM 1% HuS and samples were added (100 μl/well). After overnight incubation at 4°C, and washing the plates 4 times, the plates were incubated for 45 minutes at RT with anti-IL-5 biotin (0.25 μg/ml) diluted in PBS/10%FCS-HI (100 μl/well). After washing 6 times, poly-streptavidine-peroxidase (CLB) diluted in PBS/10% FCS-HI was added. After 30 minutes incubation at RT and washing 6 times, peroxidase activity was determined by addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Lab., Gaithersburg, MD). The reaction was stopped with 0.1 M H_3PO_4 and optical density was determined at 450 nm.

All samples were tested in duplicate. The lower detection limit was 2 pg/ml for IFN-γ, 1 pg/ml for IL-4, 2.5 pg/ml for IL-5, 2 pg/ml for IL-10 and 0.5 pg/ml for IL-13.

Statistical analyses

Statistical analyses were performed using STATA™ (Computing Resource Center, Los Angeles, CA). Parameters were tested using the Mann-Whitney test and correlation coefficients (r_s) were calculated by the Spearman sign-rank correlation. The significance level was set at $p<0.05$.

Table 1. Plasma levels of total IgE and peanut-specific IgE and IgG4 antibodies in the patient groups studied.

		peanut-specific	
Patient group	total IgE (kU/l)	IgE (kU/l)	IgG4 (ng/ml)
AD⁺PA⁺	464* (8-172000)	36 (1.0-100)	1167* (318-5761)
AD⁺PA⁻	443* (2-5910)	0.1** ND	1366* (63-6310)
HC	7 (0-137)	0.1** ND	366 (80.6-573)

AD^+PA^+ = atopic dermatitis children with peanut allergy; AD^+PA^- = AD children without peanut allergy; HC = healthy children. ND = not detectable. Results are expressed as the median (range). * $p<0.05$ as compared to HC. ** $p<0.05$ compared to AD^+PA^+.

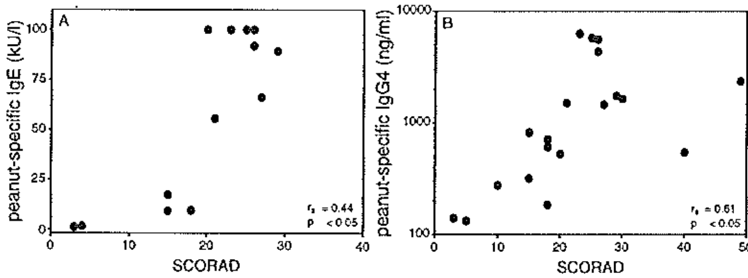

Figure 1. Correlation peanut-specific IgE (A) and peanut-specific IgG4 (B) levels with the severity of the AD (objective SCORAD). P-values were calculated by the Spearman sign-rank correlation.

RESULTS

Peanut-specific IgG4 and IgE in atopic dermatitis patients and healthy children

Total IgE levels were significantly increased in the plasma of children with atopic dermatitis, with (AD^+PA^+) or without (AD^+PA^-) a peanut sensitization, compared to HC (*Table 1*). Peanut-specific IgG4 levels in AD^+PA^+ and surprisingly also in AD^+PA^- were significantly elevated compared to HC, whereas peanut-specific IgE antibodies were only detectable in AD^+PA^+. A strong positive correlation was observed in AD^+PA^+ between the peanut-specific IgG4 and the peanut-specific IgE levels ($r_s=0.93$, $p<0.05$; data not shown). A significant positive correlation existed also between the severity of the AD, as measured by the objective SCORAD, and both peanut-specific antibody isotypes (IgE ($r_s=0.44$, $p<0.05$, *Fig. 1A*) and IgG4 ($r_s=0.61$, $p<0.05$, *Fig. 1B*)).

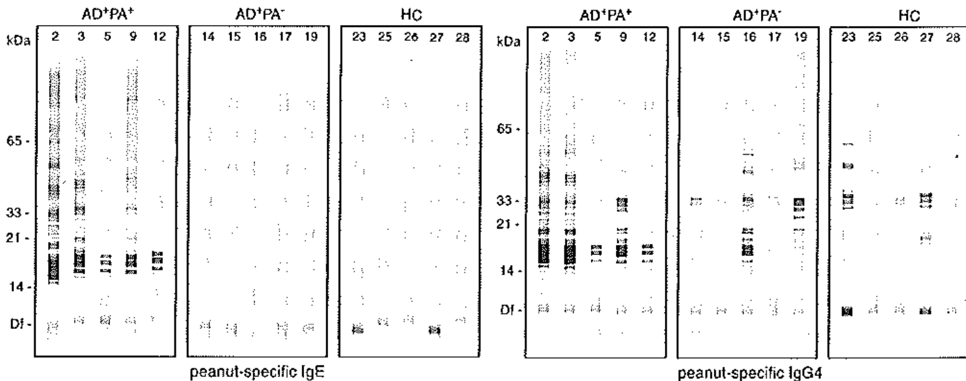

Figure 2. IgE (left) and IgG4 (right) binding patterns to peanut proteins. Blot numbers represent patient numbers. Binding patterns of 5 representive AD^+PA^+, 5 AD^+PA^-, and 5 HC were shown.

In order to detect possible differences in IgG4 and IgE binding patterns to peanut proteins, Alablots were performed for 15 AD^+PA^+, 5 AD^+PA^- and 10 HC children. In general, staining-intensity of blotting patterns paralleled measured plasma concentrations of peanut-specific IgG4 and IgE levels in the patient groups. IgE binding to peanut proteins was only found in AD^+PA^+, while in all groups IgG4 binding to peanut proteins was observed (*Fig. 2*). In AD^+PA^+ children, the individual binding patterns for IgE and IgG4 were comparable within a child, with preferential recognition of low molecular weight peanut proteins in a range between 14 and 21 kD. In contrast, IgG4 antibodies in the AD^+PA^- and HC groups recognized in general more intensely the higher molecular weight proteins in a range between 33 and 65 kD.

Table 2. Characteristics of the patient groups and the proliferative response to peanut extract stimulation of their PBMC.

Patient group	n	age[a] (months)	gender	SCORAD[a]	proliferative response[b] SI (range)
AD^+PA^+	24	39.5 (14-138)	17♂ 7♀	22 (4-44)	6.8* (1.6-24.6)
AD^+PA^-	11	28 (13-95)	3♂ 8♀	20 (3-56)	2.1 (1-5.9)
HC	21	48 (19-148)	15♂ 6♀		1.4 (1-3.8)

AD^+PA^+ = atopic dermatitis (AD) children with peanut allergy; AD^+PA^- = AD children without peanut allergy; HC = healthy children; SI = stimulation index. [a]Results are expressed as median (range). [b]PBMC were incubated for 7 days with peanut extract (500 μg/ml) after which the proliferative response was determined by [^{3}H]-thymidine incorporation. *AD^+PA^+ showed a significantly ($p<0.05$) higher proliferative response compared to both AD^+PA^- and HC.

Th2 induced cytokine response after peanut-extract specific stimulation

The proliferative responses of PBMC to peanut extract on day 7 were significantly increased in AD^+PA^+ compared to either AD^+PA^- or HC (*Table 2*). We have shown previously that the increased proliferative response in AD^+PA^+ after peanut-extract specific stimulation was preferentially due to proliferating Th2 cells [42], most likely resulting in increased production of Th2 cytokines. Therefore, we analyzed whether Th2 cytokines, such as IL-4, IL-5 and IL-13, were increased in peanut-extract specific stimulated PBMC of these patients. IL-4 and IL-5 mRNA expression levels in PBMC of AD^+PA^+ were not significantly higher compared to HC and AD^+PA^- (*Table 3*). IL-13 mRNA expression was significantly increased compared to HC, but not to AD^+PA^-. IL-4, IL-5 and IL-13 mRNA expression levels in unstimulated PBMC did not differ significantly between the patient groups (data not shown). The corresponding protein levels for IL-4 and IL-5 were generally too low to be detected by the sensitive ELISA

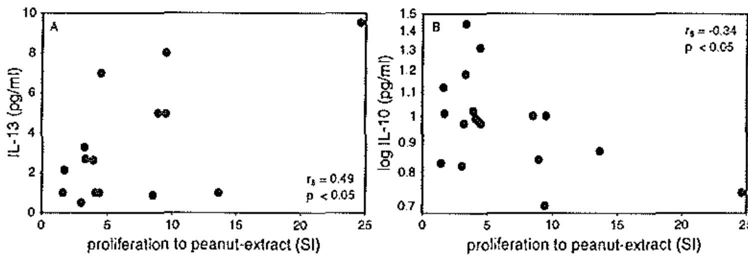

Figure 3. Correlation of IL-13 (A) and IL-10 (B) protein production with proliferation (SI) to peanut extract stimulation. Proliferative responses of PBMC of AD^+PA^+ were mesured on day 7 after peanut-extract specific stimulation. IL-13 and IL-10 production were measured in culture supernatants at day 4 after peanut-extract specific stimulation of PBMC of AD^+PA^+. P-values were calculated by the Spearman sign-rank correlation.

Table 3. Analysis of cytokine mRNA expression and protein production after peanut-specific stimulation of PBMC from AD^+PA^+, AD^+PA^- and HC.

cytokine	AD^+PA^+	AD^+PA^-	HC
IL-4 mRNA	56.7 (10-292.4)	34.5 (6-102)	46.5 (27-86.3)
IL-4 protein	<1	<1	<1
IL-5 mRNA	67.3 (0-416.7)	0 (0-197.8)	0 (0-99)
IL-5 protein	<2.5 (<2.5-29.6)	<2.5 (<2.5-12)	<2.5 (<2.5-2.7)
		P<0.05	
IL-13 mRNA	11.0 (0-424)	0 (0-163)	0 (0-55)
IL-13 protein	0.7 (<0.5-12.4)	<0.5 (<0.5-3)	<0.5 (<0.5-8)
	p<0.05		
		P<0.05	

AD^+PA^+ = atopic dermatitis children with peanut allergy; AD^+PA^- = AD children without peanut allergy; HC = healthy children. PBMC were stimulated for 4 days with peanut extract (500 µg/ml). After stimulation RNA was isolated and semi-quantitative RT-PCR was performed for the indicated cytokines. In the culture supernatants cytokines were measured with ELISA. Data represent median cytokine scan values for mRNA expression or median protein production levels in pg/ml with ranges.

methods employed, while the IL-13 protein production after stimulation was significantly increased in PBMC of AD^+PA^+ compared to AD^+PA^- and HC (*Table 3*). In AD^+PA^+, IL-13 protein production correlated positively with the proliferative responses to peanut-extract specific stimulation ($r_s = 0.49$, $p < 0.05$, *Fig. 3A*), thereby supporting the hypothesis that the Th2 cells are primarily responsible for the high IL-13 production. Furthermore, the IL-10 production levels in AD^+PA^+ were negatively correlated with the proliferative responses to

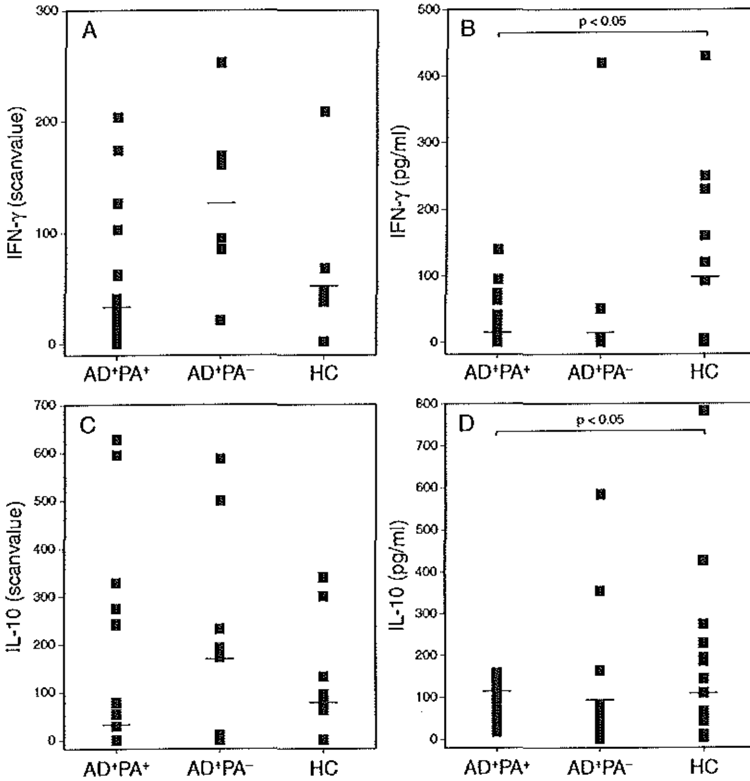

Figure 4. IFN-γ and IL-10 mRNA expression and protein production after peanut-extract specific stimulation of PBMC from children of the different patient groups. PBMC were stimulated for 4 days with peanut extract (500 μg/ml) after which RNA was isolated and semi-quantitative RT-PCR for IFN-γ (A) and subsequent radio-active hybridisation for IL-10 (C) was performed. In the culture supernatants of stimulated PBMC IFN-γ (B) and IL-10 (D) were measured by ELISA. P-values were calculated by the Mann-Whitney test. AD^+PA^+ = peanut allergic AD children; AD^+PA^- = AD children without peanut allergy; HC = healthy controls.

peanut extract (r_s= -0.34, $p<0.05$, *Fig. 3B*), suggesting a role for IL-10 in down-regulating the proliferation of Th2 cells.

Decreased IFN-γ and IL-10 protein production after peanut-specific stimulation

A dysbalance in both the Th1 and Th2 cytokine subsets was reported in children suffering from AD and food allergy [23-25,35]. We anticipated that decreased IFN-γ and/or IL-10 mRNA expression and protein production after peanut-extract specific stimulation of PBMC of the different patient groups could contribute to such a dysbalance. Surprisingly, no significant differences were observed in IFN-γ (*Fig. 4A*) or IL-10 (*Fig. 4C*) mRNA expression in stimulated PBMC of AD^+PA^+ compared to the PBMC of HC or AD^+PA^-. On the

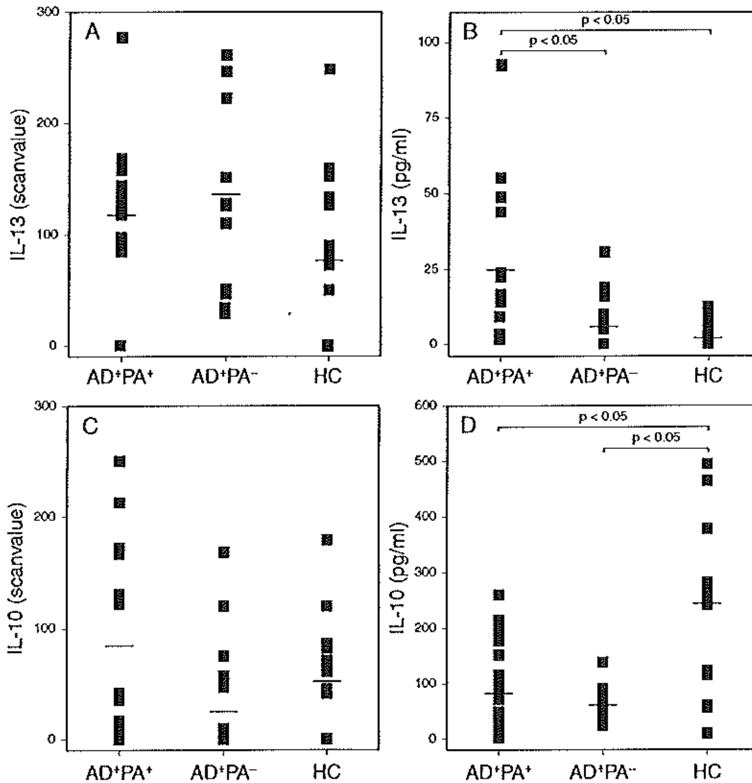

Figure 5. IL-13 and IL-10 mRNA expression and protein production after polyclonal stimulation of PBMC from the different groups of children. PBMC were stimulated for 16-18 hours with PMA and Ca-ionophore. After stimulation RNA was isolated and semi-quantitative RT-PCR for IL-13 (A) and IL-10 (C) and subsequent radio-active hybridisation was performed. In the culture supernatants of stimulated PBMC IL-13 (B) and IL-10 (D) were measured by ELISA. P-values were calculated by the Mann-Whitney test. AD^+PA^- = atopic dermatitis children without peanut allergy; AD^+PA^+ = peanut allergic AD children; HC = healthy controls.

other hand, a decreased protein production of both IFN-γ ($p<0.05$, *Fig. 4B*) and IL-10 ($p<0.05$, *Fig. 4D*) was observed in stimulated PBMC of AD^+PA^+ compared to the PBMC of HC, but not of AD^+PA^-. IFN-γ or IL-10 mRNA expression and protein production levels in unstimulated PBMC were not significantly different between the patient groups. The IL-10 production levels after peanut-extract specific stimulation of PBMC were negatively correlated with peanut-specific IgG4 antibodies both in AD^+PA^- ($r_s=-0.62$, $p=0.09$) and AD^+PA^+ ($r_s=-0.48$, $p<0.05$, data not shown), indicating a regulatory role for IL-10 in IgG4 production.

Polyclonally-induced cytokine profiles

In order to study the inherent cytokine production capacity of PBMC, cytokine profiles were investigated after polyclonal stimulation. The IL-13 protein production after stimulation was significantly increased in AD^+PA^+, but not in AD^+PA^-, compared to HC (*Fig. 5B*), while IL-13 mRNA expression by stimulated PBMC was comparable between the patient groups (*Fig. 5A*). In AD^+PA^+ after polyclonal stimulation of PBMC the IL-5 mRNA expression levels were significantly higher compared to HC and the IL-5 protein production was significantly increased compared to AD^+PA^- (data not shown). IL-4 and IFN-γ mRNA expression or protein levels after stimulation of PBMC did not differ significantly between the patient groups. After polyclonal stimulation of PBMC, the IL-10 mRNA expression was similar between the different groups (*Fig. 5C*), whereas the IL-10 protein production after stimulation was significantly decreased in both AD^+PA^+ and AD^+PA^- compared to HC (*Fig. 5D*). IL-10 production was reduced in stimulated PBMC compared to unstimulated PBMC in all patient groups (data not shown). The mRNA expression and the protein production of these cytokines in unstimulated PBMC were not significantly different between the patient groups.

DISCUSSION

In this study, we describe significantly increased levels of peanut-specific IgG4 antibodies in AD^+PA^+ and AD^+PA^- patients compared to HC, independent of the presence of a defined peanut sensitization. Peanut-specific IgE antibodies, however, were only detected in AD^+PA^+.

Also in adult AD patients increased allergen-specific IgG4 levels have been reported, but no distinction was made between food-sensitized and non-food sensitized AD patients [14]. In agreement with our findings, this group described a positive correlation between the severity of the eczema and the amount of peanut-specific IgE antibodies or peanut-specific IgG4 antibodies. Our analysis of the binding patterns of IgE and IgG4 antibodies of the different patient groups revealed IgE binding to the peanut proteins only in AD^+PA^+, while IgG4 binding to the peanut proteins was observed in all groups. IgE and IgG4 antibodies of AD^+PA^+ patients recognized especially peanut proteins with molecular weights between 14 and 21 kD. The peanut-allergens Ara hII, Ara hIII and some of the allergens described by de Jong et al. [10] are located in this area. IgG4 antibodies of AD^+PA^- and HC predominantly recognized peanut proteins with higher molecular weights, between 33 and 65 kD. These proteins probably comprise Ara hI, and some of the allergens described previously [6,10]. Possible clinical implications of the recognition of different peanut allergens by IgG4 antibodies of AD^+PA^+ or AD^+PA^- and HC remain to be studied further.

Apart from the peanut-sensitized children, most children with or without AD ingest food products containing peanuts. An explanation for the increased peanut-specific IgG4 levels in AD$^+$PA$^-$ compared to HC could be a quantitative difference in peanut allergen exposure at the mucosal surface leading to the presence of allergen-primed T cells in the circulation. Such a disturbed mucosal permeability has been suggested in adult AD patients [14,44].

In order to determine the isotype selective activity of cytokines produced after allergen-specific stimulation, we studied the presence of peanut-extract induced T cell derived cytokine profiles in PBMC of AD$^+$PA$^-$ children. After peanut-extract specific stimulation of PBMC of AD$^+$PA$^-$ no differences in the cytokine profile was found compared to HC. In contrast to the generally Th0 cytokine profile in AD$^+$PA$^-$, a polarized Th2 cytokine profile was found in the PBMC of AD$^+$PA$^+$, characterized by increased IL-5 and IL-13 levels and decreased IFN-γ and IL-10 levels compared to HC. This Th2 profile was observed after peanut-extract specific stimulation, but was also present intrinsically as revealed after polyclonal stimulation. While the decreased production of IFN-γ after peanut-specific stimulation has been described before [35;37,38], no such results have been reported for IL-5, IL-10 and IL-13.

Recently, increased IL-13 mRNA levels were described in adult AD patients [33] and an important role for IL-13 has long been suggested in atopic disease [23,32-34]. Since the production of IL-4 after allergen-specific stimulation is too low to be measured, we consider IL-13 a more convenient Th2 cytokine to discriminate between peanut-sensitized and non-peanut-sensitized children.

In PBMC of AD$^+$PA$^+$ children a decreased IL-10 protein production was found both after polyclonal and peanut-extract specific stimulation. So far, lower IL-10 protein production levels were only found after polyclonal stimulation of purified T cells of AD children [24]. Stimulation of PBMC with peanut extract results in a predominant activation of CD4$^+$ T cells [42], which suggests that Th cells are mainly responsible for the decreased IL-10 production observed in AD patients.

Besides the inhibition of cytokine synthesis by Th1 cells [26], IL-10 has been suggested to prevent allergic inflammation by inhibiting the production of Th2 cytokines, such as IL-5 and to downregulate proliferative responses [26,27]. This is in agreement with the observed negative correlation between proliferative responses and IL-10 protein production by PBMC of AD$^+$PA$^+$ after peanut-extract specific stimulation. Moreover, the observed decrease in the IL-10 protein production after peanut-extract specific stimulation of PBMC of AD$^+$PA$^+$ could be responsible for the increased IL-5 mRNA expression levels. This is supported by the negative correlation between these two cytokines after peanut-extract specific stimulation ($r_s = -0.36$, $p < 0.05$) and points to an important role of IL-5 in the inflammatory process in atopic dermatitis [24,45-48] and possibly also in peanut-sensitization.

The HC group displayed low levels of IL-4 in combination with high levels of IL-10 compared to the other two groups. IL-4 is essential to induce the expression of γ4-germline transcripts and IL-10 has been described to augment the expression of these transcripts resulting in increased IgG4 production [48,49]. The combination of low levels of IL-4 and high levels of IL-10 could result in low numbers of IgG4-secreting B cells and explain the presence of peanut-specific IgG4, but no IgE antibodies in HC.

In AD^+PA^-, similar production levels of IL-4 compared to HC will induce γ4-germline transcription. IL-10 has additionally been described to be a potent inhibitor of B cell proliferation [50]. The presence of reduced IL-10 levels in AD^+PA^- compared to HC, will enhance B cell proliferation, resulting in increased numbers of IgG4-secreting B cells. This is supported by the inverse correlation between IL-10 protein and peanut-specific IgG4 levels in this group of children. Consequently, AD^+PA^- children displayed significantly increased levels of peanut-specific IgG4 antibodies compared to HC, due to the persistent lack of downregulation of B cell proliferation by IL-10.

In contrast to HC, all AD^+PA^- children were sensitized for a food-allergen, with the exception of peanut. Peanut-specific stimulation did not result in alterations of the Th0-like cytokine profile in AD^+PA^- compared to HC. After polyclonal stimulation no increased Th2 cytokines, such as IL-5 or IL-13 were observed in AD^+PA^-, which is in contrast to the AD^+PA^+ group. Our data cannot exclude the existence of another factor being differentially activated in AD^+PA^+ and AD^+PA^-, but indicate that peanut-sensitized AD patients are different in their cytokine dysregulation than other food-allergen sensitized AD patients. The fact that peanuts are the major cause of anaphylactic reactions emphasizes the special position of peanut allergen among food allergens. Therefore peanut-allergens cannot be used as a general model representing other food-allergens to study cytokine profiles in food sensitized children.

In AD^+PA^+, besides the similar production levels of IL-4 compared to HC and decreased IL-10 levels as also observed in AD^+PA^-, we found peanut-specific IgG4 antibodies that preferentially recognized peanut proteins with low molecular weights. The differential specificity of recognized peanut proteins by AD^+PA^+ compared to HC and AD^+PA^- could be related to a difference in the route of exposure, for example a difference in gut permeability as has been noted in the case of latex allergic children [47]. Secondly, there could be a difference in the immunogenicity of the presented peanut allergens, due to a possible difference in the processing of peanut proteins by antigen-presenting cells. Finally, the differential epitope specificity of peanut-specific IgG4 antibodies in AD^+PA^- children could also result from bystander B cell stimulation through cytokines produced by activated Th0 cells. The different cytokine profiles after stimulation of PBMC from AD^+PA^- and AD^+PA^+ may results in reactivity against different (quantitatively major) epitopes in the peanut extract. In combination with a decreased IL-10-mediated inhibition of B cells, this would result in enhanced production of IgG4 antibodies with a different epitope

specificity spectrum. We can as yet not rule out any one of the proposed explanations.

In conclusion, AD children with or without a peanut sensitization and HC show a differential cytokine production profile leading to the selective activation of Th2 vs. Th0 subsets after stimulation of PBMC. This differential subset activation may result in differences in the presence of peanut-specific IgG4 and IgE antibodies and their epitope specificity. The combined analysis performed here, provides evidence for the heterogeneity in immune responsiveness between the different patient groups. Peanut sensitization does not constitute a representative model for food-allergen sensitization, but illustrates the importance of T cell cytokines, such as IL-10 in the differential regulation of IL-4-driven allergen-specific IgG4 formation.

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CHAPTER 10

LEVELS OF SOLUBLE INTERCELLULAR ADHESION MOLECULE-1, SOLUBLE E-SELECTIN, TUMOR NECROSIS FACTOR-α, SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR P55 AND P75 IN ATOPIC CHILDREN

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ABSTRACT

During inflammation membrane expression of adhesion molecules and tumor necrosis factor (TNF)-receptors (TNF-R) are increased and soluble forms of these molecules are released. This study analyzed plasma levels of sICAM-1, sE-selectin as well as TNF-α, sTNF-R55 and sTNF-R75 in non-allergic (NAA) and allergic asthma patients (AA), atopic dermatitis patients (AD) and healthy children (HC) by ELISA. Plasma levels of sICAM-1, sE-selectin and sTNF-R, but not TNF-α were detectable, but not significantly different between the patient groups and healthy children. In the AA group a significant correlation ($r_s = 0.78$, $p = 0.008$) was found between sICAM-1 and sE-selectin levels. Furthermore, a significant correlation was found between sTNF-R55 and sTNF-R75 levels in the AA group ($r_s = 0.70$, $p = 0.025$) and in the AD group ($r_s = 0.69$, $p = 0.027$). In AD patients a significant correlation was observed between sE-selectin and the disease severity, as measured by the SCORAD index ($r_s = 0.73$, $p = 0.038$). Our data demonstrate that plasma levels of sICAM-1, sE-selectin, TNF-α, sTNF-R55 and sTNF-R75 were not different between atopic and non-atopic children, during a stable phase of the disease. In AD patients, levels of sE-selectin suggested to be related to clinical severity of the disease.

INTRODUCTION

Several cytokines, including tumor necrosis factor (TNF)-α, promote the induction and upregulation of adhesion molecules, both on endothelium and on leucocyte surfaces [1]. At sites of inflammation, increased expression of intercellular adhesion molecule-1 (ICAM-1, CD54), vascular cell adhesion molecule-1 (VCAM-1, CD106) and E-selectin (CD62E) on vascular endothelium in the lung and skin have been demonstrated [2]. In skin biopsies from patients with respiratory allergy, an allergen induced upregulation of E-selectin on endothelial cells was reported during the late phase reaction after intradermal allergen (ragweed or house-dust mites) injection [3]. In allergic asthma patients an increased expression of ICAM-1 was shown on bronchial epithelial cells compared to patients with chronic bronchitis or healthy individuals [4,5]. In these patients an increased expression of both ICAM-1 and E-selectin on bronchial endothelium was observed [5]. These results indicate that adhesion molecules are up-regulated in allergic inflammation, most likely through the presence of inflammatory cytokines.

The interaction between circulating T cells and microvascular endothelial cells is a critical determinant of the homing process. In the skin, an interaction between the cutaneous lymphocyte-associated antigen (CLA) on skin homing T cells and E-selectin has been proposed to be involved in tissue-selective T cell extravasation [6,7].

During inflammation not only the expression of adhesion molecules is increased, but also soluble adhesion molecules are released [8-10]. The

presence of soluble adhesion molecules in the circulation of patients with a variety of inflammatory disease states, has raised the possibility that plasma levels may reflect disease activity. Determination of the concentration of soluble adhesion molecules could be useful in studying the process of allergic inflammation.

In patients with atopic dermatitis, but not in bronchial asthma, the plasma TNF-α concentrations were reported to be increased during inflammation [11]. In contrast, Koizumi et al. [12] reported elevated serum TNF-α levels during bronchial asthma attacks. The activities of TNF-α are mediated via TNF-receptors (TNF-R), expressed on a number of cell types [13,14]. Two different types of TNF-R have been characterized with molecular masses of 55kD (TNF-R55) and of 75 kD (TNF-R75) [15]. These receptors are also be present in soluble form in the circulation.

Cellular activation has been shown to result in increased presence of soluble TNF-R (sTNF-R) probably via shedding of cell-bound receptors [16]. These sTNF-R have retained the ability to bind TNF-α, thereby competing with cell bound TNF-R and neutralizing TNF-α activity. Normally, serum concentrations of the sTNF-R are rather low [17]. However, in a variety of pathological situations accompanied by immune activation, including sepsis [18-20], autoimmune disease [21,22] and due to reduced clearing in chronic renal failure [23], the concentrations of sTNF-R increase substantially. Hence, sTNF-R could well be released in the course of immunological activation related to allergy. Therefore, we studied TNF-α and sTNF-R levels in several groups of atopic patients.

This study analyzed whether the above mentioned markers were different between atopic and non-atopic children and whether these levels were related to disease activity. To this end, we evaluated the levels of sICAM-1, sE-selectin, TNF-α, sTNF-R55 and sTNF-R75 in plasma samples of children with (allergic and non-allergic) asthma, atopic dermatitis and healthy controls.

MATERIAL AND METHODS

Study population

We studied children suffering from atopic dermatitis (AD, n = 10) with a mean age of 29.3 months (range 7-48 months), allergic asthma (AA, n = 9) with a mean age of 36.6 months (range 12-54 months), non-allergic asthma (NAA, n = 9) with a mean age of 24.3 months (range 7-56 months) and 10 healthy children (HC) with a mean age of 32.3 months (range 8-53 months). The Medical Ethical Committee of the University Hospital Rotterdam approved this study. Informed consent was obtained from the parents of the children prior to participation in the study.

AD patients were selected on a history of mild to severe eczema according to the diagnostic criteria of Hanifin & Rajka [24]. They also had a positive scratch test or skin application food test (SAFT) [25] specific for milk,

egg, peanut, wheat, soja or codfish and a positive radio-allergo-sorbent test (RAST) [26] specific for food mix (Pharmacia, Uppsala, Sweden) or inhalation allergens (Phadiatop cap RAST, Pharmacia). The severity of the AD was determined by the modified SCORAD system (27), which is a composite index including the assessment of two items in a standardized way: a) extent and b) intensity (erythema, edema/papulation, oozing/crust, excoriation, lichenification, dryness). This scoring system reflects the modified consensus of the European Task Force on Atopic Dermatitis. Unlike the initial version of the SCORAD index [28], subjective symptoms are not included in the scoring of AD.

Children with asthma were selected using the criteria of the American Thoracic Society: coughing and/or shortness of breath, at least three times a year for a period of two weeks [29]. All patients had a positive family history of asthma and/or atopy. Asthma patients were divided in two groups, allergic and non-allergic, as judged by the presence of a positive or negative RAST (Phadiatop and food mix), respectively. All asthma patients were studied during a stable phase of their disease and there was no need for treatment with (inhalation-) corticosteroids.

The healthy controls had no history of allergy and a negative family history as judged by a questionnaire. All healthy children lacked specific IgE for inhalation and/or food allergens as measured by RAST (Phadiatop and food mix).

Collection of plasma samples

Five ml of heparinezed peripheral blood was collected by vena puncture of each subject. After Ficoll centrifugation, plasma samples were obtained and stored at -70°C until analysis. The analyses of sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75 were performed simultaneously in plasma diluted 1:1 in sterile 10 mM phosphate-buffered saline (PBS, pH 7.2).

Determination of sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75 by ELISA

sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75 were measured by enzyme-linked immunosorbent assay (ELISA) as previously described [30-32]. Briefly, 96-wells microtitre plates (Nunc Immuno Plate Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with monoclonal antibodies (mAb) to sICAM-1 (HM.2, 3 µg/ml) or sE-selectin (ENA1, 10 µg/ml) or sTNF-R55 (MR1.1, 5 µg/ml) or sTNF-R75 (MR2.2, 5 µg/ml) in PBS followed by saturation with 1% bovine serum albumin (BSA; Sigma, St.Louis, MO). Standard or diluted samples were added in 100 µl/well. After 2 hours at room temperature (RT), the plates were washed and incubated for 1 hour at RT with biotin labeled anti-ICAM mAb (HM.1), anti-E-selectin mAb (ENA2) or biotinilated rabbit anti-sTNF-R55/R75 immunoglobulin (Ig)G. After washing streptavidin-conjugated peroxidase (Dako, Glostrup, Denmark) was added. Peroxidase activity was determined by addition of TMB (3,3',5,5'-tetramethylbenzidine) substrate (Kirkegaard & Perry Lab., Gaithersburg, MD). The reaction was stopped with H_2SO_4 and read at 450 nm. The concentrations of sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75 in the samples were determined by interpolation from the standard curve. The

detection limits of the individual ELISA assays were 400 pg/ml for sICAM-1, 200 pg/ml for sE-selectin, 12 pg/ml for sTNF-R55 and 25 pg/ml for sTNF-R75. All monoclonal antibodies used in the different ELISA's were a kind gift of Dr. W.A. Buurman.

Sandwich ELISA for detection of TNF-α in plasma

Determination of TNF-α levels in plasma of patients and healthy children was performed by a sandwich ELISA as described previously [33]. Briefly, 96-wells microtitre plates (Falcon 3912, Becton Dickinson & Company, New Jersey, USA) were coated overnight at 4°C with 100 µl/well of 2.5 µg/ml mAb 61E71 in PBS. Nonspecific binding was blocked by incubating the plates with 125 µl/well PBS containing 1% BSA (Sigma) for 1 hour at RT. TNF-α standard was diluted in plasma of a suitable donor. To this end, several donors were tested and proper plasma samples were obtained. Plasma samples (1:1 diluted in PBS) and rhTNF standard (0.01-10 ng/ml; standard plasma diluted 1:1 in PBS) were added in duplicate (100 µl/well) and incubated for 2 hours at RT. Next, the plates were incubated for 1 hour at RT with rabbit anti-TNF diluted in PBS containing 0.1% BSA, followed by 1 hour incubation with a polyclonal swine anti-rabbit IgG conjugated to horse radish peroxidase. After washing the plates 5 times with bidest containing 0.1% Tween 20, bound enzyme activity was measured with 1 mg/ml 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS; Sigma) in a 1:1 mixture of 0.1 M citric acid and 0.2 M $Na_2HPO_4.2H_2O$), containing 0.003% H_2O_2 as substrate. The optical density was read at 414 nm. The lower detection limit of the ELISA was 40 pg/ml.

Statistical analyses

Statistical analyses were performed with STATA™ (Computing Resource Center, Los Angeles, CA). Statistical significance was analyzed using the Mann-Whitney test and correlation coefficients were calculated by linear regression or the Spearman sign rank correlation. P values smaller than 0.05 were considered significant.

RESULTS

Plasma levels of sICAM-1, sE-selectin, TNF-α, sTNF-R55 and sTNF-R75 in the various patient groups

We studied whether the plasma levels of sICAM-1, sE-selectin, TNF-α, sTNF-R55 and sTNF-R75 were different between atopic and non-atopic children. Table 1 summarizes the plasma levels of sICAM-1, sE-selectin, sTNF-R55 or sTNF-R75 for each patient group. No significant differences could be observed between healthy children and the various patient groups for any of the molecules analyzed. TNF-α could not be detected in any of the samples.

Table 1. Plasma levels of sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75 in various patient groups.

Patient group	sICAM-1 (ng/ml)	sE-selectin (ng/ml)	sTNF-R55 (pg/ml)	sTNF-R75 (pg/ml)
Healthy children	65.4* (43.2-101.8)	5.1 (2.4-15.5)	385 (230-870)	1620 (930-2930)
Non-allergic asthma	60.4 (49.4-85.7)	5.2 (3.4-8.5)	390 (350-520)	1370 (350-1820)
Allergic asthma	65.8 (50.6-126.4)	4.5 (2.4-15.5)	390 (270-1290)	860 (290-3150)
Atopic dermatitis	61.9 (56.0-76.8)	6.1 (2.6-8.5)	415 (220-760)	1160 (480-2340)

* Results are expressed as median concentrations (range). All samples were assayed in duplicate.

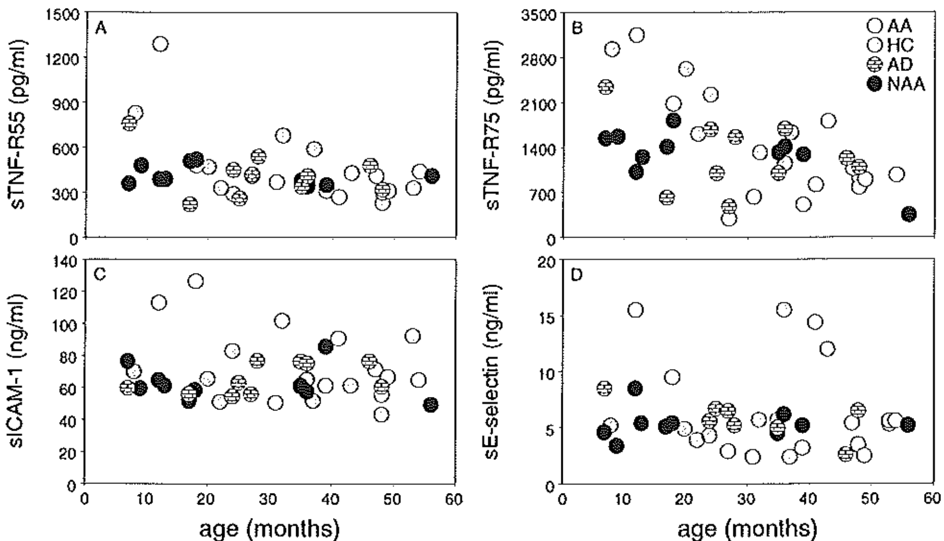

Figure 1. Plasma levels of sTNF-R55 (pg/ml; A), sTNF-R75 (pg/ml; B), sICAM-1 (ng/ml; C), sE-selectin (ng/ml; D), in relation to the age (months) of the children. Levels of sTNF-R55 ($p = 0.003$) and sTNF-R75 ($p = < 0.001$) decreased significantly with the age of the children.

Relationship between plasma levels of sICAM-1 or sE-selectin or sTNF-R55 or sTNF-R75 and age of the children

The plasma levels of sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75 were ordered in each patient group according to age, as illustrated in Figure 1, to examine whether age-related changes could be observed. As the age distribution in the patient groups were similar, the data obtained of the measured markers could be compared between the different groups. The data were analyzed by regression analysis. We evaluated whether the regression

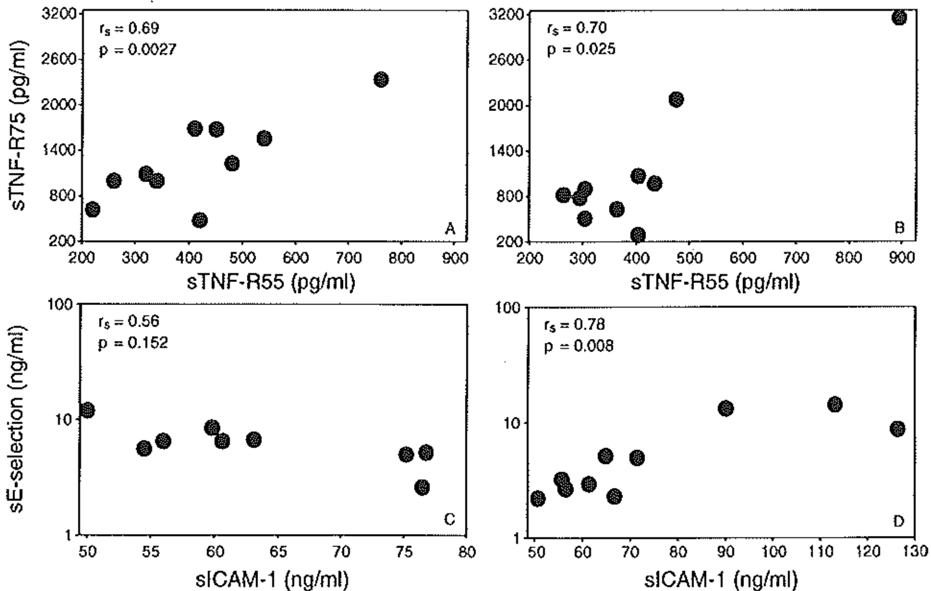

Figure 2. Correlation between sTNF-R55 (pg/ml) and sTNF-R75 (pg/ml) or sICAM-1 (ng/ml) and sE-selectin (ng/ml) in AD and AA. **(A)** Correlation between sTNF-R55 and sTNF-R75 in AD (r_s=0.69, p=0.027). **(B)** Correlation between sTNF-R55 and sTNF-R75 in AA (r_s=0.70, p=0.025). **(C)** Correlation between sICAM-1 and sE-selectin in AD (r_s=-0.56, p=0.152). **(D)** Correlation between sICAM-1 and sE-selectin in AA (r_s=0.78, p=0.008).

lines in each group showed a comparable trend with age. An age-related effect was observed for sTNF-R55 (p=0.003, Fig. 1A) and for sTNF-R75 ($p<0.001$, Fig. 1B). No significant age-related effects were observed for sICAM-1 (Fig. 1C) and sE-selectin (Fig. 1D).

Relationship between plasma levels of sICAM-1 and sE-selectin or sTNF-R55 and sTNF-R75

We analyzed whether a correlation was present between sICAM-1 and sE-selectin, or between sTNF-R55 and sTNF-R75 in each patient group. As shown in Figures 2A and 2B, there was a significant positive correlation between sTNF-R55 and sTNF-R75 levels in the AD group (r_s=0.69, p=0.027) as well as in the AA group (r_s=0.70, p=0.025), but not in the NAA group and in the HC group. The correlation coefficient between the plasma levels of soluble adhesion molecules, sICAM-1 and sE-selectin, was not significant in the AD group (r_s=-0.56, p=0.152) (Fig. 2C), but was significant in the AA group (r_s=0.78, p=0.008) (Fig. 2D).

Relationship between plasma levels of sICAM-1, sE-selectin, sTNF-R55 or sTNF-R75 and severity of the AD as assessed by SCORAD

Levels of sICAM-1 were described to be increased in sera from patients with an acute exacerbation of their atopic eczema [9]. Therefore, the relationship between the SCORAD and the plasma levels of sICAM-1, sE-selectin, sTNF-R55 or sTNF-R75 was determined. In AD patients a significant correlation ($r=0.73$, $p=0.038$) was observed between plasma levels of sE-selectin and the SCORAD (Fig. 3). No correlation could be detected between sICAM-1, sTNF-R55 or sTNF-R75 and the SCORAD (data not shown).

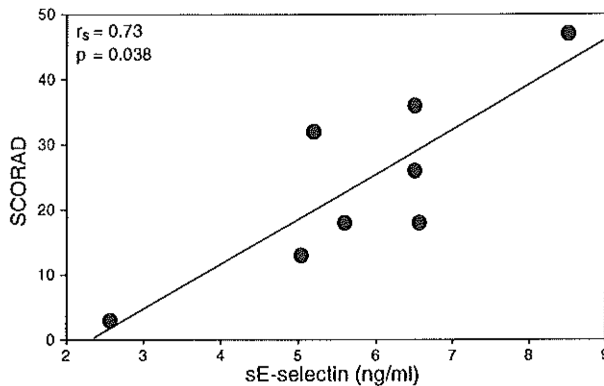

Figure 3. Correlation between sE-selectin (ng/ml) and SCORAD in AD ($n=8$), with a correlation coefficient of $r=0.73$ and $p=0.038$.

DISCUSSION

This study shows that sICAM-1 and sE-selectin can be detected in plasma of children whether or not they suffer from atopic disease. Most important, in the case of sE-selectin, but not sICAM-1 a significant correlation was found with an index of disease severity (SCORAD) in the AD group.

Inflammatory cytokines, such as TNF-α and interleukin (IL)-4, induce upregulation of several adhesion molecules, including E-selectin and ICAM-1 on endothelial cells located in the target organs of the inflammatory process. After shedding from endothelial cells, these soluble adhesion molecules retain their capacity to bind their receptors, thereby potentially limiting the inflammatory process [2]. In our study no differences could be observed in the plasma levels of these soluble adhesion molecules between individuals in any of the patient groups tested relative to healthy controls. One possible explanation is based on the heterogeneity among individuals. Furthermore, we studied patients under relatively stable conditions of their atopic disease and the disease severity may vary within the patient groups.

In the case of adult asthmatic patients, it is disputed whether sICAM-1 and sE-selectin reflect the severity of the inflammation [8] or were related with

the allergic status of the patients [5]. Our results, showing a correlation between sICAM-1 and sE-selectin in AA but not NAA patients, are more compatible with the results of Gosset et al [5], suggesting that the mechanisms and cells involved in the inflammation in non-allergic asthma are different from those found in allergic asthma. This suggests that adhesion molecules are more important in the inflammation present in allergic asthmatic patients than in non-allergic asthmatic patients.

A second explanation of the lack of differences in the levels of soluble adhesion molecules between healthy controls and the various patient groups might be the relative immaturity of the immune system of the children studied. In several respects, the immune system of newborns and infants has been described to be relatively immature as compared to adults [34]. We were not able to detect age-related differences in the plasma levels of soluble adhesion molecules. Moreover, the age distributions in all patient groups were similar. Therefore, we consider it unlikely that the immaturity of the immune system of the children included in this study, was responsible for this lack of difference.
We were not able to detect TNF-α in plasma of any patient or healthy control studied, probably due to the half-life of TNF-α in the circulation that is generally believed to be very short [35].

In this study, atopic patients were studied who were more or less in stable phases of their disease. In these patients, no disease related increases in TNF-α or sICAM-1 were detected. During exacerbation of the disease, increases in the levels of TNF-α and sICAM-1 or sE-selectin were found [8,9,11].

Soluble TNF-R in the circulation are considered to be more stable than TNF-α [19]. Therefore, it was of interest to study the levels of sTNF-R in these patients as well. We were able to detect sTNF-R55 and sTNF-R75 levels in healthy children in levels similar as observed in healthy adults [17,36]. For all patient groups, a similar age-related decrease in the levels of sTNF-R was found. For sTNF-R also no differences could be observed between atopic and non-atopic children. Serum sTNF-R levels were found to be increased only transiently in some diseases, like systemic lupus erythematosus and rheumatoid arthritis [21,22]. Therefore, it might be that increased levels of sTNF-R in plasma are present only during an asthma attack or during an exacerbation of atopic dermatitis.

Only in the groups of atopic children a correlation was found between sTNF-R55 and sTNF-R75 levels (both AD and AA group). In non-atopic children (HC and NAA) all of these molecules could be detected, but no significant correlations were found. A correlation between both sTNF-R was also described for other diseases [37-39]. Thus, variation in soluble adhesion molecules and sTNF-R levels could be a reflection of disease activity. Further study including patients during exacerbation of the atopic disease is necessary to establish such a relation.

Between plasma sE-selectin levels and the SCORAD in AD patients a linear relationship was found. The SCORAD is not a marker for a deterioration of the disease, but it is a combined symptom score collectively indicating the

severity of the disease [28]. Such a correlation between sE-selectin levels and the severity of the dermatitis has been found before in adult AD patients by Czech et al. [40]. The difference between the correlation coefficient between their and our study might be due to multiple factors (i.e. different ELISA's, children versus adults, different type of severity index). This indicates that sE-selectin is probably associated with disease activity in AD. Further analysis in larger patient groups will permit the determination of the distribution of these sE-selectin levels against severity of disease and possibly support the clinical application of this relation. The lack of consensus in a severity index for asthmatic children did not permit such an analysis for these groups of patients as yet.

In conclusion, in AD patients a significant correlation was observed between the levels of sE-selectin and SCORAD. Further study, using a larger patient population is necessary to determine how these molecules change during a deterioration of the atopic disease. These results will give a better understanding of the clinical implication and will give more insight in the inflammatory processes characteristic in these diseases.

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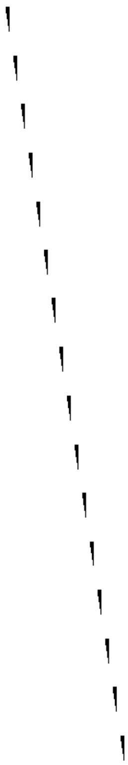
GENERAL DISCUSSION AND SUMMARY



CHAPTER 11

GENERAL DISCUSSION

In the first years of life the immune system of children is relatively immature compared to that of adults (1-4). This immaturity of the immune system creates a window period between birth and 2 years of age with an enhanced risk for allergic sensitization and subsequent atopic disease (5).

The allergen-specific T cell responsiveness, which distinguishes atopic from non-atopic children, is programmed into the T-helper (Th) cell memory compartment already during early childhood (6). In children with 'established' atopic disease a disturbed balance in Th cell subsets (Th1 vs. Th2) towards a polarized Th2 phenotype is generally accepted (7-10). The failure of atopic children to develop a long-term Th1-dependent memory has been proposed to be due to a reduced IFN-γ production (11-13). The shift towards a polarized Th2 phenotype during the process of allergic sensitization in children that develop atopy is not completely understood.

The *aim of the studies* presented in this thesis was to identify dysregulations in T cell responses (a) prospectively in children at high risk to develop atopy, and (b) cross-sectionally in children with established atopic dermatitis (AD). These T cell responses were studied both after polyclonal and allergen-specific stimulation, with emphasis on mRNA expression and protein production profiles of the relevant cytokines. Detection methods for cytokine protein production are less sensitive than for mRNA expression. Therefore, the combined analysis of mRNA expression and protein production will result in a more precise overview. Markers used for the analysis of incidence and prevalence of atopic diseases were primarily skin tests, allergen-specific IgE antibodies and clinical symptoms. Cellular markers, such as the release of mediators by blood cells, may also be useful in the assessment of atopic disease. Application of such markers, however, requires further validation, which is another hallmark of this thesis.

11.1. THE DEVELOPMENT OF ATOPIC DISEASE

Allergen challenge in an atopic patient results in the selective activation, recruitment and accumulation of the specific Th2 cells in the target organs, such as skin and lungs. The importance of Th2 cells in atopic diseases is tightly associated with the functional activities of the cytokines they produce.

In order to reverse the trend of increasing prevalence of atopic disease in young children, an understanding of the immunological processes in early life, which potentially promote Th2-polarized atopic sensitization, is necessary. Below we discuss how the investigated environmental and genetic risk factors and immunological responses could contribute to the development of atopic disease in high-risk children.

11.1.1. Influence of genetic and environmental risk factors

For the prospective study, 133 newborns at high risk for the development of atopy were selected and followed from birth up to 2 years of age. The *prevalence* of atopy in this cohort increased from 16% at 6 months to 32% at 24 months of age (chapter 4). AD was the most frequently diagnosed atopic disease. Of the children with AD at 12 months of age 22% (5/23) also had a food allergy, confirming previous findings (14,15). The combination of AD and food allergy in the first 12 months of life proved to be an important risk factor and constituted the most reliable diagnostic parameter associated with the development of asthma-like disease after the first year of life (16, and chapter 4).

All of the children included in the study had an atopic mother and 20% of the children also had an atopic father. The total prevalence of atopy during the first 2 years of life was 27% in the case of an atopic mother only and 52% in the case of two atopic parents, which is in agreement with the expected *genetic predisposition* (17). Moreover, atopy of both parents was significantly related (relative risk (RR)=2.13; chapter 5) with the development of atopy in the children at 12 months of age. This clearly confirms the role of genetic predisposition in the development of atopy.

The role of atopic siblings in the genetic predisposition of the newborns included in our cohort was difficult to interpret, since most of our newborns were first-borns. Furthermore, at the evaluation point of 2 years of age they had siblings mostly younger than 6 months, which was too young for a distinct clinical manifestation of atopy.

Besides the genetic predisposition, *environmental* factors influence the development of atopic disease. Several environmental factors such as parental smoking, bacterial and/or viral infections, feeding method (bottle vs. breast feeding), birth-weight, early exposure to household pets and inhalant allergens, have all been implicated as factors increasing the risk for the development of atopic disease (18-21). In our study, we analyzed the relationship between feeding method, exposure to household pets, house-dust mite (HDM) avoidance strategies, parental smoking and the development of atopy before 12 months of age (chapter 5). Of these factors, parental smoking was the only significantly related risk factor (RR=2.23; chapter 5) for the development of atopy in children before 12 months of age.

It was recently published that parental smoking did not increase the risk for allergic sensitization in children, after excluding asthma and other wheezing disorders (22). However, most published studies show convincing evidence that exposure to tobacco smoke increases the risk for the development of atopic disease (including wheezing bronchitis, asthma, and bronchial hyperreactivity) in unselected children as well as in children with an atopic predisposition (18-21,23-26).

As the number of cigarette-smoking young women has steadily increased in the Western industrialized countries over the last 40 years, passive smoking early in life may be one of the factors behind the increasing prevalence of atopy (27). Exposure to maternal smoking appears to have the greatest effect, probably because the mother is more in close contact and spends more time with the infant during the first year of life (23). Moreover, maternal smoking during pregnancy could affect the development of the immune system of the fetus (23,24,28). It has been documented that maternal smoking causes a significant rise in cord blood IgE levels and reduces the NK cell activity (28). Maternal smoking might also act as an adjuvant factor magnifying the underlying proces of allergic sensitization (27). Our data confirms that during the first year of life passive smoking is the best identified and the strongest environmental risk factor for the development of atopic disease. For the development of atopy at later ages other risk factors, such as exposure to household pets and HDM may become more important (20). A dose-response relation between exposure to HDM and the sensitization to HDM was recently described for children up to 3 years of age (29). The clinical implications of these markers as risk factors for the development of atopy may become more obvious during longer follow-up.

11.1.2. The immune response during the development of atopic disease

For the process of allergic sensitization, the relative immaturity of the immune system of newborns compared to that of adults could create a window period starting *in utero* and lasting up to 2 years of age with increased susceptibility to develop atopy. In order to gain more insight into the process of allergic sensitization and the subsequent development of atopic diseases, we examined the development of immunological responses in high-risk children.

11.1.2.1. T cells with a Th0, Th1 or Th2 phenotype

Optimal culture and stimulation conditions are necessary for the detection of cytokine production profiles to discriminate between the Th0, Th1 and Th2 subsets. It has been argued that subpopulations of Th cells form a continuous spectrum in which Th1 and Th2 phenotypes are only the two extremes. Whether Th0 cells represent a separate, stably differentiated T cell population, remains unclear.

In order to study the development of Th0/Th1/Th2 cytokine profiles during the process of allergic sensitization, peripheral blood mononuclear cells (PBMC) were stimulated *in vitro*. The different Th phenotypes were based on the produced cytokine levels (either protein or mRNA).

In chapter 5 differences in cytokine levels produced by stimulated PBMC of children who developed atopy relative to those who did not were used to discriminate between the Th phenotypes. A Th2 phenotype is defined

as producing relatively more Th2 cytokines (IL-4, IL-5 or IL-13), while a Th1 phenotype is defined as producing relatively more IFN-γ. The Th0 phenotype is defined as producing similar amounts of Th1 and Th2 cytokines.

In chapters 5 and 6 the different Th phenotypes were also based on the proportional production of IL-4 and IFN-γ, by correlating the production of these two cytokines. A Th2 phenotype was defined as less than 5000 pg/ml IFN-γ and more than 5 pg/ml IL-4 protein production. A Th1 phenotype was defined as more than 5000 pg/ml IFN-γ and less than 5 pg/ml IL-4 protein production, whereas the other production levels were defined as be due to Th cells of the Th0 phenotype.

11.1.2.2. Proliferative responses and cytokine production after polyclonal stimulation

Proliferative responses and cytokine profiles were studied after polyclonal stimulation to investigate whether the intrinsic capacity of T cells to give rise to a Th1 or Th2 cytokine profile was different between atopy-prone children and children who do not develop atopy within their first year of life. We used the stimulus PMA plus Ca-ionophore to achieve maximum T cell stimulation. In contrast to allergen-specific stimulation, this stimulus does not involve triggering of the T cell receptor (TCR)/CD3 complex. Moreover, this stimulus results in high production levels of both Th1 and Th2 cytokines. Retrospectively, our data shows an increased proliferative capacity of cord blood mononuclear cells (CBMC) of those children who developed atopy before 12 months of age (chapter 4), which is in agreement with findings of others (30, 31).

The vaginal delivery process of a child usually takes several hours, and during this delivery-related stress-period for the fetus the immune system may become non-specifically activated (32,33). Such a stress-period could explain the higher baseline proliferative response observed in CBMC of all newborns compared to the baseline proliferative response at 6 months of age. In addition, the cytokine mRNA expression levels, as determined by a quantitative PCR, were also increased at birth compared to 6 months of age, which provides further evidence for such a non-specific activation of the immune system of the newborn at birth.

The cytokine profile observed at birth after polyclonal stimulation of CBMC is in accordance with a predominance of Th cells of the Th0 phenotype (chapters 5 and 6). However, an underlying selective Th2 cytokine profile to specific allergens could still be present but escaping detection (34).

The Th0 cytokine profile observed at birth shifted to a Th2 cytokine profile at 6 months of age in children that developed atopy before 12 months of age (chapter 6). Several groups proposed that the development of a strong Th2 polarized memory response in children who developed atopy was mainly due to a reduced IFN-γ production (31,34-36).

The analysis of IFN-γ is complicated by the rapid degradation of the IFN-γ protein in culture supernatants (dr. H. Yssel, personal communication). Therefore, optimal culture conditions (as described in chapter 3) and the combined analysis of mRNA expression levels and protein levels are especially important for IFN-γ. In our study, the mRNA expression levels were determined by a quantitative PCR using the TaqMan technology, which is a sensitive method to detect small differences in expression levels between different patient groups (37).

During the first year of life no significant differences were observed for mRNA expression or protein levels of IFN-γ between children who did and did not develop atopy. Therefore, we do not consider an intrinsic T cell defect for the production of IFN-γ to be the cause of the selective development of the Th2 profile in atopics (chapter 6). Excluding such an intrinsic T cell defect for IFN-γ production, possible differences in cytokine production levels between atopics and non-atopics must be the consequence of differential regulation. Infections are suggested to play a negative regulatory role in the development of the Th2 cytokine profile in atopics, based on the inverse relationship between plasma IgE levels and the number of infections in children (38). In our study, we did not find a difference in the number of infections between children who did and did not develop atopy. However, the relation between infections and the process of allergic sensitization is an important and complex issue, and still not completely understood.

11.1.2.3. Proliferative responses and cytokine production after allergen-specific stimulation

We and others did not observe differences in allergen-induced proliferative responses of CBMC between newborns who did and did not subsequently develop atopy (11,39,40). In addition, newborns who developed food allergy could not be distinguished from the other atopic children by the level of proliferative responses to food stimulation. We therefore conclude that allergen-induced proliferative responses of CBMC do not significantly contribute to the *prediction* of allergic sensitization during the first year of life.

Remarkably, a Th0 rather than a Th2 cytokine profile was observed after HDM-specific stimulation of CBMC (chapter 5). This is in contrast to the weak Th2 cytokine profile at birth as originally suggested by Holt et al (11). In the latter studies no clear discrimination was made between HDM-induced cytokine profiles in atopic children with or without clinical manifestations of upper-airway allergy. The weak Th2 skewing was proposed to be the result of low levels of environmental allergens crossing the placenta in combination with a local micromilieu (progesterone, PGE2, IL-4 and IL-10) which is strongly inhibitory to Th1-associated activities (41,42).

An interesting question concerns the mechanism of processing and presentation of the allergen to the T cell compartment. At birth, an allergen-specific proliferative response of CBMC is observed, indicating previous interactions with the allergen and the necessary involvement of antigen-presenting cells (APC). The principal APC network responsible for immune surveillance is the dendritic cell (DC) network, which is functionally immature in neonates and infants (43). The capacity of peripheral tissue DC to direct the neonatal T cell responses against environmental allergens towards the non-atopic Th1 profile may play a role in the development of atopy. In adults differences in DC functions *in vitro* between atopic and non-atopic individuals have been reported (44,45). The precise role of APC/DC in the postnatal maturation of Th1-associated functions in infancy has not yet been established. Furthermore, possible differences at the gut and/or bronchial mucosal surfaces between children who do and do not develop atopy could contribute to the nature and the amount of allergens presented to the systemic T cell compartment.

The process of *allergic sensitization* is currently only discussed in the light of the Th1-Th2 dogma. However, the observed cytokine profile of relatively high IL-10 production in combination with reduced IL-4 expression after HDM-specific stimulation in children who developed atopy could also be interpreted as a T regulatory 1 (Tr1) cytokine profile. Tr1 cells are able to produce high amounts of IL-10 and low amounts of IL-4 in combination with low proliferative responses after repeated antigenic stimulation in the presence of IL-10 (46,47).

Tr1 cells and their high IL-10 production levels in the development of atopy in children may cause a negative feedback in the process of allergic sensitization. High IL-10 levels are principally able to downregulate various components of the allergic reaction, i.e. Th2 development, IgE production, mast cell and eosinophil function. This may inhibit and/or delay the development of atopy. In addition, Tr1 cells, by their production of IL-10 and TGF-β, can induce long-lasting anergy in $CD4^+$ cells, which may inhibit the antigen-specific responses (46,47). High IL-10 levels also induce a general inhibition of cytokine release by both Th1 and Th2 cells, but more profoundly inhibit the IFN-γ production (48-50). Therefore, during the development of atopy in children, Tr1 cells might downregulate the Th1 response and thereby indirectly cause a relative increase in the Th2 response.

11.1.3. Other immunological markers analyzed during the development of atopic disease in children

We also investigated the relationship between cell surface markers, markers for allergic sensitization (specific IgE levels and skin tests) and markers for inflammation (plasma sE-selectin levels and blood eosinophil counts) on the one hand and the clinical expression of atopy at 12 and 24

months of age on the other hand (chapters 4 and 5). Markers for the early diagnosis of children developing atopic disease will allow measures to be taken to prevent or diminish the clinical expression of atopic diseases.

The analysis of cell surface markers on CBMC revealed an increased percentage of $CD4^+$ and $CD4^+CD45RO^+$ (memory) cells in newborns who later developed atopy, which is in agreement with data of others (51). In accordance with the data of Miles et al. (52) we did not find a difference in the percentage of $CD25^+$ ($IL\text{-}2R^+$) T cells between newborns who did and did not develop atopy within the first year after birth.

The increased percentage $CD4^+CD45RO^+$ cells in combination with the higher baseline proliferative response of CBMC from children who developed atopy, supports the hypothesis that at the time of birth the Th cells of these children had been previously activated, underscoring that the initial allergen sensitization could already start *in utero* (32 and chapter 5).

$CD4^+CD45RO^+$ cells are considered to be the major T cell subpopulation which responds to allergen and subsequently produces cytokines (53). However, the mean percentages of $CD4^+CD45RO^+$ cells in cord blood are very low compared to the percentages found in adults (54,55), which makes it unlikely that these memory cells are the only source of the allergen-induced cytokines. As described in chapter 1, $CD45RO^+$ cells can convert into $CD45RA^+$ cells. As the $CD45RA^+$ T cell population includes naive T cells, this suggests that the $CD45RA^+$ cells are not a homogeneous population. We suggest that subpopulations of $CD4^+CD45RA^+$ cells contribute to the allergen-induced cytokine production by CBMC. This is supported by the finding that $CD45RA^+$ T cells from atopic patients produced IL-4, IL-5 and IFN-γ after stimulation with mitogens (56). Moreover, most of the $CD45RO^+$ cells in cord blood are $CD45RO^+/RA^+$ double positive cells (57), indicating that $CD45RO^+$ cells in cord blood are not equivalent to the $CD45RO^+$ cells in the peripheral blood of older children or adults.

Of the analyzed markers for allergic sensitization, a positive skin test at 12 months of age was significantly related to the clinical expression of AD and food allergy at 24 months of age. Nevertheless, a substantial number of children yielded positive skin-prick tests in the absence of clinical symptoms, which makes the practical usefulness of skin-prick testing for the diagnosis of food allergy controversial (58,59).

Food- and HDM-specific IgE antibodies were significantly associated with food and upper-airway allergy, respectively. However, of the children with food-specific IgE antibodies only 25% had a food allergy. Previous studies showed that only 30% to 40% of patients with food-specific IgE manifested clinical symptoms after ingestion of the involved food (58,60). This can be the consequence of the nature of the immune response to produce IgE antibodies against food allergens in young children (61). Introduction of solid foods to children can result in the development of

varying plasma levels of IgE antibodies against one or more common food antigens, such as cow's milk and egg. The antibody titers usually peak around 9 months of age and rapidly disappear thereafter. Only in children that develop a food allergy these titers persist beyond the age of 9 months and are generally higher than the titers measured in non-atopic children. Therefore, the practical use of food-specific IgE for the diagnosis of food sensitization or allergy in children below 2 years of age is disputable (58,59,62,63).

IgG antibodies to food antigens occur in young children before IgE antibodies to food antigens are detectable in the serum. Recently, increased food-specific IgG levels in the serum of one year old children have been proposed as a predictor for an increased risk of developing atopy (64).

After allergen challenge high expression levels of E-selectin and ICAM-1 have been found in the inflammatory infiltrate in skin biopsies of AD patients (65). We therefore analyzed plasma sE-selectin and sICAM-1 levels, as soluble markers for the local inflammatory reaction in atopic children. We did not observe, however, a relationship between these markers and the clinical expression of atopy in children below two years of age. Another marker of inflammation, blood eosinophil counts, was also not associated with the clinical expression of atopy in children below two years of age. Apparently, the clinical symptoms of atopic disease are too mild to cause the presence of these markers in the circulation. It should be noted, however, that the determinations were performed in children without exacerbations of their atopic disease at the moment of examination. Exacerbation of the atopic disease, even in children at this age, is able to increase the levels of sE-selectin and sICAM-1 and to increase the numbers of blood eosinophils (66,67).

11.1.4. Immunological markers predicting atopy at 12 months of age

As stated before, cord blood of newborns who developed atopy before 12 months of age, displayed an increased percentage of $CD4^+CD45RO^+$ cells and an increased proliferative capacity after polyclonal stimulation of CBMC. Moreover, differences in cytokine production levels were found after allergen-specific stimulation of CBMC of newborns who did and did not develop atopy. Children who developed their first atopic symptoms between 6 and 12 months of age, already displayed a Th2 cytokine profile at 6 months. Despite these differences between the subpopulations of children who did and did not develop atopy before 12 months of age, the ranges of these immunological markers within the groups were large, resulting in a substantial overlap. Therefore, it is not feasible to use these cellular markers to predict the development of atopy at the level of an individual child in this age range.

Of the investigated markers for allergic sensitization or inflammation, a positive skin test and specific IgE antibodies to food and inhalant allergens were related with, but did not clearly precede the clinical expression of atopy (chapter 4). This was also suggested in studies which analyzed children older than 2 years of age (21,68-70). Apparently, analysis at 2 years in our cohort is too early for a definitive conclusion about the predictive value of the investigated markers for atopy development at later ages.

11.2. THE IMMUNE RESPONSE IN CHILDREN WITH ESTABLISHED ATOPIC DERMATITIS

The dysregulations in immune functions as observed during the development of atopic disease could possibly persist in children who suffered clinical symptoms of atopy for longer periods. Therefore, we performed a cross-sectional study in which the expression of cell surface markers on PBMC, plasma levels of soluble adhesion molecules, T cell proliferative responses and cytokine production profiles after polyclonal and allergen-specific stimulation of PBMC were investigated both in children with 'established' AD and in healthy children (HC).

In the Netherlands, the most frequently diagnosed food allergies are directed against cow's milk, egg and peanut (71). Peanut allergy can result in severe reactions such as anaphylaxis (72). Based upon these observations, we expected to find the most profound differences in T cell responses after stimulation of PBMC with peanut allergen. Therefore, we selected children with established AD with (AD^+PA^+) or without (AD^+PA^-) a peanut sensitization to analyze T cell responses after stimulation of PBMC.

11.2.1. T cell dysregulations after polyclonal stimulation

The Ki67 antigen is a nuclear antigen expressed in actively cycling cells (73). By a double-staining method using Ki67 antigen and cell surface markers it is possible to identify the proliferating cells. In chapter 8 we show that polyclonal stimulation of PBMC resulted in a stronger proliferation of $CD8^+$ cells than of $CD4^+$ cells. Nevertheless, 25-40% of the cells that expressed the Ki67 antigen were $CD4^+$ cells.

In children with established AD, the cytokine profiles after polyclonal stimulation of PBMC were significantly different between AD^+PA^+ children compared to AD^+PA^- children (chapter 9). In AD^+PA^+, children in contrast to AD^+PA^-, a Th2 cytokine profile was observed. Other studies also reported a Th2 cytokine profile after stimulation of PBMC from children with established atopy (9,74,75). Thus, after the establishment of AD a Th2 cytokine profile is present in children with a peanut-sensitization after polyclonal stimulation of PBMC.

11.2.2. The absence of a clear Th2 cytokine profile in AD^+PA^-

The absence of a clear Th2 cytokine profile in AD^+PA^- after polyclonal stimulation of PBMC may be due to the children outgrowing their atopy. AD^+PA^- children suffer from AD and sensitization to food constituent(s), but not to peanuts. In contrast to a peanut allergy that will not resolve, most children outgrow their food allergy. This is supported by the observation that the cytokine levels of AD^+PA^- are intermediate compared to the levels found in HC (Th0 profile) and in AD children with a peanut sensitization (AD^+PA^+; Th2 profile) (chapter 9). Therefore, our AD^+PA^- group consists of a relatively small and heterogeneous group of children. Longer follow-up of these children is needed to discriminate between children who will or will not outgrow their atopy.

11.2.3. T cell dysregulations after allergen-specific stimulation

As stated before, we chose peanut-allergen to study food allergen-induced T cell responses and used this as a model to study food allergy. However, both peanut-extract specific and polyclonal stimulation resulted in T cell cytokine profiles that were significantly different between AD^+PA^+ and AD^+PA^- or HC. This indicates that the immune responses in peanut-sensitized AD patients are not representative for the whole population of food-allergen sensitized AD patients (section 11.2.1). We therefore conclude that peanut-allergen stimulation can not be used in a model system for studying cytokine profiles in food-sensitized children (chapter 9).

Children who developed clinical symptoms after HDM-inhalation or egg-ingestion could not distinguished by the *proliferative responses* of their CBMC to HDM and egg stimulation compared to children who developed another atopy or non-atopic children (chapter 5). This might be due to the low frequency of allergen-specific cells present in the cord blood of children who subsequently develop clinical symptoms after HDM-inhalation or egg-ingestion. In contrast, children above 3 years of age with clinical symptoms due to atopy to egg or HDM distinguish themselves by an increased proliferative response of their PBMC to the allergen *in vitro* (76,77). In these children the frequency of allergen-specific T cells is apparently sufficiently increased since birth.

After allergen-specific stimulation of CBMC of newborns who developed atopy before 12 months of age, a Th0 *cytokine profile* was found. This profile apparently develops into an allergen-specific Th2 cytokine profile present in PBMC of children displaying an 'established' atopy (76-79). Together with the age-related increase in the frequency of allergen-specific Th2 cells, the allergen-induced proliferative responses reflects the allergen-induced clinical symptoms.

11.2.4. Other dysregulations in the immunological markers analyzed

In children with established AD, in contrast to children developing atopy, the observed *plasma total and specific IgE levels* reflected the clinical atopic manifestation (chapters 8 and 9). Moreover, increased *peanut-specific IgG4 levels* were observed in AD (both AD^+PA^+ and AD^+PA^-) compared to HC (chapter 9). This might be due to a quantitative difference in peanut allergen exposure at the mucosal surfaces of AD children, leading to the presence of allergen-primed T cells in the circulation (80,81). The contribution of the antigenic environment to the Th1 vs. Th2 cytokine balance and subsequently to the isotype switching process in B cells is as yet unresolved.

In plasma of AD^+PA^+, peanut-specific IgE and peanut-specific IgG4 antibodies were observed (chapter 9), indicating the presence of ε^+ and $\gamma 4^+$ B cells. This is likely the result of the increased presence of peanut-specific Th2 cells *in vivo* (82). Using Western blots, we demonstrated that the IgG4 and IgE antibodies present in AD^+PA^+ children recognized similar peanut proteins. This suggests that trans-splicing is involved in the generation of IgG4- and IgE-synthesizing cells from the same $\gamma 4^+\varepsilon^+$-positive B cell clone (83).

Peanut-specific IgG4, but not peanut-specific IgE antibodies, were observed in the plasma of AD^+PA^- children. Peanut-specific T cell stimulation in these children did not result in a Th2 cytokine profile. However, the observed Th0 cytokine profile, with low IL-4 levels, could be sufficient for the switch of B cells to γ4 (84). Based on this data we propose that strong allergen-induced Th2 responses are necessary for the switch of B cells to ε, while weaker allergen-induced Th2 or even Th0 cytokine responses are sufficient for a B cell switch to γ4 expression.

The presence of increased levels of *soluble adhesion molecules* in the circulation of patients with a variety of inflammatory disease states has raised the possibility that the plasma levels of these molecules may reflect disease activity. Therefore, possible differences in plasma concentrations of soluble adhesion molecules (sICAM-1 and sE-selectin) were investigated between atopic and non-atopic children with a mean age of 4 years (chapter 10). No significant differences were observed in the plasma levels of sICAM-1 and sE-selectin between children with AD, allergic asthma, non-allergic asthma and HC. We did find, however, a significant correlation between plasma sE-selectin levels in AD children and the extent and the severity of the eczema (chapter 10), as measured by the SCORAD-index (85). This initial finding was confirmed in a larger group of 40 children (86) and has been described previously in adult AD patients (87). Although an increased plasma sE-selectin level is not specific for AD (87), it reflects disease severity. It may be a reliable marker for monitoring the long-term disease activity in young children with AD, as sE-selectin levels did not precede the improvement of the symptom scores of the severity of the eczema (88). This could also explain that plasma sE-selectin levels can not be used to discriminate

between children who will and will not develop atopy in the first year of life or between mild AD children and HC (chapters 4 and 10).

In conclusion, immunological markers analyzed at birth or during the first year of life, such as plasma total and specific IgE levels, plasma sE-selectin levels and blood eosinophil counts, were not different between children who did and did not develop atopy before 12 months of age. However, these markers were all significantly increased or related with disease severity in older children with established atopy compared to HC.

11.3. CURRENT VIEW ON ALLERGIC SENSITIZATION AND THE DEVELOPMENT OF ATOPY

11.3.1. Intrauterine sensitization

One of the mechanisms underlying the development of atopy concerns the intrauterine sensitization. It is repeatedly documented that cord blood T cells are capable of proliferating *in vitro* in response to both food and inhalant allergens (11,39,40,89). This suggests that the initial allergen-specific T cell priming may occur *in utero* as the result of transplacental transfer of small amounts of the relevant allergens, to which mothers are exposed during pregnancy. The allergens may cross the placenta as soluble or IgG-complexed peptide fragments, already processed by the APC of the mother. Recent data on cytokine production levels at birth suggests that this T cell priming may be strongest in infants with a positive family history of atopy and in infants who later develop atopy (31,90).

As stated before, allergen-induced proliferative responses of CBMC from newborns who either did or did not develop atopy before 12 months of age support the hypothesis that the initial allergen sensitization of all newborns could have already started *in utero*. Jones et al. have presented evidence that allergen-specific T cell priming may already be initiated in the 2nd trimester of pregnancy (91). Such fetal T cell priming is suggested to be dependent of the season and the extent of allergen exposure of the mother (91-93). Very low or very high maternal exposure to allergens seems to be protective against primary fetal sensitization (92). High doses of maternal allergen-specific IgG antibodies are suggested to account for the prevention of atopy development in the newborn (94). Moreover, it might well be that differences exist between the intrauterine environment of fetuses of atopic versus non-atopic mothers. For example, there is some evidence that the amniotic fluid of atopic mothers contains higher IL-10 levels than that of non-atopic mothers (95). However, the mechanisms involved, including the role of fetal APC, and the initiation of intrauterine sensitization are largely unclear and need to be studied further.

11.3.2. Reduced production of IFN-γ during the perinatal period

Another factor proposed to be involved in the mechanism of allergic sensitization is the reduced production of IFN-γ by fetal CBMC and/or neonatal T cells after mitogenic stimulation relative to adult PBMC (96). This reduced IFN-γ production appears to be more marked in newborns either at risk for atopy or who later develop atopic disease (12,13,31,40). In chapter 6 we show that there appears to be no intrinsic defect in the capacity of T cells to produce IFN-γ when children who do develop atopy before 12 months of age are compared with children who do not. Our data therefore do not support this concept. We can not exclude, however, a possible difference in the capacity to produce IFN-γ between low and high-risk newborns, because we only included high-risk children in our study.

After the establishment of AD in children, most studies show a decreased capacity to produce IFN-γ compared to healthy children. However, in chapter 9 we show that the capacity of PBMC of children with AD to produce IFN-γ after polyclonal stimulation again is not reduced compared to non-atopic children. The intrinsic capacity of cells to produce IFN-γ protein appears not to be disturbed in newborns who will develop atopy and in children with established AD compared to non-atopic children.

In contrast, a disturbance was observed in the IFN-γ production after allergen-specific (HDM and peanut) stimulation, both in newborns who later developed atopy (chapter 5) and in children with established AD and apparent peanut-sensitization (AD^+PA^+; chapter 9). This indicates that the reduced IFN-γ production is most likely the consequence of a difference in regulatory mechanisms between atopic or not-yet atopic and non-atopic children. Allergen-specific stimulation apparently reflects more closely the *in vivo* situation. Therefore, *in vivo* IFN-γ could still play a role in the mechanism underlying the development and maintenance of allergic sensitization and atopic disease.

An explanation for the reduced allergen-induced IFN-γ production by CBMC of newborns who will develop atopy may be the presence of the recently described Tr1 cells (46). After allergen-specific stimulation of CBMC from newborns who developed atopy before 12 months of age, we indeed observed a relatively large IL-10 production in combination with reduced IL-4 expression, supporting the presence of Tr1 cells (chapter 5).

However, in children with established AD Tr1 cells likely do not explain the reduced allergen-induced IFN-γ production by PBMC, as a reduced IL-10 production was observed in these children. This reduced IFN-γ production has been attributed to a 'post-transcriptional defect', as these children display a discrepancy between IFN-γ mRNA expression and IFN-γ protein production (73). However, after polyclonal stimulation with PMA and Ca-ionophore of PBMC from AD no discrepancy between IFN-γ mRNA expression and IFN-γ protein production was found (chapter 9). In addition,

the IFN-γ production is believed to be primarily transcriptionally regulated with little or no post-transcriptional regulation (97), arguing against a post-transcriptional defect. An explanation for the discrepancy between the comparable mRNA levels and the reduced allergen-induced IFN-γ production in children with established AD could be the rapid degradation of secreted IFN-γ protein in culture supernatants. Alternatively, an increased autocrine receptor-mediated consumption of secreted IFN-γ in children with established AD might account for the disturbed Th1-Th2 cytokine balance.

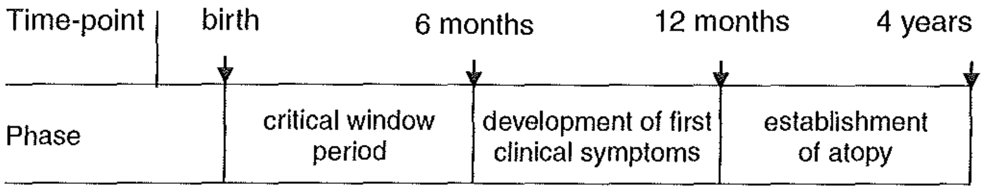
11.3.3. Proposed sequence of phases implicated in the gradual development and maintenance of atopic disease

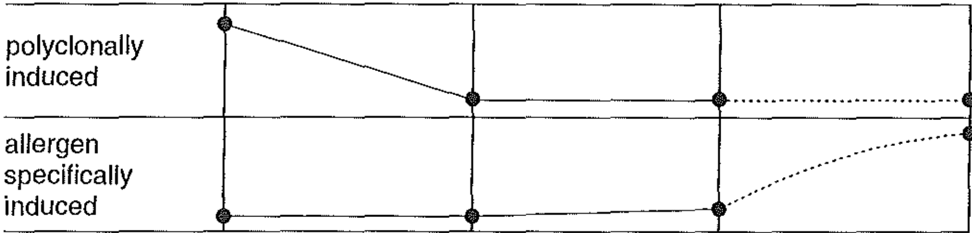
Based on the data presented in this thesis and the hypothesis for allergic sensitization at the perinatal age (section 1.6.3), we propose the following sequence of important phases implicated in the allergic sensitization and the maintenance of atopic disease during childhood (Figure 1). The data represent gradual changes in a critical set of immunological markers in children who develop atopy relative to non-atopic children. These parameters were analyzed at discrete time-points. Eventually, such data might aid in the assessment of selected immunological markers suitable in the follow-up of high risk children. Between birth and 12 months of age, data were obtained from a cohort of children participating in the longitudinal study, whereas the data around 4 years were obtained from another patient group of children participating in a cross-sectional study. Longer follow-up of the children participating in the longitudinal study should ideally confirm the data at 4 years.

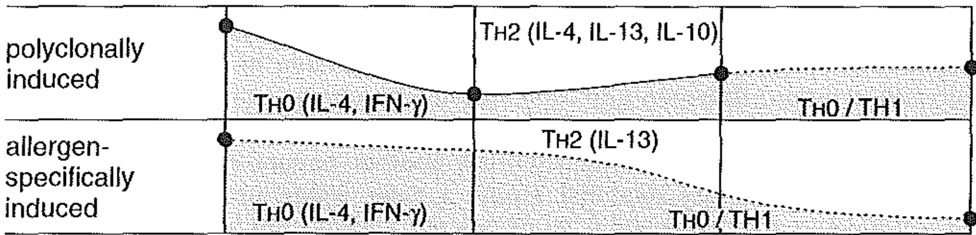
The initial T cell priming already starts *in utero* and may be stronger in infants that will develop atopy. This results in an increased proliferative capacity of cord blood T cells and a Th0 cytokine profile after polyclonal and allergen-specific stimulation of CBMC from newborns who will develop atopy. Alternatively, the increased proliferative capacity of CBMC might be a reflection of the stress-period during the delivery and will gradually disappear during the first 6 months of life.

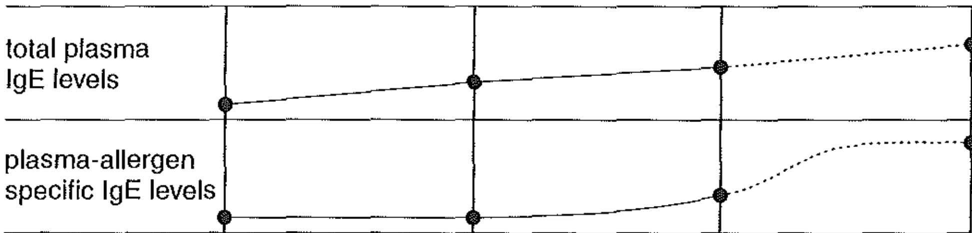
The first 6 months of life appear to be the most critical period for the induction of atopic disease (critical window period). During this period the inherent capacity to produce a Th0 cytokine profile gradually shifts to a Th2 cytokine profile in children that develop an atopy by 12 months of age. Later during infancy, allergen-specific stimulation of PBMC leads to a pronounced Th2 cytokine profile, due to an increased frequency of allergen-specific T cells in the peripheral blood.

Between 6 and 12 months of age the clinical manifestations of atopic disease usually become more pronounced. During this period a Th2 cytokine profile is still detectable after polyclonal stimulation of PBMC. In addition,


Time-point
birth
6 months
12 months
4 years
Phase
critical window period
development of first clinical symptoms
establishment of atopy


T-cell proliferative responses
polyclonally induced
allergen specifically induced


Cytokine profile
polyclonally induced
TH2 (IL-4, IL-13, IL-10)
TH0 (IL-4, IFN-γ)
TH0 / TH1
allergen-specifically induced
TH2 (IL-13)
TH0 (IL-4, IFN-γ)
TH0 / TH1


B-cell responses
total plasma IgE levels
plasma-allergen specific IgE levels

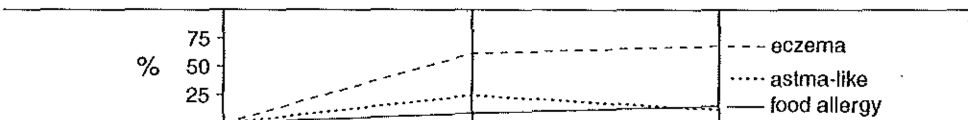

Clinical symptoms
%
75
50
25
eczema
astma-like
food allergy

Figure 1. Schematical presentation of sequence of phases implicated in the gradual development and maintenance of atopic disease. Three different phases are distinguished: (1) the critical period: between birth and 6 months of age, (2) the development of the first clinical symptoms: between 6 and 12 months of age, and (3) the establishment of atopy: between 12 months and 4 years of age. Data for phase 1 and 2 was obtained from samples of children included in a longitudinal study and data for phase 3 was obtained from samples of children included in a cross-sectional study. The development of detectable T cell proliferative responses, Th cell cytokine profiles, B cell responses and clinical symptoms are schematically shown during the different phases of allergic sensitization and the establishment of clinically overt atopic disease during childhood. Data represents gradual changes of the immunological markers with emphasis on the time sequence. Distinction is made between polyclonally induced and allergen-specifically induced T cell proliferative responses and cytokine profiles. Moreover, the B cell response is indicated by total IgE as well as allergen-specific IgE plasma levels.
• =value of analyzed data, — = extrapolated course, ... =expected course

with increasing age allergen-specific stimulation of PBMC leads to a more pronounced Th2 cytokine profile. In the same period, the non-specific B cell response, as determined by total plasma IgE levels, becomes more prominent. Between 12 months and approximately 4 years of age the first clinical symptoms of atopy tend to develop into an established atopic disease. By that time, an allergen-specific proliferative response and a pronounced Th2 cytokine profile is to be observed after stimulation of PBMC. During this period, allergen-specific IgE appears in the circulation and also plasma total IgE levels become more representative for atopic children. Thereafter a more stable phase starts in these children possibly persisting into adulthood, although variations may well occur (98-100).

11.3.4. Th2 polarization in the first years of life

During the different phases of the development of allergic sensitization and the establishment of the atopic disease in childhood, particular cytokines can be used to discriminate between children who will and will not develop atopy. Around 6 months of age, a short lasting strong increase in the IL-4 protein production after polyclonal stimulation of PBMC is observed in children who will develop atopy (chapter 6). This IL-4 is likely responsible for the initial development of Th2 cells (101). During all phases, small differences in IL-5 mRNA expression and IL-5 protein production are observed after stimulation of PBMC from children who will develop atopy compared to those who will not (chapters 6 and 9). Probably, IL-5 is more related to the local tissue inflammation and therefore the presence of this cytokine in the peripheral blood is less pronounced than that of other Th2 cytokines, such as IL-13.

IL-13 is a key cytokine for atopic diseases that is useful to discriminate between atopic and non-atopic children during all different phases (chapters 6 and 9). Moreover, IL-13 is described to be related with the presence of specific IgE and to be the most predictive cytokine for the

presence of a Th2 pool independent of the age of the child (10,98).

During the establishment of the atopy the reduced production of IL-10 and IFN-γ protein becomes gradually more prominent (chapter 9). As IL-10 is a strong immunosuppressive cytokine (50), the reduced IL-10 production may result in a lower degree of immunosuppresion and subsequently in the maintenance of the allergic inflammation. The imbalance between Th1 and Th2 cells in atopy, with a skewing towards Th2 cells, most likely causes the relatively reduced IFN-γ protein levels.

In summary, during allergic sensitization and the establishment of atopic disease in childhood, the Th0 cytokine profile as detected at birth gradually shifts to a Th2 cytokine profile. Polyclonally induced responses of circulating cells can reveal atopic sensitization at an earlier age than allergen-induced immune responses. Similarly, allergen-specific T cell responses precede allergen-specific IgE antibodies in the circulation. The allergen-specific IgE antibody formation correlates with the appearance of clinical symptoms of atopy.

11.3.5. Practical implications

With reference to the sequence of phases of atopy development, we propose some risk factors with practical implications for preventive measures.

Pregnancy: So far none of the primary preventive measures (allergen-free diet, reduction of allergen exposure) taken during pregnancy have been shown to be effective (102,103). One of the explanations could be that these preventive measures were not started before the third trimester of pregnancy, which is probably too late to prevent allergic sensitization in the fetus.

Critical window period (0-6 months): Passive smoking is the most pronounced risk factor in this period. Other environmental factors, such as HDM, pets and other allergens, will also influence the allergic sensitization during this period, although these were not significantly associated with atopy development at 12 months in the children in our cohort study. Probably, these factors cause a dose-dependent risk and their effect will become more clear at later ages (20,29).

Development of first clinical symptoms (6-12 months): During this period a strict alertness on clinical symptoms of a developing atopic disease (primarily AD and food allergy) is important in order to start intervention as soon as possible.

Establishment of atopy (12 months-4 years): In our cohort the combination of AD and food allergy at 12 months of age appeared the strongest risk factor for the development of asthma-like disease at 24 months of age (chapter 4). Other risk factors were a positive skin test, the presence of allergen-specific IgE in plasma and a Th2 cytokine profile of the PBMC.

For a definitive conclusion about the value of these markers in the prediction of atopic disease and the maintenance of atopy, more research will

be necessary. In this and other studies, the high-risk children should be followed up for a longer time period and during the first year of life at shorter time intervals, because as yet no reliable early predictive factors are available atopy development in high-risk children.

11.4. CONCLUSIONS

The studies described in this thesis lead to the following conclusions relating to the research questions described in the section Aims of the study (section 1.7).

- In high-risk newborns who do not develop atopy before 12 months of age, polyclonally induced and allergen-specific proliferative responses of CBMC to cow's milk, egg and HDM can be found at birth. Moreover, the CBMC possess an inherent capacity to produce a Th0 cytokine profile.
- Children who develop atopy before 12 months of age, have CBMC capable of producing a Th0 cytokine profile. After birth the PBMC gradually develop the potency to produce a Th2 cytokine profile, which is established within the first 6 months of life. This selective development of a Th2 cytokine profile appears to be independent of the capacity to produce IFN-γ.
- After the establishment of the atopic disease (AD), a Th2 cytokine profile was present after stimulation of PBMC. The B cell response leading to the production of total and specific IgE is associated with, and probably underlies, the clinical atopic manifestations.
- A positive skin test, specific IgE to food or inhalant allergens and a Th2 cytokine profile were related to the clinical expression of different atopic diseases. More research is necessary for a definitive conclusion about the value of each of these markers for the *prediction* of atopy development in children before 2 years of age.

We conclude that the first 6 months of life are a critical time window for the immunological changes reflecting the early allergic sensitization, which is clinically detectable at 12 months of age. Future studies should focus on the development and the regulation of cytokine profiles, specific IgE levels and skin tests during the first 6 months of life, as markers for early allergic sensitization.

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SUMMARY

The development of atopic diseases, such as atopic dermatitis (AD), asthma, and food- or upper-airway allergy, is the result of complex interactions between genetic susceptibility and environmental factors. These interactions can induce allergic sensitization and subsequently inflammation in the target organs, resulting in clinical symptoms of atopy. In the first years of life, the immune system of children is relatively immature compared to adults. This immaturity of the immune system may create a window period between birth and 2 years of age, in which the risk of allergic sensitization and the subsequent development of atopic disease is increased.

Helper T lymphocytes (Th cells) play a central role in the allergic sensitization and the development of atopic disease. Based on their cytokine production profile, these Th cells can be divided into a Th1 (e.g. producing interleukin (IL)-2 and interferon (IFN)-γ), a Th2 (e.g. producing IL-4, IL-5, IL-10 and IL-13) and a Th0 subset (producing all these cytokines). In children with established atopic disease, a disturbed balance in Th cell subsets towards a polarized Th2 phenotype is generally accepted. However, the shift towards a polarized Th2 phenotype during the process of allergic sensitization is not yet completely understood. A reduced IFN-γ production has been proposed to be responsible for the failure to develop a profound Th1 response in children who will develop atopy.

The aim of the studies presented in this thesis was to prospectively identify dysregulations of T cell responses in children at high risk to develop atopy (*chapters 4-6*) and cross-sectionally in children with established AD (*chapters 7-10*). These T cell responses were studied both after polyclonal and allergen-specific stimulation, with emphasis on cytokine mRNA expression and protein production profiles. Definitions for the various atopic diseases in early childhood (atopy, AD, asthma, food- and upper-airway allergy and allergic sensitization) and the employed clinical tests are described in *chapter 2*. The development and optimization of different techniques for the analysis of proliferative responses, cytokine mRNA expression and protein production, both after polyclonal and allergen-specific stimulation, are described in *chapter 3*.

For the prospective study, 133 high-risk newborns were selected, the mothers of whom all had an atopic disease. These children were followed up for the development of atopy from birth to 2 years of age. The prevalence of atopy, mostly AD, increased from 25% at 12 months to 32% at 24 months of age.

We examined whether markers of sensitization (total and specific IgE levels, allergen-induced proliferative responses and skin tests) and markers of inflammation (plasma sE-selectin and blood eosinophil numbers) were related to the clinical expression of atopy during the first 2 years of life and whether they preceded the development of atopic disease in these children (*chapter*

4). Positive skin tests and specific IgE to food or inhalant allergens were related to the clinical expression of different atopic diseases. The combination of AD and food allergy at 12 months of age reflected the strongest risk factor for the development of asthma-like disease at 24 months of age. However, none of the investigated markers for allergic sensitization or inflammation preceded atopic manifestations at 2 years of age.

In *chapter 5* we provide evidence that environmental and genetic risk factors and immunological responses at birth contribute to the development of atopic disease before 12 months of age. Two atopic parents, parental smoking and atopic dermatitis of at least one of the parents was significantly related to the presence of atopy in their children at 12 months of age. In cord blood of newborns who developed atopy, an increased percentage of $CD4^+CD45RO^+$, but not $CD25^+$ T cells, was observed. In addition, a significant correlation was observed between IFN-γ and IL-4 protein production by cord blood mononuclear cells (CBMC) of newborns who developed atopy, both after polyclonal and house-dust mite-specific stimulation. This suggests the presence of a previously activated Th0 population in newborns who subsequently develop atopy.

As described in *chapter 6*, we found that at birth the CBMC of all children had the capacity to produce a Th0 cytokine profile. In children who had developed atopy at 12 months of age, this profile shifted gradually to a Th2 profile within 6 months. This was detected at the protein level and at the mRNA expression level, the latter using the TaqMan PCR technology. This selective development of a Th2 cytokine profile was independent of the production of IFN-γ, as no significant differences were observed in IFN-γ production between children who did and did not develop atopy. We conclude that the first 6 months of life form the critical period for the initiation of immunological changes related to the allergic sensitization, which was clinically detectable at 12 months.

In *chapters 7-10* we focus on T cell responses and soluble adhesion molecules in children with established atopy. In *chapter 7* we show that peak proliferative responses occurred at day 7 after stimulation of peripheral blood mononuclear cells (PBMC) with 500 μg/ml peanut-extract. Moreover, after peanut-extract stimulation IFN-γ mRNA expression was maximal at day 4 of culture. Peanut-extract-induced proliferative responses of PBMC from children with AD and a peanut-sensitization (AD^+PA^+) were significantly higher compared to non-peanut sensitized children with AD (AD^+PA^-) and healthy children (HC) (*chapter 8*).

By a double staining method using Ki67 and lymphocyte cell surface markers, we demonstrated that 80-100% of the proliferating cells after peanut-extract stimulation of PBMC from AD^+PA^+ children were $CD4^+$ cells. The principal responding population of T cells after polyclonal stimulation of PBMC

from AD^+PA^+ were, however, $CD8^+$ cells. As described in *chapter 9*, in AD^+PA^- no profound Th2 cytokine production profile was observed after peanut-extract or polyclonal stimulation, while in AD^+PA^+ children increased IL-5 mRNA, IL-13 mRNA and protein, and decreased IFN-γ protein production were observed after polyclonal and peanut-extract stimulation. These results show that the T cell cytokine profile after polyclonal stimulation of PBMC from AD^+PA^+ is significantly different from that in AD^+PA^-, indicating that peanut sensitization does not constitute a representative model for other food-allergen sensitization. Furthermore, significantly increased levels of peanut-specific IgG4 antibodies and decreased IL-10 levels were observed in children with AD compared to HC, independent of the presence of a peanut sensitization. IL-10 is a potent inhibitor of B cell proliferation. We propose that the observed decreased IL-10 production in AD children results in enhanced B cell proliferation and subsequently in increased peanut-specific IgG4 antibody levels in these children.

In *chapter 10* we describe that during the stable phase of atopic diseases, plasma levels of soluble intercellular adhesion molecule-1(sICAM-1), sE-selectin, tumor necrosis factor (TNF)-α, sTNF-receptor (R)55 and sTNF-R75 were not different between atopic and non-atopic children. In AD children levels of sE-selectin did, however, correlate with the clinical severity of the disease.

In conclusion, the CBMC of all high-risk newborns had the capacity to produce a Th0 cytokine profile at birth. In children who developed atopy before 12 months of age, this Th0 cytokine profile had shifted to a Th2 cytokine profile within 6 months. This selective development of a Th2 cytokine profile was independent of the production of IFN-γ. After the establishment of the atopic disease, a Th2 cytokine profile was present after stimulation of PBMC.

Allergen-specific proliferative responses of CBMC to cow's milk, egg and HDM were found at birth in all children, but were not significantly different between children who did and did not develop atopy. In children with established atopy, however, peanut-specific proliferative responses were significantly increased in AD^+PA^+ compared to AD^+PA^- and HC.

A positive skin test, specific IgE to food or inhalant allergens and a Th2 cytokine production profile of PBMC, but not plasma sE-selectin levels were related to the clinical expression of different atopic diseases. More research will be necessary to reach a definitive conclusion about the use of one of these markers for the prediction of atopy development in children before and after 2 years of age.

SAMENVATTING

Er bestaan verschillende atopische ziekten, zoals atopische dermatitis (AD), astma, voedselallergie en allergie van de bovenste luchtwegen. De ontwikkeling van deze ziekten wordt bepaald door complexe interacties tussen de genetische predispositie van een persoon en de omgevingsfactoren waarmee hij/zij wordt geconfronteerd. Deze interacties kunnen een allergische sensibilisatie en vervolgens een ontsteking van het doelorgaan veroorzaken, met als gevolg klinische symptomen van een atopische ziekte. Deze atopische ziekten, vooral AD en voedselallergie, openbaren zich vaak al in de eerste levensjaren.

Tijdens de eerste levensjaren is het immuunsysteem van kinderen relatief weinig ontwikkeld (onrijp) vergeleken met dat van volwassenen. Door deze onrijpheid van het immuunsysteem is er vanaf de geboorte tot de leeftijd van 2 jaar een periode, waarin het risico van allergische sensibilisatie, en dientengevolge het ontwikkelen van een atopische ziekte, verhoogd is.

Helper T lymfocyten (Th cellen) spelen een centrale rol in de allergische sensibilisatie en het ontwikkelen van een atopische ziekte. Deze Th cellen kunnen worden verdeeld in een Th1 subset (produceert bijvoorbeeld interleukine (IL)-2 en interferon (IFN)-γ), een Th2 subset (produceert bijvoorbeeld IL-4, IL-5, IL-10 en IL-13) en een Th0 subset (produceert al deze cytokinen). Th1 en Th2 cellen reguleren elkaars activiteit door de verschillende cytokinen die zij uitscheiden. Zo hebben Th1 cytokinen, vooral IFN-γ, een remmende werking op de functie van Th2 cellen en andersom. Het wordt algemeen aangenomen dat kinderen met een atopische ziekte een verstoorde balans van hun Th subsets hebben, die gekenmerkt wordt door een relatieve overmaat van Th2 cellen (Th2 polarisatie). Hoewel de oorzaak van de ontwikkeling van de Th2 polarisatie tijdens een allergische sensibilisatie grotendeels onbekend is, wordt vaak verondersteld dat een verlaagde IFN-γ productie mogelijk daarvoor verantwoordelijk is.

De vraag die ten grondslag ligt aan het onderzoek dat beschreven wordt in dit proefschrift, is of een verstoorde regulatie van het Th cel compartiment een rol speelt bij de ontwikkeling en handhaving van een atopische ziekte. Om deze vraag te beantwoorden is een longitudinaal onderzoek uitgevoerd met pasgeborenen die een genetisch verhoogd risico hebben om een atopische ziekte te ontwikkelen, en een transversaal onderzoek met kinderen die een klinisch bevestigde AD hebben.

De definities van de verschillende atopische ziekten op de vroege kinderleeftijd (atopie, AD, astma, voedselallergie, allergie van de bovenste luchtwegen en allergische sensibilisatie) en de uitgevoerde klinische bepalingen zijn in *hoofdstuk 2* beschreven.

De ontwikkeling en optimalisatie van de gebruikte technieken voor de analyse van proliferatieve responsen, cytokinen-mRNA expressie en de productie van cytokine-eiwit, zowel na polyklonale als na allergeen-specifieke stimulatie, staan beschreven in *hoofdstuk 3*.

Voor de longitudinale studie (*hoofdstukken 4-6*), werden 133 pasgeborenen met een verhoogd risico op de ontwikkeling van een atopie geselecteerd. De moeders van deze kinderen hadden een atopische ziekte. Deze kinderen zijn door ons vanaf de geboorte gedurende twee jaar gevolgd. In deze groep nam de prevalentie van atopische ziekte, meestal AD, toe van 25% op de leeftijd van 12 maanden naar 32% op de leeftijd van 24 maanden. Wij onderzochten of parameters voor sensibilisatie (totale en allergeen-specifieke IgE waarden, allergeen-geïnduceerde proliferatieve responsen en huidtesten) en parameters voor ontsteking (plasma sE-selectin en aantallen eosinofiele granulocyten in bloed) gerelateerd waren aan de klinische expressie van atopische ziekte gedurende de eerste twee levensjaren (*hoofdstuk 4*). Positieve huidtesten en/of specifiek IgE gericht tegen voedsel- of inhalatie-allergenen bleken inderdaad gerelateerd te zijn aan bepaalde atopische ziekten op het moment van klinische presentatie.

De combinatie van AD en voedselallergie op de leeftijd van 12 maanden was de sterkste risicofactor voor de ontwikkeling van een astma-achtig ziektebeeld op de leeftijd van 24 maanden. Echter, voorafgaand aan de atopische symptomen op de leeftijd van 24 maanden konden we nog geen evidente veranderingen waarnemen in de onderzochte parameters die voorspellend waren voor de allergische sensibilisatie en ontsteking.

In *hoofdstuk 5* laten wij zien dat zowel omgevings- en genetische factoren als het patroon van immuunreactiviteit bij de geboorte bijdraagt aan de ontwikkeling van atopie op de leeftijd van 12 maanden. Het hebben van twee atopische ouders, één of beide ouders die roken en tenminste één ouder met AD, waren allen risicofactoren voor de ontwikkeling van een atopie die reeds op de leeftijd van 12 maanden bij de kinderen waarneembaar was. Zo werd een verhoogd percentage van $CD4^+CD45RO^+$ cellen, maar niet van $CD25^+$ T cellen, waargenomen in het navelstrengbloed van de pasgeborenen die later een atopie ontwikkelden. Bovendien werd een significante positieve correlatie gevonden tussen de productie (op eiwitniveau) van IFN-γ en IL-4 door mononucleaire cellen geïsoleerd uit navelstrengbloed (CBMC) en het ontwikkelen van een atopie. Deze gegevens wijzen op de aanwezigheid van een voorheen reeds geactiveerde Th0 populatie in die pasgeborenen.

In *hoofdstuk 6* wordt beschreven dat de CBMC van alle kinderen in staat waren een Th0 cytokinenprofiel te produceren na polyklonale stimulatie. In de fractie mononucleaire cellen geïsoleerd uit het perifere bloed (PBMC) van kinderen die op 12 maanden een atopie ontwikkeld hadden, verschoof dit Th0 profiel binnen 6 maanden na de geboorte geleidelijk naar een Th2 profiel. Deze selectieve ontwikkeling van een Th2 cytokinenprofiel was onafhankelijk van de productie van IFN-γ. Er werden namelijk geen significante verschillen gevonden in de productie van IFN-γ tussen PBMC afkomstig van kinderen die een atopie ontwikkelden en PBMC van kinderen die geen atopie ontwikkelden. Wij concluderen uit deze gegevens dat reeds in de eerste 6 levensmaanden de

initiatie plaatsvindt van de immunologische veranderingen, waaronder de transitie van een Th0 naar een Th2 cytokinenprofiel. Deze immunologische veranderingen zijn betrokken bij, en wellicht verantwoordelijk voor, de allergische sensibilisatie die klinisch op de leeftijd van 12 maanden bij een aantal van de onderzochte kinderen kan worden vastgesteld.

In de transversale studies, die beschreven worden in de *hoofdstukken 7 tot en met 9*, concentreren wij ons op de T cel responsen in de kinderen met een klinisch vastgestelde atopie. In deze studies werden kinderen met AD en een pinda-sensibilisatie (AD^+PA^+), kinderen met AD maar zonder een aantoonbare pinda-sensibilisatie (AD^+PA^-), en gezonde leeftijdsgenootjes (HC) met elkaar vergeleken.

In *hoofdstuk 7* laten wij zien dat na stimulatie van de PBMC met pinda-extract *in vitro*, de sterkste proliferatie op dag 7 werd gevonden, terwijl de IFN-γ mRNA expressie maximaal was na 4 dagen kweken. Bovendien bleek dat de pinda-extract geïnduceerde proliferatie van de PBMC van AD^+PA^+ kinderen significant hoger was dan die van de PBMC van de AD^+PA^- kinderen en de HC (*hoofdstuk 8*). Door gebruik te maken van een dubbelkleuring van de cellen met Ki67 (een marker voor prolifererende cellen) en lymfocyt-oppervlaktemarkers, hebben we aangetoond dat 80-100% van de delende cellen na pinda-extract stimulatie van de PBMC van de AD^+PA^+ kinderen $CD4^+$ cellen zijn.

In *hoofdstuk 9* wordt beschreven dat in AD^+PA^- kinderen geen uitgesproken Th2 cytokinenprofiel gevonden werd na polyklonale of pinda-extract stimulatie, terwijl in de AD^+PA^+ kinderen na polyklonale en pinda-specifieke stimulatie een verhoogde IL-5 mRNA, IL-13 mRNA en eiwitproductie, samen met een verlaagde IFN-γ eiwitproductie, waargenomen werd.

Deze resultaten laten zien dat het T cel cytokinenprofiel na polyklonale stimulatie van de PBMC van AD^+PA^+ kinderen significant verschillend is van dat van AD^+PA^-. De AD^+PA^- kinderen hadden een voedsel-sensibilisatie anders dan voor pinda. Dit maakt duidelijk dat pinda-sensibilisatie niet representatief is voor voedsel-sensibilisatie.

In de kinderen met AD werden ten opzichte van de gezonde kinderen, onafhankelijk van de aanwezigheid van een pinda-sensibilisatie, significant verhoogde concentraties van pinda-specifieke IgG4 antistoffen en verlaagde IL-10 waarden waargenomen. IL-10 is een krachtige remmer van de B cel proliferatie. Wij veronderstellen dat de waargenomen verlaagde IL-10 productie in de kinderen met AD resulteert in een versterkte B cel proliferatie en vervolgens in een verhoogde productie van de pinda-specifieke IgG4 antistoffen in deze kinderen.

In *hoofdstuk 10* beschrijven wij het onderzoek naar de aanwezigheid van oplosbare adhesiemoleculen in plasma van atopische en niet-atopische kinderen. Het doel is na te gaan in hoeverre deze moleculen, die vrijkomen tijdens inflammatie, in de circulatie voorkomen en het ziektebeloop

representeren. Gedurende de stabiele fase van een atopie, waren de plasmawaarden van oplosbaar intercellulair adhesie molecuul-1 (sICAM-1), sE-selectin, tumor necrosis factor (TNF)-α, en de oplosbare TNF receptoren sTNF-R55 en sTNF-R75 niet verschillend van die in de niet-atopische kinderen. Echter, binnen de groep kinderen met AD waren de sE-selectin waarden wel positief gecorreleerd met de ernst van de aandoening.

Uit ons onderzoek concluderen wij dat alle pasgeborenen met een verhoogd risico op de ontwikkeling van een atopie bij de geboorte een Th0 cytokinenprofiel laten zien na polyklonale stimulatie van de CBMC. In kinderen die een atopie ontwikkeld hadden op de leeftijd van 12 maanden was dit Th0 cytokinenprofiel al binnen 6 maanden na de geboorte verschoven in de richting van een Th2 cytokinenprofiel. Deze selectieve ontwikkeling van een Th2 profiel was onafhankelijk van de productiegrootte van IFN-γ.

Allergeen-specifieke proliferatieve responsen van de CBMC gericht tegen koemelk, ei en HDM werden gevonden bij de geboorte, maar bleken niet verschillend tussen kinderen die wel of niet een atopie ontwikkelden. Echter, in AD^+PA^+ kinderen waren de pinda-specifieke proliferatieve responsen *in vitro* wel significant verhoogd ten opzichte van die in AD^+PA^- kinderen en gezonde kinderen.

Een positieve huidtest, specifiek IgE gericht tegen voedsel- of inhalatie-allergenen en een Th2 cytokinenprofiel van de PBMC zijn gerelateerd aan de klinische expressie van de verschillende atopieën. Meer onderzoek is nodig voor een definitieve conclusie over de bruikbaarheid van één van deze parameters voor het voorspellen van de ontwikkeling van een atopie bij kinderen in de leeftijdsgroep tot 2 jaar.

Op basis van ons eigen onderzoek en gegevens uit de literatuur zouden de volgende preventieve maatregelen overwogen kunnen worden om de ontwikkeling van een atopische aandoening bij kinderen te voorkomen:

Zwangerschap: preventieve maatregelen tijdens de zwangerschap, zoals een allergeen-vrij dieet en een gereduceerde blootstelling aan allergenen, zijn tot nu toe niet effectief gebleken. Veelal werd echter pas gestart met deze maatregelen tijdens het derde trimester van de zwangerschap. Dit is mogelijk te laat, omdat de allergische sensibilisatie al gedurende het tweede trimester van de zwangerschap kan starten.

Periode 0 tot 6 maanden: de meest uitgesproken risicofactor tijdens deze periode is passief roken. Vermoedelijk beïnvloedt de mate van blootstelling aan huisstofmijt, huisdieren en andere allergenen ook de ontwikkeling van atopie gedurende deze periode. In onze studie waren deze factoren echter niet significant geassocieerd met de ontwikkeling van een atopie vóór de leeftijd van 12 maanden. Waarschijnlijk geldt voor deze factoren een dosis- en tijdsafhankelijk risico, waardoor het effect vaak pas op latere leeftijd duidelijk wordt.

Periode 6-12 maanden: deze periode is een belangrijke fase voor het verschijnen van de eerste klinische symptomen van atopische ziekte bij de kinderen. Daarom is een nauwlettende controle op deze eerste atopische symptomen gewenst, zodat eventuele interventie zo vroeg mogelijk kan starten.
Periode na 12 maanden: zoals hierboven reeds vermeld, hangen een positieve huidtest op voedsel, de aanwezigheid van allergeen-specifiek IgE en een Th2 cytokinenprofiel samen met de klinische expressie van een atopie op de leeftijd van 24 maanden. De combinatie van AD en een voedselallergie op de leeftijd van 12 maanden bleek in ons cohort de sterkste risicofactor voor de ontwikkeling van astma-achtige klachten op de leeftijd van 24 maanden.

Uit het bovenstaande blijkt dat meer kennis en inzichten nodig zijn voor goed geargumenteerde maatregelen die genomen kunnen worden om het risico op de ontwikkeling van een allergie bij kinderen te verkleinen.

ABBREVIATIONS

Abbreviation	Meaning
AA	allergic asthma
AD	atopic dermatitis
$AD^{+}PA^{-}$	AD children without a peanut sensitization
$AD^{+}PA^{+}$	AD children with a peanut sensitization
AIMV	serum free lymphocyte medium
APC	antigen-presenting cells
BSA	bovine serum albumin
CBMC	cord blood mononuclear cells
CI	confidence interval
CLA	cutaneous lymphocyte-associated antigen
CM	cow's milk
cpm	counts per minute
DC	dendritic cells
DTH	delayed-type hypersensitivity
DZ	dizygotic
ELISA	enzyme-linked immunosorbent assay
EW	egg-white
FA	food allergy
FcεRI	high affinity IgE receptor
FCS	fetal calf serum
HC	healthy children
HDM	house-dust mite
HI	heat inactivated
HPRT	human hypoxanthine phosphoribosyl transferase
HuS	human serum
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
RT	room temperature
LFA	lymphocyte function associated antigen
mAb	monoclonal antibodies
MHC	major histocompatibility complex
MZ	monozygotic
n	number in study or group
NAA	non-allergic asthma
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PGE2	prostaglandin E2
PMA	phorbol-12-myristate-13-acetate
Pr-Pr	prick-prick test
RAST	radio-allergo-sorbent test
RR	relative risk
rs	Spearman's rank correlation coefficient
RSV	Respiratory Syncytial Virus
RT-PCR	reverse transcriptase-polymerase chain reaction
SAFT	skin application food test
SCORAD	severity scoring of atopic dermatitis
SI	stimulation index
SD	standard deviation
Tc	T-cytotoxic
TCR	T-cell receptor
TGFβ	Transforming Growth Factor-β
Th	T-helper
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TNF-R	TNF-receptor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Tr	T-regulator
UA	upper-airway allergy
VCAM	Vascular Cell Adhesion Molecule
VLA	Very Late Antigen
YM	Yssel's medium

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