T AND B CELL ACTIVATION IN CHILDHOOD ALLERGY

a cross-sectional study of cytokines and immunoglobulins

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T EN B CEL ACTIVATIE BIJ KINDEREN MET ALLERGIE

een transversale studie over cytokinen en immunoglobulinen

PROEFSCHRIFT

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"Dubitando ad veritatem pervenimus"
Cicero

"Door te twijfelen komen we tot de waarheid"

Ter nagedachtenis aan mijn vader
Voor mijn moeder

T AND B CELL ACTIVATION IN CHILDHOOD ALLERGY

a cross-sectional study of cytokines and immunoglobulins

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CHAPTER 1.1

DEVELOPMENT OF IMMUNE FUNCTIONS, RELATED TO ALLERGIC MECHANISMS, IN YOUNG CHILDREN

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ABSTRACT

The newborn immune system differs quantitatively and functionally from that of adults. Development of the immune system has important implications for childhood diseases. The immaturity of the immune system in the first years of life may contribute to failure of tolerance induction and in the development of allergic disease. T-cell function is diminished, especially the capacity to produce cytokines; production of interferon-γ (IFN-γ) and interleukin-4 (IL-4) is strongly reduced. IFN-γ has been found to be even lower in cord blood of newborns with a family history of atopy. Differences in other cell types (natural killer cells, antigen presenting cells and B cells) could also play a role in the development of allergic disease.

Current data suggest that irregularities in immunoglobulin E (IgE) synthesis, helper T cell subsets (T_h1, T_h2, CD45RA and CD45RO), cytokines (IL-4, IFN-γ) and possibly other cell types may play a role in the development of allergy in childhood. Moreover, the role of cell surface molecules, like co-stimulatory molecules (CD28, CD40L), activation markers (CD25) and adhesion molecules (LFA-1/ICAM-1, VLA-4/VCAM-1) is also discussed. These variables are modulated by genetic (relevant loci are identified on chromosome 5q, 11q and 14) and environmental forces (allergen exposure, viral infections and smoke).

The low sensitivity of current predictive factors for the development of allergic diseases, such as cord blood IgE levels, improve in combination with family history and by measurement of *in vitro* responses of lymphocytes and skin reactivity to allergens. New therapeutic approaches are being considered on the basis of our current understanding of the immunopathology of allergic disease, for instance cytokine therapy and vaccination with tolerizing doses of allergen or peptides.

INTRODUCTION

Growth and development which are critical in children, are also relevant for immunopathology in relation to allergy. Important developments in various compartments of the immune system occur in young children, which are different from the adult situation (Table I). These differences could contribute to the widely held view that during the first years of life there is an enhanced risk of allergic sensitization leading to development of allergic diseases in childhood and later on in life (1). It is generally accepted that the prevalence of both asthma and hay fever is increasing in children worldwide (2). This increase could be the result of better diagnostic capabilities and better recognition of childhood asthma, air pollution and environmental risk factors and socio-economic factors.

Table I. Differences of the immune system of newborns and infants as compared with adults in relation to allergy

Compartment	Differences	References
T cells	Strongly decreased production of IL-4, IFN-γ and other cytokines by neonatal T cells	119-121
	Age related switch from $CD45RA^+$ to $CD45RO^+$ phenotype	106
B cells	Strongly diminished immunoglobulin secretion by neonatal B cells	153,154
	Doubling of the proportion of B cells expressing CD23 between infancy and adulthood	102
Macrophages	Neonatal macrophages are functionally immature; phagocytosis and chemotaxis are impaired	156,157
Natural killer cells	NK cells in cord blood are less active and have reduced IFN-γ production	102,159-161

Here we discuss the interaction between the development of the immune system and genetic and environmental factors considered to be involved in the pathogenesis of allergic diseases. Risk factors for the development of allergic diseases, cytokine patterns in allergic children and cell surface markers thought to be involved in allergy are discussed. Finally, we present possible mechanisms of allergy development and new approaches for therapy.

Predictive factors for allergy development

Genetic factors

In newborns, a positive family history has been shown to be an important risk factor for the development of atopic disease (3,4). If a child has one allergic

parent, the risk to become allergic is approximately 20%, which is increased to 60% with two allergic parents, whereas without allergic parents the risk is around 10% (5). Family history could be very useful as an early marker for allergic disease, with an acceptable sensitivity (61%) and specificity (83%) (6). Allergic diseases are presently considered as at least a two locus inheritance, with expression being influenced by environmental variables, resulting in heterogeneous expression. Moreover, an individual's likelihood of developing allergic disease is not constant over time, even if the general propensity is genetically determined.

Recently, evidence of linkage of high levels of total serum immunoglobulin E (IgE) to loci on chromosome 5q31.1, including the cytokine gene cluster, was reported (7,8). It was also demonstrated that elevated serum IgE is coinherited with a trait for bronchial hyperresponsiveness and that a gene governing bronchial hyperresponsiveness is located near a major locus that regulates serum IgE levels on chromosome 5q (9). Further support for genetic influences, comes from several studies reporting significantly higher cord blood IgE concentrations in males than in females (10-13), although conflicting information exists (14,15). The gender influence on cord blood IgE can hypothetically be due to differential expression of the gene encoding for atopy in males (13). An increased number of atopic boys as compared to girls is in line with this observation (16). Cookson *et al.* found a strong linkage between a marker on chromosome 11, localized at 11q13, with atopic IgE responses in large pedigrees (17). This finding was confirmed in 60 nuclear families (18). Abnormalities in the chromosome 11q region account for 60% of allergy in British families. It was also noticed that this linkage was only detected with maternally derived alleles (19). A genetic mechanism, known as genomic imprinting, in which a paternal 'atopy gene' is suppressed, may explain this (20). A maternal inheritance will mean that unaffected mothers may still carry the trait if they have inherited an abnormal gene from their father. One specific site on chromosome 11q accounts for 20% of the 11q-associated allergy. This site represents a single amino acid change in the β chain of the high affinity IgE receptor ($Fc_{\varepsilon}RI$) (21). Furthermore, genetic linkage was found between a gene (or genes) in the T-cell receptor α region, encoded on chromosome 14, and specific IgE responses (22).

Neonatal IgE levels

The principal feature so far that distinguishes atopic from non-atopic individuals is their predisposition to develop antigen-specific IgE antibodies upon repeated exposure to low doses of foreign proteins (*i.e.* pollen, animal products, house dust mite, food, etc.) (23). This results in higher serum IgE levels in atopics than non-atopics. However, twin studies strongly suggest that although the tendency to IgE production is genetically determined, the antigenic specificity is governed most likely by environmental influences (24). These results

exemplify our current understanding of allergy development, in which we consider the interaction between genetic makeup (genotype) and environmental factors as determining the development of disease. Thus it is important to be able to discern children at risk for allergy development as early as possible and to determine those environmental factors, which subsequently should be avoided.

Another prediction factor is measurement of umbilical cord blood IgE levels (16,25-27). Elevated cord blood IgE concentrations may be genetically determined (genetic high IgE responders) (28) and/or be due to intra-uterine sensitization (25,27). The latter alternative seems to be only rarely the case. The IgE antibodies present in cord blood seem to be more the consequence of nonspecific spontaneous IgE production, perhaps lack of suppression, than of antigenic stimulation. However, as discussed later (see "T_h Cell subsets") some antigen-specific T-cell responses were recorded in cord blood.

Furthermore, the mother's, more than the father's, atopic history had a significant correlation with cord blood IgE levels (13,29). Presumably, cord blood IgE levels are mainly determined by the fetal immunologic reaction to intrauterine allergens and other factors (such as smoke), based upon genetic determinants. Controversy exists about the possible placental transfer of maternal IgE.

Several considerations support the use of cord blood IgE levels, since a fetus can make IgE from the eleventh week of gestation (30); IgE cannot cross the placental barrier (31); and a well-established correlation exists between a high IgE responder and the clinical tendency to develop atopic disease for adults as well as for children (32).

Although the specificity of cord blood total IgE levels in predicting allergy is relatively high (94%), the sensitivity is quite low (26%)(3). The predictive value is not very high, hence relevance of elevated cord blood total IgE levels as the sole marker for predicting atopic disease has been questioned (33-37). Furthermore, a rapid decrease of IgE levels during the first weeks occurred in half of the infants born into allergic families (33). Hattevig *et al.* (38) reported a transient IgE response to food proteins, which occurred relatively frequently, even in infants who did not develop atopic diseases. Thus, young children may show temporarily positive results, developing IgE antibodies against food allergens. Persistently high IgE concentrations, however, were more strongly related to the development of atopic disease (38). This transient IgE production is an integral part of the normal immune response, because at the time of initial contact(s) with an allergen not previously encountered, the immune system initially "recognizes" the allergen and responds to it. In immunologically normal (non-atopic) children and animals, these initial responses are self-limiting and spontaneously switch off, after a few weeks or months, despite continuing allergen exposure. The initial response is replaced by a state of life-long allergen-specific immunological tolerance (unresponsiveness). This is supported

by animal studies showing that in healthy (non-atopic) animals the natural response of the respiratory mucosal immune system to inhaled allergens involves initial recognition, accompanied by transient low-level IgE production due to selective suppression, resulting in a state of tolerance (39-41). Tolerance induction functions poorly in the pre-weaning period (42), presumably due to delayed postnatal maturation of one or more key elements of the mucosal immune function that are rate-limiting in inducing tolerance (1). Therefore allergen exposure in the very early phase of infancy primes for subsequent T-cell reactivity (43,44). This results in a persisting IgE response, leading to a state of clinical hypersensitivity.
High total IgE levels at two and six months after birth showed no better predictive values than cord blood IgE (45,46). However, cord blood IgE levels in conjunction with a positive family history (especially in the mother) can be useful for identifying infants at risk for early development of atopic disease.

Allergenic reactivity
Measurement of allergen reactivity can add to the prediction of allergy (47). A positive skin reaction to egg in young children, resulted in a specificity of 54% and a sensitivity of 54% in the prediction of atopy at a later age. Recently, Sigurs *et al.* (48) confirmed this finding by showing that atopic disease appeared before the age of 4 years in 80% of the 40 children who had IgE antibodies to egg white at 9 months of age. A similar sensitivity to that with skin testing was obtained by Kobayashi *et al.* by measuring *in vitro* proliferative responses of cord blood lymphocytes to food allergens (egg and cow's milk) (49). Combining the proliferative response values with cord blood total IgE concentrations results in a rise in sensitivity to 79%.
Hattevig *et al.* (50) showed that specific IgE antibodies to inhalant allergens appeared in increasing frequency starting at the age of 2 years, later than to food allergens. This agrees with the difference in clinical and immunological expression of the atopic status in infancy and childhood. In infancy food allergen sensitization and atopic dermatitis predominate. Beyond that age range inhaled allergen sensitization and respiratory tract symptoms become important (10).
New specific immune factors need to be considered. Possibly, differential cytokine expression may be relevant in this respect (see "Cytokines").

The role of environmental factors in allergic sensitization
The identified risk factors are presented in Table II. Smoking during pregnancy has been shown to be associated with increased cord blood IgE levels (51) and the subsequent development of asthma (52). Other studies have failed to demonstrate this (12,14). Paternal smoking did neither influence cord blood IgE levels nor infant allergy (51). After birth exposure to concentrations of

Table II. Potential risk factors for the development of allergy

Source	Risk factor	References
Genetic	A positive family history for atopy	3-5
Immunopathology	A positive skin test to egg or egg-specific IgE antibodies early in life	47,48
	Raised proliferative responses and defective IFN-γ production by cord blood MNC after stimulation with β-lactoglobulin	133
	Reduced PHA-induced IFN-γ production by cord blood MNC	124
	Elevated cord blood IgE levels	16,25-27
	Elevated IL-4 levels in serum in the first months of life	132
Environment	Low-birth weight	52,59
	Maternal smoking	51,52
	Viral infections	65-68
	Degree of allergen exposure	178-180
	Month of birth	56-58

pollutants indoors when people are smoking may be far in excess of outdoor concentrations of pollutants that are found regularly (53,54).
Outdoor pollution, by combustion by-products (CO_2, NO_x, SO_2, CO), solid particles, ozone, etc., is potentially able to enhance allergic sensitization. The actual contribution of pollutants acting as adjuvants in allergy development is, however, controversial. The prevalence of allergic diseases in the more polluted former East Germany was found to be less than that in West Germany, against expectations (55).
The month of birth was studied by de Groot *et al.* (56) in a group of 45.000 patients in relation to the presence of IgE antibodies to inhalant allergens. Children born between December and February had a slight but significantly higher chance to become sensitized to grass pollen. On the other hand children born in autumn (August - November) had a higher chance to become sensitized to indoor allergens, like house dust mite and animal danders (56). This was in agreement with earlier studies of Björksten and Suonemi (57) and Businco *et al.* (58). Such information suggests that in the first three months after birth an increased sensitivity for allergic sensitization is present.
Low birth weight has been found a highly significant risk factor for allergic disorders probably due to immaturity of the immune system that renders infants more liable to allergic sensitization (52). The effect was more pronounced than that of an atopic family history. These children were also at risk to develop house dust mite allergy by the age of 2 years (52) (see below, "Immunopathology of Allergy Development in Childhood). Recently, an association has been suggested between impaired or disproportionate fetal growth and raised serum IgE levels as an adult (59). Other studies have not seen any indication for prematurity as a risk factor for atopic sensitization (5).

In addition, Martinez *et al.* (60) established a relationship between impaired neonatal lung function and development of wheezing respiratory illness in infants, however, the allergic state was not taken into account.

Viral infections

Viral infections in childhood are thought to have a modulating role on the immune system, which can be stimulatory, as well as inhibitory. Viral infections may cause epithelial injury, inflammation, enhanced mediator release and sensitization of IgE (61). Variations over time in an individual's susceptibility to sensitization might be due to respiratory tract infection, thereby occurring more easily in early life. Respiratory syncitial virus (RSV) and parainfluenza virus are the predominant viruses isolated from infants and children less than 6 years of age, whereas *Mycoplasma pneumoniae* and rhinovirus predominate in older children (62). The occurrence of recurrent wheezing and asthma after RSV-bronchiolitis in infancy is frequently reported (63,64). Thus, respiratory infections are an important cause of wheezing and RSV is the predominantly associated organism. In subjects with existing allergic disease, RSV-infections promote the development of inflammatory response in the lungs, *i.e.* the late allergic response.

In infants with confirmed RSV infections during the first six months of life, the frequency of persistent wheezing up to seven/eight years was directly related to the level of RSV-specific IgE in naso-pharyngeal secretions during the initial period (65). Moreover, allergy in childhood often occurs shortly after an RSV infection (66). Only 28% of infants with undetectable titers of RSV-IgE had subsequent episodes of wheezing. In contrast, 70% of infants with high RSV-IgE titers experienced wheezing (67). Recently, a link has been shown between viral infections and the T helper 1 (T_h1)-like and T helper 2 (T_h2)-like profile. RSV contains an attachment protein called glycoprotein G, which could skew the immune response towards a T_h2 phenotype (68). In this way, RSV infections could induce or enhance the development of allergy in a hypersensitive period of life. However, peripheral blood mononuclear cells (PBMC) from 22 infants previously infected with RSV usually had RSV-specific increases in T_h1 cytokine specific mRNA (interferon-γ (IFN-γ) and interleukin-2 (IL-2)). This was not observed in PBMC from RSV antibody-negative children, indicating that naturally acquired RSV normally induces a T_h1 memory response (69). This is in accordance with the hypothesis formulated by Martinez that recurrent early life infections render a preferential selection of T_h1 clones with subsequent inhibition of allergic sensitization (70). Furthermore, long-time persistence of RSV genome and protein in an animal model has recently been shown (71), which may result in latent and persistent viral infections. Another possible mechanism is the effect of virus infections upon adhesion molecules which regulate eosinophilic and neutrophilic migration. Intercellular adhesion molecule-1 (ICAM-1) permits eosinophilic and neutrophilic migration and it has

been demonstrated that it functions as the major human rhinovirus receptor (72). However, the precise relationship between respiratory virus infections and the development of allergic diseases may well depend upon host factors, the period in life and virus characteristics, which should be further studied (73,74).

Immunopathology of allergy development in childhood

T_h cell subsets

Based on their cytokine production profile, $CD4^+$ T_h cells can be divided into a T_h1 (*e.g.* IL-2 and IFN-γ), a T_h2 subset (*e.g.* IL-4, IL-5 and IL-10) and a naive T_h0 subset (producing all of these cytokines) (75,76). The intricate cytokine network provides the most appropriate and convincing basis for intercellular communications and control. Several factors, most of them cytokines themselves, may specifically promote T_h1 or T_h2 outgrowth. IFN-γ and IL-12 strongly promote the generation of T_h1-like cells, whereas IL-4, IL-10 and prostaglandin E_2 (PGE_2) promote the generation of T_h2-like cells (77-79) (Figure 1). Since IL-12 and PGE_2 are produced by antigen presenting cells (APC),

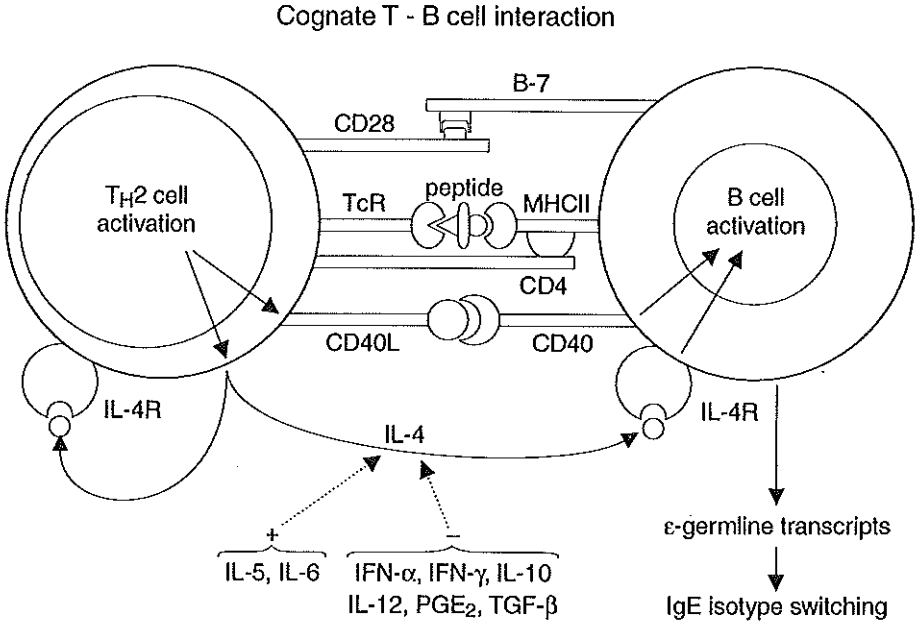

Figure 1. Schematic representation of T-B cell interaction. B cells acting as APC present peptides of the allergen to the specific T cell receptor, expressed on $CD4^+$ T_h2 cells. Subsequently B7 expression is upregulated on the APC and interacts with CD28 expressed on the T cell. Signals through the T cell receptor and the CD28 co-stimulatory molecule are obligatory for the activation of the T cell. Upon activation the T cell transiently expresses CD40L and secretes IL-4. 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. These 2 signals result in B cell activation and subsequent isotype switching to the sythesis of IgE. This process of isotype switching can be modulated by several cytokines interacting with IL-4 directed processes.

eventually the balance between different APC populations may determine the selective outgrowth of T_h1 and T_h2 cells. On the other hand, T cell derived cytokines, like IL-4 and IFN-γ may be important for the maintenance of T_h1 and T_h2 responses. Naive T cells require for their activation inductive signals from dendritic cells, while sensitized memory T cells can respond to processed antigen presented by virtually any cell type expressing class II major histocompatibility complex (MHC) molecules on the surface. While monocytes can act as APC for memory T cells, resident alveolar macrophages strongly suppress the APC function of dendritic cells. Hereby, alveolar macrophages might induce a state of tolerance reflected in decreased (local) memory T cell expression and suppression of IgE formation (80). The immunological outcome of initial T cell activation events after inhaled allergens can be detected as T cell anergy or tolerance (80). In most individuals, repeated exposure to an allergen results in the induction of such tolerance. This is less likely to occur in atopic persons. The eventual outcome of individual encounters with inhaled antigens depends to an important extent on the functional capacity of the APC which initially removes the antigen. The balance between the potential immunoregulatory APC populations is a key factor in hypersensitivity and the pathogenesis of immunoinflammatory diseases (80).
An antigen also can determine to some extent the preferential outgrowth of T_h1 *versus* T_h2 type cells. Very low doses of peptide presented by dendritic cells, lead to selective priming of T_h2-like cells that produce IL-4 and not IFN-γ on restimulation *in vitro*. Priming with much higher doses of antigen causes selective outgrowth of IFN-γ producing T_h1 cells. Low doses of allergens can therefore, induce T_h2 responses that lead to IgE production, while higher doses, used for desensitization, could convert this response to inhibitory T_h1-like cells (81).

Induction of IgE synthesis
Cytokines play a crucial role in the regulation of IgE synthesis (Figure 1). IL-4 is a key cytokine in the production of IgE by naive B cells (82). This is based on its ability to induce expression of ε-germline transcripts, indicating that IgE synthesis reflects Ig isotype switching (83). Another T-cell derived cytokine, IL-13, can also induce germline-ε expression in B cells, but it is two- to five-fold less potent than IL-4 in inducing IgE synthesis (84). For this activation by IL-4 a physical contact between activated $CD4^+$ T cells and B cells is required, called cognate interaction (82) (Figure 2). Antigen-activated T-B cell interactions require binding of the T cell receptor to MHC-class-II-peptide complexes on antigen-presenting B cells, which results in T cell activation and cytokine production. Once T helper (T_h) cells are activated and express the CD40 ligand (CD40L), they interact with B cells that constitutively express CD40 and induce proliferation and differentiation in an antigen- independent fashion (85). The costimulatory signal provided by $CD4^+$ T cells, can be replaced by anti-CD40

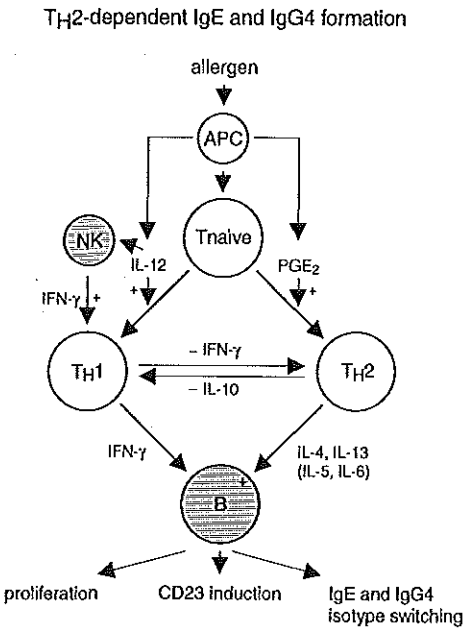

Figure 2. T_h2-dependent IgE and IgG4 formation. Allergen exposure in atopic individuals leads to the activation of na $CD4^+$ T cells. Depending on different types of APC and the local micro-environment, these T cells differentiate into functio T_h1 or T_h2 effector cells. This micro-environment dictates the selective outgrowth of T_h1 or T_h2 cells, primarily by t presence of IL-12 (T_h1) and PGE_2 (T_h2) produced by monocytes. IL-12 can either directly induce T_h1 development indirectly via upregulating the IFN-γ production by NK-cells. Activated T_h2 cells induce in activated B cells, through t process of cognate T-B cells interaction (see Figure 1), proliferation, upregulation of CD23 and isotype switching to IgE a IgG4. IFN-γ produced by T_h1 cells inhibits these processes, while other cytokines (like IL-5, IL-6 and IL-13) further enhan the IL-4 activity. The ratio of T_h1 *vs* T_h2 cells therefore determines the final outcome of the B cell response. Cross-regulati between T_h1 and T_h2 cells by IFN-γ and IL-10 further modifies this ratio.

monoclonal antibodies (86), infection of B cells with Epstein-Barr virus (EBV (87) or the addition of hydrocortisone to T cell depleted PBMC cultures (88). CD40-CD40L interaction causes the induction of B7 on B cells. The membrane antigen B7.1, expressed on activated B cells, monocytes and dendritic cells interacts with CD28 on T cells. This interaction leads to higher IL-4 production resulting in higher IgE synthesis (89,90). CTLA-4 can also act as a ligand fo B7.2 and is expressed on activated T-lymphocytes (91,92).
Another important interaction in IgE regulation takes place between CD23 (lov affinity receptor for IgE ($Fc_\varepsilon RII$)) expressed on many haemopoietic cells an CD21 (receptor for EBV and complement receptor-2) on B cells, T cells an follicular dendritic cells (93,94). Triggering of CD21 specifically increased IL- induced IgE production from PBMC (93). Thus compared to other interactions the CD21-CD23 pairing controls IgE synthesis in an isotype-specific manner.

The activating effect of IL-4 is promoted by IL-5 and IL-6, but inhibited by IFN-α and -γ, transforming growth factor-β (TGF-β) and PGE_2 (95-98). Moreover, PGE_2, IFN-α and IFN-γ also inhibit IL-4- induced expression of CD23 on B-cells, indicating that there is an association between CD23 expression and IL-4-induced IgE production (99,100). Furthermore, IL-10 has a down-modulating effect on the production of IFN-γ (101).

Cell Surface Markers

$CD4^+/CD8^+$ T cells

White blood cell and lymphocyte counts are at their highest values at birth and decline with age. Proportions of lymphocyte subsets vary, but the percentage of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells progressively increases over time (102).

The number of CD4 and CD8 T lymphocytes in the peripheral blood of adult asthmatic patients did not differ from those in the normal control subjects (103). Gemou-Engesaeth *et al.* (104) studied a group of atopic asthmatic children (7-16 years), compared with age-matched atopic non-asthmatic children, in which also no differences were found in the percentages of $CD4^+$ and $CD8^+$ T lymphocytes.

$CD45RA^+/CD45RO^+$ T cells

Neonates are immunologically naive, because exposure to micro-organisms and foreign antigens has been prevented to some extent by the placental barrier. Indeed, most (91%) neonatal $CD4^+$ T cells express CD45RA, a well accepted marker of naive T cells. This $CD45RA^+$ population of neonatal T cells is phenotypically identical to the $CD45RA^+$ naive T cells in adult peripheral blood (105). These cells are gradually replaced by CD45RO (marker of activated or memory T cells) positive cells, presumably as a result of repeated antigen exposure (102). A plateau phase of $CD45RO^+$ cells is reached during puberty (106). The switch from $CD45RA^+$ to the $CD45RO^+$ phenotype appears to reflect an age-related accumulation of memory cells in the circulation. These phenotypic changes are associated with functional changes, such as an increased proliferative response to IL-2 and an increased production of IFN-γ (106). *In vitro* $CD45RO^+$ T lymphocytes show enhanced help for IgE synthesis (107).

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

In adult asthmatic patients the percentage of $CD4^+CD45RO^+$ T cells was significantly elevated and that of $CD4^+CD45RA^+$ T cells significantly reduced

compared to healthy controls (103). In a study of asthmatic children (1-17 years), who were largely above 10 years of age, it was shown that the percentage of $CD4^+CD45RA^+$ T cells in peripheral blood was not significantly increased compared with the percentage in atopic non-asthmatic children. Three different patient groups (asthmatic patients, atopic non-asthmatic patients and patients with atopic dermatitis) did however show an increased expression of CD45RA as compared with non-atopic children (110). This suggests that at least in children, elevated percentages of peripheral $CD4^+$ T lymphocytes expressing CD45RA were associated with the atopic state rather than specifically with asthma. This may, however, be caused by accumulation of $CD45RO^+$ T cells in the target organs.

$CD25^+$ T cells

The IL-2 receptor (CD25) is generally expressed on activated T cells, B cells, natural killer (NK) cells, monocytes and macrophages. The IL-2 receptor is useful as a marker of activation, but not for discrimination between virgin and memory cells, as the expression is lost when cells return to the resting state (111).

In adult patients with acute severe asthma, it was demonstrated that the percentage of $CD4^+$ T cells expressing the IL-2 receptor was elevated (112). Interestingly, percentages of peripheral blood $CD4^+$ T lymphocytes expressing the activation markers CD25 and HLA-DR, and of $CD8^+$ T lymphocytes expressing CD25 were elevated in asthmatic children compared with atopic non-asthmatic children (104). This suggests that T lymphocyte activation may be physiologically associated with asthma and not simply with the atopic state *per se*.

CD40/CD40Ligand

Recently, two comparable studies were published, both suggesting an association between ineffective expression of CD40L on cord blood T cells and the low Ig production in the newborn (113,114).

Cord blood mononuclear cells (MNC) have no (113) or decreased (114) expression of CD40L as compared to adult MNC. This defect can be largely attributed to the lack of antigenic exposure in the neonate, since *in vivo* primed cord blood lymphocytes could express substantial amounts of CD40L upon appropriate stimulation (priming and restimulation with PMA and ionomycin) (113). This ineffective CD40L expression by cord blood $CD4^+$ T cells, together with their failure to produce a number of costimulatory cytokines, might be regarded as the primary cause for the poor Ig production in the neonate (114).

The CD40 molecule may also play a role in allergic disease. In adult patients with atopic dermatitis, the mean fluorescence intensity of CD40 expression on B cells was increased when compared with expression on PBMC from nonatopic donors (115).

$CD23^+$ B cells

CD23, the low-affinity receptor for the Fc portion of IgE, is also a presumed activation antigen. The number of B cells that express CD23 nearly doubles between infancy and adulthood (102). Allergic young children (0-2 years) showed a significantly higher percentage of $Fc_\varepsilon RII$ positive cells than nonallergic young children. $Fc_\varepsilon RII$ positive lymphocytes may play a regulatory role in IgE synthesis in allergic young children (116).

Cytokines

Neonatal cytokine production differs from the adult situation and may play a role in the increased sensitivity in the neonatal phase to allergic sensitization. Since cloning procedures potentially distort the pattern of *in vivo* cytokine production, cytokine mRNA expression and production should be studied in freshly isolated and short-term cultured T cells obtained from peripheral blood (117). In this way the intrinsic capacity of T cells to produce cytokines can now be studied precisely, because possible influences, such as cytokine production by other cell populations, are prevented (118).

IFN-γ

In neonatal T cells IFN-γ production is strongly decreased to as low as 10% of the adult IFN-γ production, while production of IL-2 and expression of IL-2 receptor are comparable to those in adults (119). Several causes of this low IFN-γ production have been suggested: an increased sensitivity of infant T cells to the inhibitory effect of PGE_2 (120), an intrinsic deficiency in the capacity of infant T cells to synthesize IFN-γ (119) or an inefficient accessory cell activity on the part of infant macrophages (121). This reduced capacity to produce 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 allergic disease (122).

After polyclonal stimulation IFN-γ production by cord blood MNC was found to be reduced in newborns with a positive atopic family history, compared to those without familial risk. This was independent of cord blood IgE levels. IL-4 production was mostly below the detection limits of the assay and could not be analyzed (123). Production of other T_h2-derived cytokines exerting enhancing effects on IgE synthesis (IL-5, IL-6) did not differ significantly, neither between adults and newborns, nor between newborns in different familial risk groups (123).

In a study using stimulated $CD4^+$ T cell clones from infants genetically 'at risk' for atopy, both the IFN-γ- and the IL-4-producing capacity of clones from babies born into allergic families were significantly reduced relative to clones from normal infants (124). This suggests that in children who develop allergy initial immune responses to environmental allergens in early childhood occur against a background of maturational $CD4^+$ T cell dysfunction, resulting in abnormal

immunological responsiveness.

IL-4

IL-4 production is also strongly decreased in neonatal T cells (125). In adults all of the IL-4 and most of the IFN-γ are produced by CD45RO$^+$ T cells, while IL-2 is produced by both CD45RO and CD45RA positive cells. Therefore, the acquisition of memory T cells may be a major determinant of maturation permitting both IL-4 and IFN-γ production (126). However, given the appropriate stimulation, *in vitro* neonatal T cells were able to express the lymphokine gene repertoire characteristic for adult T cells (IL-3, IL-4, IL-6, IFN-γ and granulocyte macrophage colony stimulating factor were measured). This suggests that T cells generated from neonatal blood by a primary stimulation *in vitro* are functionally indistinguishable from the T cells in adult blood (127). If previous *in vivo* activation is a major determinant for the capacity of T cells to produce IL-4 and IFN-γ, then the neonatal T cells, which presumably have had a minimal exposure to exogenous antigens, will exhibit a profile of lymphokine production similar to that of adult virgin T cells. Therefore, the cytokine production profile of neonatal T cells may reflect their antigenically naive status (125). These differences comprise a diminished capacity to provide help for, or actual suppression of, immunoglobulin production by B cells, a diminished generation and activity of cytotoxic T cells and a decreased capacity to activate macrophages (125).

Tang and Kemp (128) studied the production of IL-4 in PHA-stimulated PBMC cultures in healthy neonates, children and adults. *In vitro* IL-4 production was found to be significantly reduced in neonates and children under 10 years of age as compared with adults, and to increase progressively with age. These age-dependent variations in IL-4 production may reflect differences in naive and memory T cell populations. Interestingly, the addition of plasma from neonates to PBMC cultures from adults at a final concentration of 10%, resulted in approximately 50% inhibition of IL-4 production. This points into the direction of inhibitory factors present in the plasma of neonates, the nature of which is as yet unknown.

A large body of evidence suggests that allergen-specific T_h2-like cells are expanded in atopic subjects, particularly at the level of target tissues, like skin and lungs (129). Already at birth, allergen-specific T_h2 cells can be expanded, suggesting that aero-allergen sensitization can occur during fetal life (intrauterine sensitization) (130). Apart from IL-4, overexpression of other genes of the IL-4 gene family, like IL-3, IL-5 and granulocyte macrophage colony stimulating factor also appears to be present in atopics. Several genetic alterations could be present at the level of signal transduction pathways or complexes formed by transcription factors and their corresponding promotor regulatory elements. Alternatively, deficient production or regulatory activity of the cytokines responsible for the inhibition of T_h2 cell development (*e.g.* IFN-γ)

might induce such T_h2 cytokine overexpression (131).
Recently, Borres *et al.* (132) demonstrated that IL-4 levels in serum are associated with development of allergic disease in infancy. Elevated levels were recorded before onset of clinical symptoms, suggesting that atopic disease is associated with a primary disorder in T-cell function.

Allergen-induced cytokine profiles
In order to establish the relationship between cytokine production, exposure to environmental allergens and development of atopic disease, it is interesting to study *in vitro* allergen-induced cytokine profiles. Warner *et al.* (133) showed that the development of atopic eczema, with positive cow milk skin prick tests at 1 year of age, was associated with increased proliferative responses and defective IFN-γ production to stimulation with beta-lactoglobulin at birth. The deficient IFN-γ response was specific for certain allergens, including a generalised maturational delay in T cell function.
However, data on IFN-γ production show conflicting results. The group of Tang *et al.* (134-137) found in children with atopic dermatitis (mean age 3.5 years) a higher percentage of IFN-γ producing cells by intracellular staining in unstimulated PBMC cultures compared with those of healthy control children. This indicates *in vivo* activation of T cells in the atopic group. IFN-γ secretion after stimulation, however, was significantly reduced in children with atopic dermatitis, compared with controls, while the percentage of IFN-γ-producing cells was not different (134). Thus the reduced ability of atopic children to secrete IFN-γ *in vitro*, does not relate to a lack of IFN-γ-producing cells, but to a difference in the regulation of IFN-γ production beyond the stage of gene transcription and translation. This was confirmed by analyzing IFN-γ mRNA expression in children with atopic dermatitis in whom a constitutive expression of IFN-γ mRNA was found. In controls this could only be detected after stimulation (135). In the same group of children spontaneous IL-4 mRNA expression was detected in 4 out of 8 severe atopic dermatitis patients, while this was absent in healthy children (136). This suggests *in vivo* activation of T cells in children with atopic dermatitis. Additional evidence is provided by reports of increased serum levels of soluble IL-2 receptors (138). As T cells from patients with atopic dermatitis have been shown to have an intrinsic defect of IFN-γ secretion, the imbalance of IL-4 and IFN-γ secretion documented in atopic dermatitis could reflect general activation of T cells in the presence of an intrinsically defective IFN-γ secretion (136).

Adhesion Molecules
Leucocyte-endothelial adhesion molecules are thought to be involved in the initial stages of selective recruitment and migration of inflammatory cells from the circulation to the sites of inflammation. Several cytokines, including IL-4 and IFN-γ, promote induction and upregulation of adhesion molecules, both on the

endothelium and on leucocyte surfaces (139). At the sites of allergic inflammation increased expression of ICAM-1 and E-selectin (endothelial leucocyte adhesion molecule-1 (ELAM-1)) on vascular endothelium have been demonstrated (140,141). The selective accumulation of eosinophils and lymphocytes in allergic responses has been hypothesized to be generated by a VLA-4 (very late antigen-4)/VCAM-1 (vascular cell adhesion molecule-1) pathway induced by the release of IL-4 and IL-5 from T_h2 lymphocytes (142). This is supported, by the marked expression of VLA-4 on basophils, eosinophils and lymphocytes, but not on neutrophils and minimally on monocytes (143,144). Furthermore, it was identified that IL-4 selectively upregulates VCAM-1 expression on vascular endothelium (144,145). However, it has been demonstrated that although the initial attachment of eosinophils and lymphocytes to endothelial cells is mediated by LFA-1 (Lymphocyte function-associatied antigen-1)/ICAM-1 and VLA-4/VCAM-1, the subsequent transendothelial migration process relies heavily on ICAM-1 and LFA-1 (146,147). IFN-γ is the strongest cytokine enhancer of epithelial ICAM-1 expression, with IL-1β, tumor necrosis factor-α (TNF-α) and IL-4 also being effective (148).
In allergic asthmatic patients, adhesion molecule expression on endothelium was correlated with eosinophil and total leucocyte infiltrate (149). This suggests that adhesion molecule expression in the vessels of the bronchi may be involved in leucocyte and particularly in eosinophil infiltration.
Adhesion molecules can also be present in a circulating form (150). In adult allergic asthmatic patients increased serum levels of soluble ICAM-1 and E-selectin during asthma attacks have been demonstrated (151). This may reflect the up-regulation of ICAM-1 and E-selectin expression in allergic inflammation. The soluble form of these adhesion molecules may be a useful marker for the presence of allergic inflammation (151). However, in a study in asthmatic children who underwent allergen provocation, no differences were observed in soluble ICAM-1 expression (152). These soluble adhesion molecules might slow down the allergic response by binding to ligands on leucocyte surfaces and, thereby, blocking adherence to tissue ligands and limiting the inflammatory process (139). Since little is known about the role of adhesion molecules in the pathophysiology of allergy in children, more research in this field is necessary.

Other cell types
Neonatal B-lymphocytes secrete minimal amounts of Ig in response to stimulation with anti-CD3 in contrast to adult cells. This deficiency could be corrected by the addition of IL-2, IL-4 or IL-6, resulting in secretion of all isotypes (153,154). There were no differences in the distribution of Ig isotypes secreted in response to the cytokines alone or in combination. Deficient production of these cytokines is likely to contribute to the decreased capacity of neonatal lymphocytes to generate an Ig response. Maturation of B

lymphocytes into antibody-producing plasma cells occurs gradually during the first weeks of life (155).
An abnormal monocyte/macrophage function may contribute at least partly to deficient neonatal immune responses. Specifically, the newborn infant's monocytes/macrophages, when studied *in vitro*, are permissive for intracellular multiplication of herpes simplex virus and have impaired phagocytosis and chemotaxis, compared with monocytes/macrophages of adults (156). The decreased ability of neonatal cells to produce IFN-γ appears to be primarily related to the immature function of the neonatal macrophage (157). Cord blood macrophages cultured with adult T cells show only a minimal production of IFN-γ. Adult macrophages cultured with cord blood T cells, however, give rise to high IFN-γ production (158). This points to an important limiting role of macrophages in young infants.
NK cells in cord blood are less active than those in adults, for example lysis of bound targets and the production of NK cytotoxic factor and IFN-γ are both reduced (159-161). This may reflect their immaturity or the absence of cytokines necessary for full NK cell activation. A deficient NK cell function could play an additional role in the development of allergy, because they produce IFN-γ among other cytokines.
Thus developmental aspects in non-T cells, namely macrophages and NK cells, may contribute to the generation of atopy by compromised IFN-γ production, although specific abnormalities related to allergy are not yet documented.

Therapeutic approaches
The progress in our understanding of allergic mechanisms in children gives rise to new therapeutic options. Interference can take place on different levels. Firstly, primary prevention of sensitization (allergen avoidance), secondly, at the level of factors regulating the disease process (several forms of immunotherapy) and thirdly, suppression of inflammatory mechanisms (corticosteroids and others).

Allergen avoidance
A variety of studies have investigated the possible benificial role of allergen avoidance. The concept of intrauterine sensitization led to studies evaluating the effects of maternal diets. It has decisively been demonstrated that maternal allergen avoidance diets (cow milk´s or egg) during pregnancy fail to affect the frequency of atopic disease, serum IgE levels and food sensitization, from birth up to 5 years of age in offspring at high risk for atopy (162,163). Elimination diet during lactation was beneficial for high-risk infants and reduced the development of atopic eczema (164-166). These studies, however, need to be proven, since no double-blind placebo controlled food challenges were performed. However, breastfeeding, with or without diet restriction, has given a favourable outcome in many studies (167,168), but without effect in others

(169,170). The recently published results of a 17-year follow-up study (171) show that the prevalence of manifest atopy throughout follow-up was highest in the group who had little or no breastfeeding. The original groups, however, were not selected by randomization. In addition, the studied population was not selected for a positive family history of atopy. The combination of exclusive breastfeeding with maternal lactation and infant elimination diets has given the most benificial effect (164,172,173). The effect was limited to the first or second year of life and the reduction of eczema, while the incidence of respiratory symptoms and allergic manifestations appearing later was not affected. These results are not surprising due to the antigen-specificity of the allergen avoidance protocol that will not protect against sensitization to inhaled allergens or food allergens not avoided. In an European Society of Pediatric Allergy and Clinical Immunology position paper, extensively hydrolysed formula is recommended for avoidance of cow's milk in cow's milk-allergic infants in order to prevent cow's milk allergy and associated atopic dermatitis in high-risk infants, but not for prevention of respiratory symptoms. Soy is not beneficial in this respect, considering its immunogenicity and allergenicity (174). Late introduction of solid food besides breastfeeding seems to have long-lasting benefits on the development of atopic diseases in high risk children (175-177). This needs to be confirmed in a randomized study. The addition of mite reduction to the food avoidance results in a reduction of respiratory allergy (178). The Isle of Wight study showed that the dual approach of avoidance of food antigens and a reduction of exposure to house dust mite can reduce the manifestations of atopy in the first two years of life (13% instead of 40%) (179). Sanda *et al.* (180) demonstrated a similar beneficial effect of house dust mite avoidance in children with atopic dermatitis.

Immunotherapy

Peptides that represent epitopes on allergens can downregulate T cells, as suggested in cell culture and animal studies (181). In this context it is important not to rely on a single synthetic peptide, but to focus on the identified immunodominant regions of the allergens (182). It has been reported that peptide analogues based on known immunogenic T-cell epitopes which maintain the necessary MHC binding sites but have altered T cell receptor contact residues, may alter the effector response of T cells both *in vitro* and *in vivo* (183). In adult allergic patients clinical trials with such altered peptide ligands inducing a state of long-term non responsiveness are currently under way.

Recent adult trials, as reviewed by Bousquet and Michel, suggest a benificial effect of traditional subcutaneous immunotherapy in allergic rhinitis, conjunctivitis and more recently in allergic asthma (184). The study of Creticos *et al.*, did not show a practical benefit of immunotherapy for allergic asthma (185). A limited number of double blind placebo controlled trials of specific oral immunotherapy in children have been reported. Studies using birch pollen

(rhinoconjunctivitis) (186) have shown clearly demonstrable effects, whereas studies with grasspollen (pollen asthma and/or rhinitis) (187) and *Dermatophagoides* (perennial asthma and/or rhinitis) (188) have failed to demonstrate efficacy. A major challenge for successful immunotherapy is to convert an existing T_h2 response into a T_h1 response that is beneficial, or at least not pathological. Allergen immunotherapy of adult allergic patients caused a remarkable reduction in the quantity of IL-4 production by their allergen-specific $CD4^+$ T cells (189). Furthermore, repeated parenteral challenge with tolerizing doses of grass pollen allergen in atopic adults selectively stimulates allergen-specific T_h1 like T cell reactivity (190). This may be even more effective in children who do not have an established T cell memory (191). Therefore, vaccination with a cocktail of relevant inhalant allergens at the appropriate time in childhood, especially in combination with an Th_1-selective adjuvant, may provide a method to strengthen a population of appropriate T_h1 cells (191). Further research is needed to investigate whether this approach is useful, as well as the time sequence and profile of children who would benefit from it.

Cytokine therapy

When considering cytokine therapy, IL-4 is a highly attractive target, because of its critical role in regulating both IgE isotype switch and the commitment of T cells to a Th_2cytokine pattern. Key regulatory units in the IL-4-gene-promotor structure have been identified, but most of the nuclear factors that bind to these regulator sequences are common to many related genes (like IL-5). However, there are indications of specific regulatory proteins, offering the possibility for selective inhibitors (192). Inhibition of the IL-5 response might prevent terminal differentiation of eosinophils and abolish the eosinophilic component of inflammation in asthma and allergy (193). The removal of specific cytokines in animal models often had more profound and informative effects than cytokine addition (192). This suggests that cytokine antagonists may often be more valuable in patients than the addition of cytokines.

Recombinant IFN-γ (rIFN-γ) therapy over a 12 week period in adult patients with severe atopic dermatitis turned out to be safe, well accepted and effective in reducing inflammation, clinical symptoms and eosinophilia (194). Despite clinical improvement, no change in serum IgE levels was found, although spontaneous *in vitro* IgE synthesis decreased. This was not unexpected, since serum IgE levels reflect IgE synthesis by terminally differentiated B cells, which may no longer be responsive to rIFN-γ. Two 4- and 5-year old children suffering from refractory atopic dermatitis were treated with rIFN-γ, which was well tolerated. Clinically, some benefit was observed in one child, while no beneficial effect was seen in the other, although marked immunological changes were noted (195). Further studies are needed before IFN-γ can be recommended for therapy of pediatric atopic eczema. Considering the pleiotropy and the redundancy in

the cytokine network, an approach targetting combinations of several involved cytokines seems appropriate.

Glucocorticoids
Glucocorticoids are widely used as the most effective agents currently available in the treatment of atopic diseases. The anti-inflammatory actions of topical corticosteroids may depend partly, on downregulation of cytokine synthesis. *In vitro* it was demonstrated that corticosteroids inhibit the production of IL-4 and also downregulate the transcription of IL-4 mRNA in PBMC cultures (196,197). Furthermore, dexamethasone suppressed IL-5 production in atopic human PBMC's through an inhibitory action on its gene expression (198). This suppression of IL-5 gene expression is one of the most important mechanisms by which glucocorticoids inhibit eosinophil functions in the treatment of atopic diseases.

Anti-IgE
Anti-IgE forms a prophylactic/treatment agent designed to decrease the amount of IgE by targetting and subsequent lysis of IgE-secreting B cells. Furthermore, anti-IgE treatment could decrease the biological activity of IgE by initiating IgE clearance and by preventing IgE binding to receptors on effector cells. For anti-IgE to be safe, a monoclonal antibody was selected that did not bind to receptor-bound IgE (either CD23 or $Fc_{\varepsilon}RI$). This antibody was humanized and is being currently evaluated in trials with adult atopics, but not yet in young allergic children (199).
Disodium cromoglycate also has an established role in the prophylactic treatment of allergic disease. The possible mode of action of cromolyn sodium was thought to be stabilization of mast cells and subsequent prevention of mediator release after antigen challenge (200). More recently, it was shown that disodium cromoglycate inhibits T cell-driven IgE synthesis by human B cells, by inhibiting deletional switch recombination from μ to ε (201). It was also demonstrated that disodium cromoglycte inhibited the recruitment of inflammatory cells, particularly eosinophils, possibly by affecting the generation of cytokines and the expression of leucocyte-specific adhesion molecules (202) These results suggest novel potential mechanisms for the prevention of allergic disease by disodium cromoglycate.

CONCLUDING REMARKS

In the understanding of the development of allergic diseases in childhood, progress is rapidly evolving with respect to the contribution of genetic, environmental and immunopathological factors. The key factors currently considered relevant in the development of allergic diseases at a young age are:

1) With respect to genetic markers the linkage of genes located on chromosome 5q31.1, encoding the cytokine gene cluster, to total serum IgE levels, is now the subject of many investigations. The linkage and relationship of genetic markers on chromosome 14 (T cell receptors α/δ) to allergen-specific IgE levels, and the linkage between genes on chromosome 11q ($Fc_{\varepsilon}RI$) and allergy, however, still remain to be further unraveled .This genetic propensity can be further modified by environmental factors, like allergen exposure, pollution and viral infections, whose implications remain controversial and in need of further study. *2*) Concerning cytokines, a reduced allergen-induced IFN-γ production, as well as elevated IL-4 serum concentrations, before the onset of allergic symptoms in infants point to a developmentally early skewing toward a T_h2 rather than a T_h1 response. *3*) Transiently elevated IgE levels in healthy infants could be followed by development of immunological tolerance, whereas persistently elevated IgE levels in atopic children could be associated with failure in immunological tolerance. This might be due to the presence or absence of suppresive factors, possibly IFN-γ. Further research to establish the role of other factors involved is needed. In addition T_h2-derived stimulatory factors, like IL-4 or IL-13 are likely to be present in higher levels in atopic individuals. *4*) Failure of tolerance induction based on or accompanied by immaturity of the immune system, may create increased sensitivity to allergic sensitisation in early infancy. T cells in combination with APC most likely play an important role in allergy development. This role is probably largely due to genetic factors. *5*) Abnormalities at the level of B cells, manifested by an increased CD40 expression, resulting in an enhanced responsiveness to CD40L, may also contribute to disease. It remains to be established whether elevated IgE levels in atopics are based on increased precursor frequencies of IgE secreting B cells, differences in activation requirements of B cells or differential activity of regulatory T cells (either cytokines or membrane-bound molecules). *6*) The contribution of the various immunopathological factors and their possible interaction in the clinical heterogeneity of allergic diseases, like atopic dermatitis, food allergy, allergic asthma and allergic rhinitis, awaits further elucidation. Currently, we and other groups are investigating the role that these factors may play in the immunopathologic changes in young potentially atopic children, which probably will result in detection of disease markers and new therapeutic approaches.

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CHAPTER 1.2

PATIENT GROUPS

In this thesis we investigated children between 0 - 4 years of age suffering from asthma or atopic dermatitis and compared them with healthy control children. Below the characteristics of these diseases are presented, with emphasis on early childhood. Moreover, we discuss the criteria we employed to include the children in the studies.

Asthma

Bronchial asthma constitutes one of the most common chronic and disabling diseases in childhood (1). Several reports indicate that the prevalence of asthma in childhood in increasing (2-4).

Asthma is a disease which is difficult to define. The current working definition is: "asthma is a lung disease with the following characteristics: (1) airway obstruction that is reversible (but not completely so in some patients) either spontaneously or with treatment; (2) airway inflammation; and (3) increased airway responsiveness to a variety of stimuli" (5).

The airway obstruction observed in asthma is due to increased mucus production, enhanced bronchial smooth muscle contraction, and increased vascular permeability with mucosal edema. Chronic inflammation of the airway walls of asthmatic patients has been demonstrated by histology (6).

Both by analyzing lavage fluids and bronchial biopsies it was demonstrated that even patients with mild disease have lung inflammation (7,8). Limited lung biopsies from children also revealed airway inflammation (9). In addition, airway responsiveness in children between 6 and 16 years of age who had stable asthma correlated closely with increases in eosinophil and macrophage counts in bronchoalveolar lavage fluid (10).

The increased airway hyperresponsiveness characteristic for asthma can be defined as a decreased threshold of airway narrowing in reaction to various non-specific stimuli (11), such as viral infections (12), tobacco smoke (13), ozone (14), physical stimuli (exercise (15), cold air (16)) and inhaled pharmacologic agents (metacholine (17,18), histamine (19)). Airway hyperresponsiveness can also be caused by exposure to specific stimuli, such as allergens (20). It has been reported that infants considered to be predisposed to asthma, on the basis of family history or parental smoking, are more responsive to inhaled histamine than normal infants (21).

Asthma is often associated, especially in childhood, with atopy manifested by positive skin tests, a clinical history of allergen-induced noisy breathing episodes, or concomitant eczema or allergic rhinitis (22). However, some children and many adults with asthma are apparently not atopic and are

considered to have non-allergic disease (23,24). It was demonstrated that the prevalence of airway responsiveness rose, as serum immunoglobulin E (IgE) levels increased, strongly suggesting the critical part that allergy plays in facilitating the development of both airway hyperresponsiveness and clinical asthma (25,26). Whereas allergen interaction with specific IgE is a prerequisite for allergic asthma (24,27), the etiology of non-allergic asthma is largely unclear to delineate (27,28) and various pathogenic mechanisms have been postulated (29-31). A prominent feature of both types of asthma is the presence of eosinophilia (32).
Studies in children with asthma suggest that those with multiple positive skin tests are more likely to have potent responses to allergens and more persistent and severe disease, possibly because of the presence of an ongoing inflammatory process (33).
With increasing knowledge of the pathogenesis of asthma, the eventual aim will be primary prevention. This will require identification of the at risk group, avoidance of *in utero* factors that facilitate allergic sensitization, and avoidance of postnatal environmental inducers so that the inflammatory process leads to tolerance for environmental allergens rather than sensitization (34).
In the work presented in this thesis we studied children with allergic and non-allergic asthma. Children with asthma were selected using the following criteria of the American Thoracic Society: coughing (especially nocturnal) and/or shortness of breath, at least 3 times a year, for a period of at least 14 days (35) without fever and hypersecretion, and a positive family history of asthma and/or atopy, as well as absence of treatment with (inhalation-) corticosteroid. Retrospectively, the reaction to other medication was taken into consideration. The group of children with allergic asthma was further defined by the presence of specific IgE (>0.35 kU/l) in their serum for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The children with non-allergic asthma were selected according to the same criteria as the allergic asthmatic children, but at the time of the study they had no measurable specific IgE to inhalation and/or food allergens.

Atopic dermatitis
Atopic dermatitis is common in childhood. The prevalence of atopic dermatitis has, as have all other atopic manifestations, increased during the last decades and now affects 10% to 15% of the population at some point during their lifetime (36). Sixty percent of all cases already suffer during their first year of life and 85% during the first 5 years (37).
Atopic dermatitis is an intensely pruritic inflammatory skin disease (38). The term atopic dermatitis was first introduced in 1933, by Wise and Sulzberger (39), to emphasize the close association between atopic dermatitis, asthma and allergic rhinitis. Several observations suggest that IgE and allergens play an important role in atopic dermatitis. Patients with atopic dermatitis have the

highest serum IgE levels of all the atopic disorders (40). Serum IgE levels are elevated in 80-85% of patients with atopic dermatitis, and are highest in patients with coexisting allergic rhinitis and/or asthma (41). Furthermore, 70% of atopic dermatitis patients have a personal or family history of atopy and approximately 85% of patients have positive immediate skin tests to a variety of food and/or inhalant allergens (42). However, a direct relationship between immediate skin-test reactivity and the chronic course of atopic dermatitis has been difficult to establish (43). The difficulty in establishing a direct role for IgE in the pathogenesis of atopic dermatitis may, in part, be explained by studies demonstrating that IgE-dependent mast cell and basophil degranulation form only one component of its chronic inflammatory response. In this process T_h2 cells producing interleukin-4 (IL-4) and IL-5, and eosinophils are thought to play an important role in sustaining the chronic allergic inflammation (reviewed in 44).

Our study enclosed children between 0 and 4 years old, with atopic dermatitis, who were included according to the diagnostic criteria of Hanifin and Rajka (45). The main diagnostic criteria were pruritis, typical skin lesions and localization, a chronic recurrent course and the presence of other atopic diseases or atopic relatives. Children with atopic dermatitis had specific IgE (>0.35 kU/l) for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The severity scoring of atopic dermatitis (SCORAD) index was used as a measure for disease severity (46). This index is a well accepted method for assessment of the severity of atopic dermatitis, combining extent, severity and subjective symptoms.

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CHAPTER 1.3

AIMS OF THE STUDY

Children between 0 - 4 years are often in the most sensitive period of their life with regard to the risk of developing an allergic disease. Therefore, we investigated children in that particular age-group.
The first aim of the studies presented in this thesis was to develop techniques for analysis of cytokine gene expression and production from T cells and the isotype switching potential and production of B cells from small volumes of peripheral blood of young children. Therefore, cell separation techniques were optimized in order to purify B- and T cells from the peripheral blood mononuclear cells (PBMC) fraction and a semi-quantitative polymerase chain reaction (PCR)-technique was developed to detect cytokine and germline-ε mRNA expression. These methods are described in detail in chapters 2.2 and 2.3.
The hypothesis on which this thesis is based is:

> *"The production and the in vivo reactivity of cytokines known to play a role in the regulation of IgE synthesis is altered during the induction and development of allergic diseases in infancy".*

We therefore analyzed in a transversal study of patients with allergic and non-allergic asthma, atopic dermatitis and healthy controls, whether T cells and/or B cells display intrinsic differences that can be detected with these methods. Since elevated immunoglobulin E (IgE) levels have been found to be associated with allergic diseases, the question remains to be answered whether these increased IgE levels are caused by intrinsic properties of B cells of allergic children or that B cells of allergic children are intrinsically normal, but under the control of an altered regulation. Therefore, we compared germline-ε mRNA expression and immunoglobulin production in B cells from children with allergic and non-allergic asthma, children with atopic dermatitis and healthy control children.
In studies analyzing cytokine expression in adult patients with allergic diseases, such as asthma and atopic dermatitis, a different T cell cytokine profile from that of healthy individuals has been demonstrated, mainly based on an altered interleukin-4 (IL-4) and interferon-γ (IFN-γ) production. We tried to answer the question whether differences exist in T cell activation resulting in differential cytokine mRNA expression and protein production, between children with allergic and non-allergic asthma, children with atopic dermatitis and healthy control children. The cytokines we investiged in these studies were next to IL-4 and IFN-γ also important cytokines like IL-5, IL-10 and IL-13. The results of the analyses of B- and T cell-derived mRNA expression and protein production are

described in chapters 3.1, 3.2 and 3.3.
Adhesion molecules are also thought to play a role in the pathogenesis of allergic diseases. Several cytokines induce the expression of adhesion molecules and their subsequent release as soluble adhesion molecules. We therefore investigated whether differences in concentrations of soluble adhesion molecules occurred in the serum of children with allergic and non-allergic asthma, children with atopic dermatitis and healthy control children. The soluble adhesion molecules analyzed in this study were sICAM-1 (soluble intercellular adhesion molecule), sE-selectin, and sTNF (tumor necrosis factor)-receptor 55 and 75. The results obtained are described in chapter 4.
Finally, we investigated whether the developed techniques to study cytokine mRNA expression and protein production could also be applied to characterize diseases like Comèl-Netherton syndrome, a disease often associated with elevated IgE levels, allergic symptoms and eczema. The comparison of cytokine mRNA expression and protein production in two patients with Comèl-Netherton syndrome, with that in healthy controls and children with atopic dermatitis, is described in chapter 5.
Finally, in the General discussion we discuss the data obtained in the experimental studies in the context of relevant literature, with emphasis on the impact of the immunopathological analysis for establishing atopic disease in young children.

CHAPTER 2

CELL SEPARATION AND ANALYSIS OF CYTOKINE AND IMMUNOGLOBULIN mRNA EXPRESSION AND PRODUCTION

CHAPTER 2.1

ROLE OF THE IMMUNE SYSTEM IN ALLERGIC CHILDREN

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Patients suffering from allergic diseases like allergic asthma and atopic dermatitis, generally develop an immunoglobulin E (IgE)-mediated immune response of which the intensity is highly variable. The induction of this IgE synthesis is thought to be based on two signals: one being provided by the cognate interaction between T cells and B cells and the other signal is provided by the cytokine interleukin-4 (IL-4). The heterogeneity in clinical symptoms of allergy is to a large extent reflected in the magnitude of the allergen-specific IgE response. We therefore, aimed to study the distinctive patterns of IgE production and cytokine synthesis during the development of allergic diseases.

Characteristics of the neonatal immune system

The immune system of young children displays several quantitative and qualitative differences as compared to that of adults. With respect to the T cell compartment profound differences have been observed: the absolute number of $CD2^+$ T cells is increased, the lifespan of the cells is shorter, the memory compartment is less well developed and the $CD8^+$ T cells are less active than in adults (1). The cytokine production potential of the T cells is also markedly different: whereas the production of IL-2 and the expression of the IL-2 receptor are comparable with those in adults, the production of IL-3, granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) is reduced to 50% and the production of interleukin-4 (IL-4) and interferon-γ (IFN-γ) is only 10% of adult production at the level of individual secreting cells. This is presumably not due to deficient signalling through the T cell receptor (TCR)-CD3 complex, but is a consequence of intrinsic T cell immaturity. The T cell compartment is further characterized by a high CD4/CD8 ratio up to 2 years of age, which is mainly due to increased numbers of $CD4^+$ T cells (2). The B cell compartment in peripheral blood of children is normally increased as compared to adult blood. Because of the lack of B cell help by T cells, there is a limitation in isotype

switching favouring IgM responses in combination with impaired immunity to parasites. Besides these cells of the specific immune system, also the non-specific immune system is less well developed in children: the natural killer (NK) cell compartment is relatively decreased and certain subpopulations of monocytes appear to be not yet fully matured. Therefore, an impaired defence against intracellular pathogens was noted in young children.

T cell subsets and cytokines

Based on their cytokine production profile $CD4^+$ helper T cells can be divided in at least two effector populations. T helper 1 (T_h1)-like cells that produce IL-2 and IFN-γ, but not IL-4, IL-5, IL-6 and IL-10, and T helper 2 (T_h2)-like cells that produce IL-4, IL-5, IL-6 and IL-10, but not IL-2 and IFN-γ (3). However, not all T helper cell clones can be classified within the T_h1 and T_h2 subsets according to their cytokine secretion pattern. Cells exist that secrete both T_h1 and T_h2 cytokines (4). These cells termed T helper 0 (T_h0) cells could be the common precursor of T_h1 and T_h2 cells, or could be a third effector T helper cell population. Though originally described in mice, these subtypes of T cells have been shown to exist in humans and regulate IgE production in a manner similar as in mice. Cytokines are involved in the regulation of the different T_h subsets. IFN-γ inhibits the proliferation of T_h2 clones, whereas it does not influence the cytokine production by these cells. Currently, IL-12 was described as a cytokine that has powerful effects on T_h1 cells. IL-12 is produced by monocytes, macrophages and B cells and is required for optimal IFN-γ production both *in vivo* and *in vitro* (5). Moreover, it was shown that IL-12 is an obligatory factor for the generation of T_h1 cells. These results indicate that IFN-γ and IL-12 serve as immune regulatory molecules through which T_h1 cells can develop and interfere with the clonal expansion and effector function of T_h2 cells. Besides the capacity to downregulate T_h1 cytokine production IL-4 directs the development of T_h0 cells to T_h2 cells resulting in an increased IL-4 production (6). In the end, the balance of IL-4 and IL-12 determines the differentiation of T_h0 cells in either T_h1 or T_h2 cells.

Studies that used established T_h1 and T_h2 cell clones revealed that the proliferation of these cells is optimally stimulated by different antigen presenting cells (APC). Whereas T_h2 cell clones optimally proliferate upon stimulation with purified B cells, T_h1 cells need stimulation with adherent spleen cells for optimal proliferation. However, also exogenous IL-1 is necessary for B cells to stimulate optimal T_h2 cell clone proliferation, whereas T_h1 cell clones, stimulated with either macrophages or splenic B cells, do not need IL-1 for optimal proliferation (7).

Unravelling the role of helper T cells and their cytokines in the regulation of isotype production has also led to the understanding that a perturbed balance

of helper T cell subsets may be involved in human disease. Allergic asthma is classsified as a T_h2 type disease in which overexpression of T_h2 type cytokines contributes to the major presentation forms of the disease: IgE production (based on IL-4), eosinophilia in blood and lungs (based on IL-5, GM-CSF and IL-4) and mast cell hyperplasia in several tissues (based on IL-3 and IL-4). All of these cytokines are produced by activated T_h2 type cells.
A number of important agents that show regulatory effects on the development of T_h subsets in established antigen-specific T cell clones are currently known. Factors that shift towards T_h1 comprise transforming growth factor-β, IFN-α, IL-12, IL-1 receptor antagonist, and influenza virus. Alternatively, factors steering towards T_h2 are: IL-4, anti-IL-12 antibodies, progesteron and prostaglandin E_2. These agents most probably work by inhibiting the expression of certain cytokine genes and not by deleting these genes (8).

T-B cell cognate interaction
Antigen-specific IgE responses are thought to be generated only after cognate interaction between the specific B cell and a T_h cell activated by an antigen-presenting cell (9). B cells themselves can act as APC by recognizing specific antigen through their Ig-receptors, degrading and processing the antigen internally and presenting peptides to surrounding T cells in the context of major histocompatibility complex (MHC)-class-II molecules. B cells recognize antigens by virtue of their antigen receptors, generally with high affinity thereby selectively act as APC when antigen is presented in only low amounts. Moreover, B cells will take up antigen complexed to the antigen receptor and the antigen will be protected better against proteolytic degradation in the lysosomal compartment. Therefore, B cells will generally present different peptide fragments of an antigen than professional antigen-presenting cells, like dendritic cells, do. During these processes B cells do not become activtated but upregulate their B7 expression along with several other cell surface proteins (CD23, MHC-class-II molecules, etc). T_h cells can recognize through their receptor-specific peptides presented on class II molecules, a process in which the CD4 molecule acts as a costimulator by binding non-polymorphic parts of the MHC molecule and probably even interacts with Vα far out of the peptide-binding site. Together with costimulatory interactions between T cell (CD28, CD21) and B cell (B7, CD23), the T cells become activated. Activation leads to the induced expression of cytokine genes resulting in high rate cytokine production and a transient upregulation of membrane proteins like gp39 (CD40 ligand) and cytokine receptors. The interaction between gp39 and CD40, which is constitutively expressed on the B cells, further stabilized by several interactions between pairs of adhesion moleules (intercellular adhesion molecule-1, lymphocyte function associated antigen-3) provides a strong

activation signal for the B cell. Subsequent binding of cytokines to their receptor on the B cell will induce further activation, proliferation, isotype switching and Ig synthesis.

Significance of IL-4 in IgE responses

IL-4 is obligatory for the induction of IgE synthesis (reviewed in 10) as evidenced by its ability to induce the expression of ε-germline transcripts, the inhibition of parasite-induced IgE responses by neutralizing antibodies against IL-4, the absence of IgE responses in nematode-infected mice that were made IL-4 deficient by gene-targetting and IgE hyperproduction in IL-4 transgenic mice. Functional studies have indicated that IL-4 is a switch-inducing factor by altering the chromatine structure of the Sγ1 region and induces the accumulation of γ1 and ε germline transcripts. By the exposure to IL-4, B cells will activate DNA binding transcription factors that bind to the 179 bp conserved region upstream of the germline ε promotor. All these events are associated with class switching. IFN-γ inhibits the induction of γ1 germline transcripts and the production of IgG1 and IgE, whereas it induces IgG2a synthesis. IL-4 inhibits the IFN-γ induced IgG2a synthesis, illustrating the concept of cytokine-dependent reciprocal regulation of isotype production.

Also in humans class switching was reported by comparing Sμ-Sε junctions in IL-4 treated B lymphoblastoid cells (11). These composite switch regions contained Sγ sequences, indicating that sequential switching had occurred.

Since IL-4 and IFN-γ are the key cytokines that represent major subsets of helper T cells (IFN-γ producing T_h1 and IL-4 producing T_h2 cells), the ratio of activated T_h1 and T_h2 cells *in vivo* determines to a large extent the major isotype produced upon challenge of the immune system. As the isotype production profile is reflected in differential effector functions of the specific antibodies, it is clear that Th subsets and the cytokines they produce determine to a large extent the immune response.

Immune system in infancy

Primary allergic sensitization occurs in genetically predisposed individuals during childhood, in particular during the high risk time window in early infancy (12). As is clear from the discussion so far, T cells and the cytokines they produce play an important role in the induction and the development of allergic diseases in infancy. The main questions we asked initially were: are intrinsic defects present in the T cell compartment in atopic children in relation to their cytokine production profile? Moreover, we were interested whether intrinsic B cell defects exist in allergic children in relation to their isotype production profile. Several methods had to be adopted permitting the proper analysis of the parameters necessary for the study of the immune system in early childhood.

Isolation of cell populations

The Medical Ethical Committee permits the taking, after informed consent, of a small volume of peripheral blood from young children ranging from 0 to 4 years. We therefore started optimizing the techniques by starting from 4 to 5 ml of heparinized peripheral blood of healthy control children of ages between 6 months and 4 years. After Ficoll separation (13) and washing, a fraction of mononuclear cells was obtained, ranging usually between 1.35 x 10^7 - 3.3 x 10^7 cells isolated from 5 mls of blood. Subsequently, B cells were isolated by labeling with CD19 specific monoclonal antibodies (mAb) coupled to paramagnetic beads, and separated from all other cell populations by magnetic activated cell sorting (14). The remaining cells were incubated with a combination of mAb specific for: CD14, CD16 and CD56. After the coupling of Dynabeads, the NK cells and monocytes were removed by means of a magnet. The resulting population were T cells as confirmed by fluorescence activated cell scanner (FACScan) analysis (15). The purity and yield of these cell populations were analyzed by FACScan analysis (Tables I, II).

Table I. Cell numbers isolated from PBMC

	total cell number (x 10^6)	distribution (%)
PBMC	20.8 (6.4)	100
T cells	10.7 (3.6)	51
B cells	4.24 (1.6)	20

Cell numbers obtained after Ficoll isolation. Results are expressed as arithmetic mean (± 1SD) (n = 10).

Despite the generally low numbers of peripheral blood mononuclear cells (PBMC) available after Ficoll separation and the low numbers of purified T cells and B cells, the numbers are generally sufficient to permit the start of several cultures. This is largely due to the fact that the cell fractions obtained were relatively pure (Tables I, II).

Table II. Purity and yield of isolated cell populations from PBMC

	purity (%)	total cell number (x 10^6)	yield (%)
T cells	85.4 (8.4)	4.45 (2.17)	41 (12)
B cells	95.6 (1.5)	0.98 (0.34)	23 (6.2)

Total cell number is determined after cell separation from PBMC.
Results are expressed as arithmetic mean (± 1 SD) (n = 10).

B cell isotype production

In order to study the isotype production potential of B cells, purified B cells of healthy control children were put in culture at a density of 5000 B cells per well. For polyclonal stimulation a T_h0 clone (50,000 T cells per well) is used that produces all the relevant cytokines (IL-4, IL-5, IL-6, IL-10, IFN-γ) (16). This T cell clone will support induction of IgE synthesis only when harvested 5 days after the activation by antigen (tetanus toxin), or feeder cells plus PHA (phytohemagglutinin). Cultures were performed in Yssel's medium supplemented with 10 % fetal calf serum (17). To permit isotype switching to IgE to occur, the activity of IFN-γ has to be counteracted by the addition of exogenous IL-4 (200 U/ml). B cells cultured for 10 days and stimulated with the T_h0 clone give rise to substantial isotype production of all classes, including IgE when measured quantitatively by enzyme-linked immunosorbent assay (ELISA) (Table III). As is clear from our initial results, stimulation of the B cell cultures resulted mainly in enhanced production of IgM and total IgG, but not IgA, IgG4 or IgE.

Table 3. Isotype production by B cells stimulated with the B21 T_h0 clone

	B cells	
isotype (ng/ml)	unstimulated	stimulated
IgM	11 ± 11	326 ± 284
IgG	145 ± 38	545 ± 195
IgA	110 ± 40	182 ± 75
IgG4	67 ± 12	69 ± 16
IgE	12 ± 17	30 ± 38

ELISA results are expressed as arithmetic mean ± 1SD (n = 5).

T cell cytokine gene expression and production

T cells were purified by negative selection in order to avoid activation during the isolation procedure. From these purified T cells an aliquot was taken for mRNA extraction and determination of cytokine gene expression. Subsequently, the purified T cells (at 1 x 10^6/ml) were put in culture and either or not stimulated with Ca-ionophore (500 ng/ml) and phorbol ester (TPA (12-O-tetradecanoylphorbol-13-acetate), 1 ng/ml). This stimulation was carried out overnight at 37°C. This particular combination for stimulation was chosen based on optimal expression of all the relevant cytokine genes when compared to other modes of stimulation. The optimization of the mRNA extraction, copy DNA (cDNA) synthesis and reverse transcriptase-polymerase chain reaction have been described (18).

In the supernatant of these cultures the production of cytokines was quantified by ELISA. The values obtained were correlated to the amount of cytokine gene expression and the relative production of isotypes from the B cell cultures.

CONCLUSION

Allergy and atopic diseases in small children are intimately associated with the developmental status of the immune system. Several quantitative and qualitative parameters of both the specific and non-specific immune system are inherently different in infants. We therefore designed protocols permitting the proper analysis of several parameters of both B cells and T cells in children. Firstly, T and B cells have to be isolated in a reproducible way with high yield and purity and without activation during handling. Next, T cells have to be analyzed for their cytokine gene expression and production potential with and without stimulation. B cells should be analyzed for their switching potential *in vitro* by stimulation through cognate signalling with T_h cells and the cytokines that are relevant. The strategy described in this paper permits analysis of intrinsic defects in the T and B cell compartment in the peripheral blood of allergic children in comparison to healthy controls. Moreover, it allows longitudinal follow-up of high risk children for the early detection of the onset of allergic diseases.

ACKNOWLEDGEMENT

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CHAPTER 2.2

ANALYSIS OF CYTOKINE GENE EXPRESSION IN STIMULATED T CELLS OF SMALL CHILDREN BY SEMI-QUANTITATIVE PCR

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ABSTRACT

Only limited amounts of peripheral blood samples can be obtained from small children. Therefore, a polymerase chain reaction (PCR) aided analysis of cytokine gene expression by peripheral blood mononuclear cells (PBMC) or T cells is a valuable tool. We present a combination of procedures to obtain an accurate estimation of the expression of the cytokines interleukin-4 (IL-4) and interferon-γ (IFN-γ). This can be performed on T cells purified from blood samples of up to 5 ml in volume from children aged 0-4 years with allergic asthma and atopic dermatitis. This procedure includes multiple sampling of PCR products to determine the linear phase of the PCR; inter-experiment correction using a helper T-cell clone, expressing both IL-4 and IFN-γ; inter-patient correction by comparing the expression of a housekeeping gene (HPRT); and finally the development of specific software to analyze densitometric data obtained by scanning photographs of agarose gels, separating PCR products. In this way it is possible to study cytokine gene expression from a very small amount of material.

INTRODUCTION

Allergic diseases such as allergic asthma and atopic dermatitis are generally characterized by increased immunoglobulin E (IgE) levels in the serum (1-3). Cytokines play an important role in the regulation of IgE synthesis (4). Interleukin-4 (IL-4) is an inducer of IgE synthesis, while interferon-γ (IFN-γ) suppresses IgE synthesis (5,6). Other cytokines play an additional role in the regulation of IgE. IL-5 has an enhancing effect on the stimulatory activity of IL-4 (7) and IL-10 has a down-modulating effect on the production of IFN-γ (8). IL-13 is a recently discovered cytokine, produced by different T cell subsets, with IgE inducing activities comparable with IL-4 (9,10).
The presence of IL-4 and IFN-γ has been demonstrated in cultured peripheral

blood mononuclear cells (PBMC) from healthy individuals, atopic subjects and patients with the hyper-IgE syndrome (11). Many studies have analyzed cytokine production profiles of cultured and stimulated PBMC of allergic patients by enzyme-linked immunosorbent assay (ELISA) (12,13), or production of cytokines by cloned T cells (14,15). However, only a few studies have described cytokine mRNA expression (16,17). Ehlers *et al.* studied cytokine expression in neonatal T cells, compared with adult T cells, by polymerase chain reaction (PCR) analysis. These authors showed that cord blood T cells are able, upon stimulation *in vitro* to transcribe IL-2 mRNA. However, their capacity to transcribe mRNA for IL-3, IL-4, IL-5, IL-6, IFN-γ and granulocyte macrophage colony stimulating factor was markedly reduced in comparison with that in adults (16).

Our main interest is the role of cytokines in the development of allergy during infancy, since the immune system of children is generally considered to differ from the system in adults (19). The number of T cells is relatively low and IFN-γ production by the T-cells is decreased, in comparison with IFN-γ production in adults (18,19).

We chose to analyze cytokine gene expression in young children (0-4 years), who are developing allergic asthma or atopic dermatitis, in order to study the pathogenesis of these diseases. We aim to correlate the mRNA expression levels in PBMC and T cells with the cytokine production profile and clinical manifestations.

To make such studies feasible, T cells were purified from peripheral blood (20). The amount of peripheral blood obtained from young children is usually small, and the number of T cells that can be purified is therefore limited. Moreover, cytokine gene expression is usually transient and cytokine mRNA levels generally occur at low abundance. Therefore, such analysis requires sensitive procedures (21). The PCR (22,23) is a sensitive method for the detection of gene expression. Here we describe optimizations of the PCR method to analyze cytokine gene expression occurring at low abundance and from a limited number of cells, in a semi-quantitative way (24,25).

MATERIALS AND METHODS

Purification and stimulation of T cells

PBMC from a maximum of 4 ml heparinized blood of children, were purified by density centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden (26). The children ranged from 0-4 years of age and were healthy or with allergic asthma. These PBMC were incubated (30 min, 4°C) with monoclonal antibodies specific for monocytes (My4, 100 µl (40 µg/ml) per 1 x 10^7 pelleted cells, Coulter Cytometry, Hialeah, FL), B cells (CD19, 100 µl (100 µg/ml) per 1 x 10^7 pelleted cells , Coulter) and natural killer (NK) cells (CD16 and CD56PE, both 50 µl (100 µg/ml) per 1 x 10^7 pelleted cells, Becton Dickinson, San Jose, CA). Subsequently, the samples were

incubated (30 mins, 4°C on a roller bank) with sheep-anti-mouse Dynabeads (IgG M450, Dynal, Oslo, Norway). T cells were negatively selected by using a magnet (Dynal). The purity of the T cell fraction was between 85% and 95%, as determined by fluorescence activated cell scanner (FACScan) analysis, after staining with CD3-specific antibodies (Leu-4-FITC, 50 µl (2.5 µg/ml) per 5 x 10^5 pelleted cells, Becton Dickinson). The contribution of NK cells was always less than 3.5 %.
T cells (1 x 10^6 cells/ml) were cultured 16-18 h in Yssel's medium (27) containing 1% human serum with and without the addition of 4-bromo-calcium-ionophore (A23187, final concentration 500 ng/ml, Sigma, St.Louis, MO) and TPA (12-O-tetradecanoylphorbol-13-acetate), final concentration 1 ng/ml, Sigma) at 37°C, 5% CO_2.

RNA isolation and cDNA reaction
After spinning down the T cells, RNA was isolated from the cells by the RNAzol B (Cinna-Biotecx Laboratories Inc., Houston, TX) method (28). Briefly, per 1 x 10^6 pelleted cells 200 µl RNAzol (minimum 300 µl) 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 phenol:chloroform (1:1) was added. After mixing and centrifugation, an equal volume of chloroform was added to the aqueous phase. Following centrifugation, an equal volume of isopropanol was added to the aqueous phase. After incubation at 4°C for at least 2 h, the RNA was pelleted by centrifugation and washed with 20 µl 70% ethanol per 1 x 10^6 cells (minimum 50 µl). The RNA was resuspended in 10 µl water and the OD_{260}/OD_{280} were determined by spectrophotometry (Ultrospec III, Pharmacia-LKB). Twenty µg glycogen (Boehringer Mannheim, Germany) was added during the phenol:chloroform extraction as a carrier. copy DNA (cDNA) synthesis (29) was performed starting with 1 µg RNA, after heating for 10 min at 65°C. Random hexamer primers were used to ensure all RNA was represented equally in the cDNA pool (30). The reaction mixture contained 2 µl of 10x AMV-RT buffer (0.5 M Tris-HCl, 0.1 M $MgCl_2$, 0.5 M DTT, 10 mM EDTA and 100 µg/ml bovine serum albumin; pH 8.3), 0.25 mM of each deoxynucleotide triphosphate (dNTP), 1 mM salmon spermine HCl (Sigma), 40 units of RNAsin (Promega, Madison, WI), 2.5 OD $(dN)_6$ (Pharmacia), 0.2 µg oligo$(dT)_{12-18}$ (Boehringer) and 5 units reverse transcriptase (from avian myeloblastosis virus; Boehringer). The total reaction volume was 20 µl. Incubation was performed for 1 h at 41°C. Afterwards the cDNA was diluted to 200 µl and stored at -70°C.

Polymerase chain reaction
For the PCR, a mixture was prepared containing 50 ng cDNA (in 10 µl) and 10 µl of 10 x Taq polymerase buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$ and 1 % gelatin; pH 8.3), 0.03 mM of each dNTP and 1 unit of Taq polymerase (Ampli Taq, Perkin Elmer Cetus, Norwalk CT). One µl of the following sense- and anti-sense primers (10 OD/ml) were used:

HPRT sense: 5'-GTGATGATGAACCAGGTTTATCACCTT-3' (exon2)
antisense: 5'-CTTGCGACCTTGACCATCTTTGGA-3' (exon 6)
(product size 454 bp) (31)

IL-4 sense: 5'-ACTCTGTGCACCGAGTTGACCGTAA-3' (exon 2)
antisense: 5'-TCTCATGATCTGCTTTAGCCTTTCC-3' (exon 4)
(product size 300 bp) (32)

IFN-γ sense: 5'-TTTAATGCAGGTCATTCAGATG-3' (exon 1-2)
antisense: 5'-CAGGGATGCTTCTTCGACCTCGAAAC-3' (exon 4)
(product size 388 bp) (32)

We developed the HPRT (human hypoxanthine phosphoribosyl transferase) primerset from the genomic structure, by selecting primers for exons 2 and 6. In this way, the product including the intron sequences will be too large (3.5 kB) to be amplified by PCR. The IL-4 and IFN-γ primersets were kindly donated by Dr. R. de Waal Malefijt (DNAX Research Institute, Palo Alto, CA). It was verified by Southern blot analysis that both primersets did not amplify genomic DNA (data not shown).
The total reaction volume was 100 µl. As a negative control 50 ng RNA in 10 µl water, which had not undergone the cDNA reaction, was used from six patient samples per PCR. One sample of the cDNA mixture was also used as a negative control.
The PCR protocol started with an adaptation of the hot-start (33), to prevent aspecific primer annealing. After preparing the PCR mixture on ice, the reaction tubes were placed for 3 min in the PCR-block (DNA Thermal Cycler, Perkin Elmer Cetus), which was preheated at 94°C. Subsequently, the first 25 PCR cycles (HPRT) were started: denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 1 min. The linear phases of HPRT, IL-4 and IFN-γ were found during a different range of PCR cycles (data not shown). To cover these linear phases, the initial number of PCR cycles was 30 for IL-4 and 20 for IFN-γ. After 25 PCR cycles (HPRT) the block was cooled to 4°C, 10 µl of PCR-product was removed from the reaction tubes and the tubes were taken out of the machine. Before re-introducing the reaction tubes, a hot-start was performed again, but now only for 1.5 min, to prevent too much loss of activity of the Taq enzyme. The half-life of Taq enzyme at 94°C is 35 min (34). After five additional PCR cycles, 10 µl was collected again. This was repeated three times. The five time-point samples were analyzed on agarose gel and subsequent Southern transfer (35). The Southern transfer was performed to determine the specificity of the different primer sets and to ensure that no genomic DNA was amplified.
For analysis of patient samples, the procedure was ended by taking a photograph of the gel. B21 cDNA was used as a positive control in all PCR reactions, since the B21 T helper 0 (T_h0) clone produces among others IL-2, IL-4 and IFN-γ (36). The B21 T cell clone was kindly provided by Dr. H. Yssel (DNAX Research Institute, Palo Alto, CA)

Analysis of PCR-products
After the PCR amplification, the samples were loaded on a 1.2% agarose gel (Sea Kem LE agarose, FMC Bio Products, Rockland, ME), stained with 75 µg/ml ethidium bromide (Boehringer). One µl PhiX174 (HaeIII digest, 0.1 µg/µl, New England Biolabs, Beverly, MA) was used as a marker. The bands of the PCR products on the gel were visualised with UV light.
A life-size photograph was taken and used for scanning. Each row (30 mm x 200

mm) of the photograph was scanned separately with a handscanner (Colorscanner 2^{24}, Highscreen, Würselen, Germany) at a resolution of 100 dots per inch. Computer software was developed to analyze the intensity of the bands. A rectangle was drawn automatically around each band of a row. In each rectangle the intensity was measured on all 20 spots, from which the mean value was calculated by integrating the values. Two rectangles of the same size were drawn automatically directly next to the first row of rectangles. In this way the background was measured on two subsequent positions of the gel. After extrapolation, a correction could be made for a possible drift in the background staining of the gel. The mean background value was subtracted from the mean band value. The resulting value will be subsequently called the 'scan value'.

Analysis of scan values

The data collected with the handscanner and the software were analyzed in a spreadsheet (Quattro Pro for Windows, Borland International Inc., Scotts Valley, CA).

I. Calculation of the mean scan values of B21 cDNA for standardization of inter-experimental variation

HPRT, 35-40 cycles; IL-4, 40-45 cycles; IFN-γ, 30-35 cycles

II. On each gel with patient samples, one B21 sample run in the same PCR was applied. A correction factor could be calculated for B21, by dividing by the highest B21 scan value of the first three independent PCR runs, which were analyzed on six different gels. Such B21 dependent correction was determined for every individual cytokine or HPRT

$$\frac{\text{B21 gel scan value HPRT/IL-4/IFN-}\gamma}{\text{highest B21 scan value HPRT/IL-4/IFN-}\gamma} = \text{B21 correction factor HPRT/IL-4/IFN-}\gamma$$

III. Correction of HPRT mRNA expression of a patient sample for B21 mRNA expression

$$\text{Scan value sample HPRT} * \frac{1}{\text{B21 correction factor HPRT}}$$

IV. All patient scan values were corrected for HPRT by dividing by the highest HPRT scan value of five experiments, in order to correct for inter-patient variation

$$\frac{\text{HPRT scan value sample}}{\text{highest HPRT scan value}} = \text{HPRT correction factor}$$

V. Calculation of the mean values of HPRT of each patient sample after 35 and 40 PCR cycles

VI. Correction of IL-4 and IFN-γ mRNA expression for B21

$$\text{IL-4/IFN-}\gamma\text{ scan value sample} * \frac{1}{\text{B21 correction factor IL-4/IFN-}\gamma}$$

VII. Calculation of mean values of each sample

IL-4, 40-45 cycles and IFN-γ: 30-35 cycles

VIII. Calculation of the end values corrected for B21 and HPRT

$$\text{Mean sample value IL-4/IFN-}\gamma * \frac{1}{\text{correction factor HPRT}}$$

Southern blotting
Subsequently, the gel was put into an acid solution (0.25 M HCl) for 10 min, a denaturating solution (1 M NaCl, 0.5 M NaOH) twice for a period of 15 min, and a neutralizing solution (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.0) twice for 20 min. After this a Southern transfer (35) was performed, by which the PCR products were transferred overnight to a Nytran filter (0.45 μm, Schleicher and Schuell, Dassel, Germany). DNA was crosslinked to the filter in a UV crosslinker (120 mJ) (UV Stratalinker 2400, Stratagene, La Jolla, CA).
Probes to the inner region of the amplification target were end-labeled with ^{32}ATP (see Reference 37) and hybridization was carried out (38). The following probes were used:

HPRT: 5'-GAAGAGCTATTGTAATGACCAGTCA-3' (exon 3-4) (31)
IL-4: 5'-CAGTTCCACAGGCACAAGCAG-3' (exon 3) (32)
IFN-γ: 5'-TGACTAATTATTCGGTAACTGACTTGAATG-3' (exon 3-4) (32)

RESULTS

Semi-quantitative PCR analysis of cytokine gene expression

Initially, three different housekeeping genes, HPRT (31), ß-actin (39) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (40), were tested on cDNA of different cell populations of healthy control children. The HPRT primer set was considered the most reliable, since it did not amplify genomic

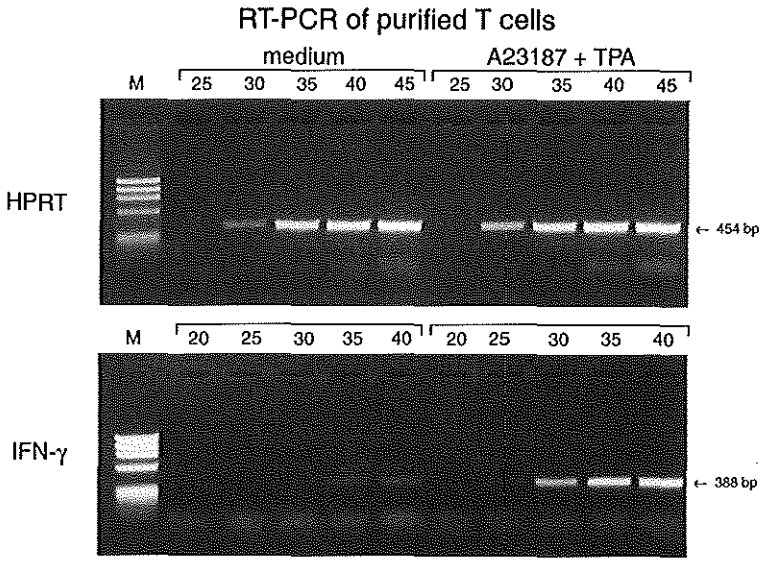

Figure 1. RT-PCR of purified T cells. RNA was isolated from purified T cells of a healthy child. These cells had been cultured overnight in medium alone or stimulated with A23187 (Ca-ionophore) and TPA. After reverse transcription into cDNA, a PCR was performed for HPRT and IFN-γ. Samples were collected from the reaction tube after different numbers of cycles. For HPRT this was done at 25, 30, 35, 40 and 45 cycles and for IFN-γ at 20, 25, 30, 35 and 40 cycles. The HaeIII digest of PhiX174 was used as a molecular weight marker (M).

DNA, as tested with Southern blot analysis and hybridization. Furthermore, HPRT had a stable expression level in the same range as the cytokine levels studied (data not shown).

Next, T cells of a healthy control child were purified and cultured overnight in medium alone or stimulated polyclonally by the addition of Ca-ionophore and TPA. Reverse transcriptase-PCR (RT-PCR) analysis was performed using primer sets specific for HPRT and IFN-γ. A representative result is shown in Figure 1. After 25 cycles (HPRT) or 20 cycles (IFN-γ), 10 μl samples were taken from the PCR mixture and subsequently every five cycles up to 45 or 40 cycles, respectively. The PCR products were made visible by means of agarose gel electrophoresis.

Visual analysis enabled qualitative observations like IFN-γ mRNA production by T cells after stimulation with Ca-ionophore and TPA. A differential mRNA expression was observed when comparing IFN-γ expression in T cells cultured in medium alone or stimulated with Ca-ionophore and TPA. The IFN-γ mRNA signal in the stimulated T cell fraction was higher (already visible after 25 PCR cycles) than in the T cell fraction cultured with medium alone (visible only after 35 cycles). In the same experiment, the HPRT mRNA appeared to be present in both samples in equal amounts.

It is possible to quantify these products, by scanning the photographs and assigning values to the intensities of the different bands. To this end, the photograph of the agarose gel (Figure 1) was scanned with a scanner and the image was analyzed by the described software. The corresponding scan values are shown in Table I. These results also reflect the differential expression shown in Figure 1, but now in absolute numbers. For example, the scan values of the IFN-γ mRNA signal after 40 PCR cycles in stimulated T cells *vs* T cells cultured in medium alone, were 1048 compared to 77 (a 13.6-fold increase). Both types of analyses show that the PCR reaction for HPRT reached a plateau phase after 40 PCR cycles. For IFN-γ a plateau level was reached after 35 PCR cycles, but only in the stimulated T-cell fraction.

Table I. Scanvalues of HPRT and IFN-γ mRNA expression in purified T cells

	Number of cycles				
HPRT	25	30	35	40	45
T cells medium	0	349	1480	1753	1880
T cells A23187 + TPA	11	858	1643	1772	1763
IFN-γ	20	25	30	35	40
T cells medium	0	0	0	77	234
T cells A23187 + TPA	0	47	843	1048	1119

The photograph shown in Figure 1 was scanned with the hand scanner and analyzed by computer. Scan values are corrected for background.

Linearity of the PCR

It is crucial to compare the results in the linear phase of the PCR (24), in order to permit comparison in cytokine mRNA expression between different patients and patient groups. Therefore, we tested which part of the PCR for HPRT and the relevant cytokine genes was linear. To this end, one large batch of cDNA of the B21 T-cell clone was used in the PCR for HPRT, IL-4 and IFN-γ. In Figure 2 the mean scan values are shown of B21 cDNA run in 3 independent PCR reactions, while the PCR products were analyzed on six different agarose gels. The linear phase of the HPRT PCR was consistently found between 25 and 35 cycles, after which a plateau level was reached. Similar findings were made for IFN-γ. The linear phase of the IL-4 PCR was found between 35 and 45 cycles. Thus, different samples can only be compared by analysing data obtained at these predetermined numbers of PCR cycles.

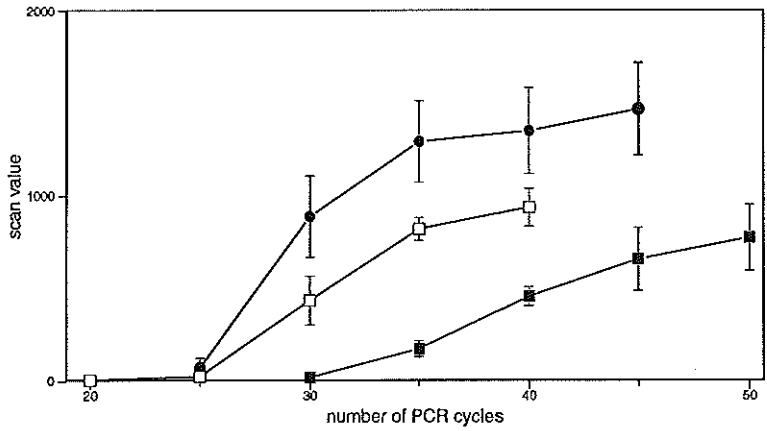

Figure 2. Cytokine mRNA expression in stimulated B21 cells. cDNA of B21 was used for inter-experiment correction, using primers for HPRT (●), IL-4 (■) and IFN-γ (□). PCRs for HPRT were run from 25 to 45 cycles, for IL-4 from 30 to 50 cycles and for IFN-γ from 20 to 40 cycles, all with intervals of five cycles. The X-ordinate indicates the number of cycles. The Y-ordinate indicates the mean scan values. The data represent the mean of six different experiments ± S.D. of the obtained scan values.

Standardization of differences in cytokine mRNA production between different patients

HPRT was used as a housekeeping gene to standardize the cytokine mRNA expression of each patient, enabling analysis of inter-patient variation within the PCR reaction. However, for obvious reasons it was not possible to perform all the PCR reactions at the same time. Therefore, an inter-experiment standard was considered necessary. To this end the B21 T cell clone was used. This clone produced, among others, the cytokines IL-4 and IFN-γ (36). From a large number of B21 cells, RNA was isolated and transcribed into cDNA. Aliquots of this batch were used for all PCR experiments. Figure 2 shows the kinetics of the PCR reaction of B21 cDNA. HPRT and IFN-γ reached a plateau relatively early (HPRT after 35 PCR cycles and IFN-γ after 40), while the linear phase of IL-4 was longer (plateau only after 45-50 PCR cycles). The variation in B21 scan values, obtained from independent PCR runs and analyzed on different gels, was relatively small (less than 10%), resulting in a generally small inter-experiment variation.

Analysis of the scan values

To optimize the reproducibility of data, photographs were processed in a similar way. Special attention was paid to obtaining gel pictures of the same intensity, contrast and paper quality.

The reproducibility of the data generated with the scanner and the computer

software were also examined. On a photograph displaying amplified PCR products one lane was scanned ten times. The results are depicted in Table II. The variation in the data collected was always less than 10%.

Table II. Reproducibility of the handscanner

band	mean	SD	n
1	665.2	43.57	10
2	1380.8	98.43	10
3	1514.2	169.35	10

One lane of an 1.2% agarose gel with 75 µg/500ml ethidium bromide was scanned ten times. Arithmetic mean and S.D. were calculated for three bands with different levels of intensity.

As described in 'linearity of the PCR', comparisons have to be made in the linear phase of the reaction. To correct for possible fluctuations, mean scan values were calculated from the two PCR cycle times in the linear phase of the PCR. This phase which was determined for each gene studied.
Correction factors were calculated for B21 mRNA. The mean correction factor for B21 HPRT was 0.78 (± 0.12), for B21 IL-4 0.75 (± 0.14) and for B21 IFN-γ 0.74 (± 0.14). The relatively small standard deviations show that the reproducibility of the PCR of B21 cDNA was high. For this reason only small inter-experiment corrections (mean 0.77 ± 0.14) were generally necessary. The mean correction factor for HPRT mRNA expression of 20 samples was 0.81 ± 0.21. In case the correction factors for B21 and HPRT were smaller than 0.5, or greater than 1.5, we considered the data not to be reliable, because of possible overcorrection. The results of such experiments (in the case of B21) or samples (in the case of HPRT) were excluded from further analysis.

Application of the technique to patient samples
T cells were purified from peripheral blood of healthy and asthmatic children. The T cells were cultured overnight in medium alone or stimulated with Ca-ionophore and TPA. After RNA isolation and cDNA synthesis, PCR was performed for HPRT, IL-4 and IFN-γ during the described number of PCR cycle times. In Figure 3 a representative example is shown of one healthy child and one asthmatic child. The shape of the curves for HPRT were comparable, both for T cells cultured in medium and for stimulated T cells. All curves reached a similar plateau level (1766 ± 74). In both the healthy control (lower left) and the asthmatic child (lower right) there was a marked difference in IFN-γ mRNA expression between stimulated and unstimulated T cells. In stimulated T cells from the healthy control, the plateau phase of IFN-γ PCR was reached earlier (35 PCR cycles) than in the stimulated T cells of

the asthmatic child (40 PCR cycles), indicating a higher expression of IFN-γ mRNA. In the unstimulated condition, no IFN-γ expression was found in the T cells from the asthmatic child, whereas after 35 and 40 PCR cycles IFN-γ expression could be detected in T cells from the healthy control. The linear range of the PCR as assessed in B21 cells, was also applicable to HPRT and cytokine gene expression in patient samples of stimulated and unstimulated T cells.

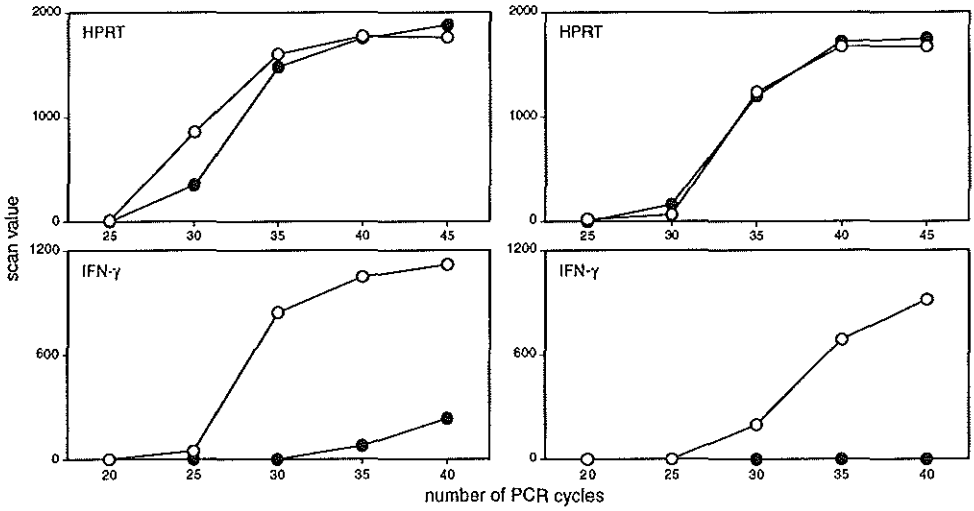

Figure 3. Analysis of HPRT and IFN-γ mRNA expression in a healthy and an asthmatic child. T cells were purified from peripheral blood of a healthy and an asthmatic child. T cells were cultured overnight in medium alone (•) or with the addition of A23187 (Ca-Ionophore) and TPA (○). RT-PCR was performed for: a healthy child for HPRT (upper left), an asthmatic child for HPRT (upper right), a healthy child for IFN-γ (lower left) and an asthmatic child for IFN-γ (lower right).

Table III. Correction factors for HPRT and IFN-γ for B21 clone

Subjects	B21 correction factor	
	HPRT	IFN-γ
Healthy	0.76	0.53
Asthma	0.70	0.99

Correction factors were calculated for B21 mRNA expression of HPRT and IFN-γ, as described in 'Analysis of scanvalues'.

In Table III the correction method is applied on the scan values of the healthy and asthmatic children presented in Figure 3. First, the inter-experiment variation was determined by calculating the B21 correction factors for HPRT

and IFN-γ. As shown in Table III, the inter-experiment correction factor for HPRT was similar for the healthy and the asthmatic child samples (0.76 *vs* 0.70). For IFN-γ this correction factor differred more (0.53 *vs* 0.99). For the subsequent inter-patient correction, the HPRT correction factors were minimal, as shown in Table IV. The end values for IFN-γ could be calculated with these correction factors (Table IV). The qualitative differences as observed in Figure 3 were also reflected in these end values, but now in a semi-quantitative way (1673.69 *vs* 444.68). This analysis allows comparison of cytokine expression between different cell populations of larger patient groups.

Table IV. HPRT correction factors and IFN-γ end values for purified T cells

Subjects	Correction factor HPRT		End values IFN-γ	
	Control	Stimulation	Control	Stimulation
Healthy	1.01	1.06	71.31	1673.69
Asthma	0.99	0.99	0.00	444.68

Correction factors were calculated for purified T cells of a healthy and an asthmatic child for HPRT mRNA expression and end values were calculated for IFN-γ, as described in 'Analysis of scan values'. T cells were cultured overnight in medium (control) or stimulated with TPA and Ca-ionophore (stimulation).

DISCUSSION

Our studies have shown that semi-quantitative analysis of cytokine gene expression is possible in small peripheral blood samples. This was achieved by adapting the RT-PCR method and combining it with an easy and reliable way of analysis of the amplified product. For small numbers of cells, other methods of studying cytokine gene expression, such as Northern blot analysis, cannot be employed because of the low amount of isolated RNA. PCR enables analysis of gene expression in a highly sensitive and specific way (41).

Several adaptations of the PCR procedure were necessary to permit the required analysis. The analysis of the expression of a housekeeping gene is generally considered essential to correct for inter-patient variation (42). The requirements for a suitable housekeeping gene are a stable expression, a similar detection range as the expression of the genes to be studied and no amplification of genomic DNA (43). A HPRT primer set was developed, which meets the above criteria as verified by Southern blot analysis and hybridization. DNAse treatment (44), often used when RNA is contaminated with genomic DNA, resulted in a significant loss of RNA and could therefore

not be used in our studies.
Determining the linear phase of the PCR was important because at the plateau phase the quantified amount of amplified product is no longer proportional to the starting amount of target molecules (24,25). The PCR is linear only during a limited number of cycles, after which the amount of product reaches a plateau (24). There are a number of factors that contribute to this plateau phenomenon, including substrate saturation of enzyme, product strand reannealing and incomplete product strand separation (25). The linear phase of the PCR has to be determined for each gene to be analyzed, because the primers used to amplify their products differ in G/C versus A/T content and thus in annealing temperatures.
Preliminary experiments indicated that the described approach could also be applied to the analysis of PCR products, that are detected by autoradiography. This way the sensitivity of the analysis was further increased. Therefore, the algorithm developed here can analyze films of Southern blots as was found to be necessary for IL-5 and IL-10.
Visual analysis of the photographed gels enabled us to make qualitative comparisons between different patient samples. However, the appearance of a positive signal after a certain number of cycles did not necessarily reflect the original mRNA concentration of the relevant gene. Two different samples could reach a detectable PCR signal after the same number of PCR cycles, while the intensity of the bands was different and another plateau level was reached. Therefore, quantitative analysis of the bands was considered necessary. This was achieved by using scanning in combination with newly developed software for data analysis.
Another necessary step in the standardization of the procedure was the use of B21, a T_h0 clone, which produces upon activation all cytokines relevant for this study (36). B21 cDNA gave reproducible data for HPRT, IL-4 and IFN-γ expression in different experiments. The inter-experiment correction was thus generally small (0.77 ± 0.14). This correction had to be determined for every individual cytokine. The variation of HPRT from different patient samples was in the same range (0.81 ± 0.21) as for B21, therefore a similar inter-patient correction was necessary. When the variation is too large, over-correction can occur. We consider a correction factor smaller than 0.5 or larger than 1.5 too extreme to detect the cytokine response in the sample reliably.
The analysis developed here is a reliable and simple alternative to other methods based on the use of radioactivity, like phosphor imaging (45). The variation found in cytokine expression within a patient group of, for example, healthy children of the same age is relatively high. The accuracy of the semi-quantitative method described here has sufficient discernment in relation to this extent of variation.
Recently, true quantitative methods have been developed. The method

developed by Gilliland *et al.* (46) uses an internal standard with the same primer requirement, but differing in the size of PCR product. However, the accuracy of quantification may be affected by sequence differences between the DNAs used for the standard and the sample (47).
When studying the intrinsic capacity of cells to express a particular cytokine gene, it is necessary to analyze highly purified cell populations. However, many studies examine the significance of cytokines in patients by analysing the total PBMC fraction only (11,13).
When endogenous cytokine gene expression is to be studied in purified T cells, it is important to note that the T cells should not be stimulated during purification. Indeed, negative selection during purification of T cells was found to be adequate to avoid stimulation. It is also important that for some cytokines, e.g., IFN-γ, cells need to be stimulated to obtain detectable expression levels. In our study, we used a polyclonal stimulus, Ca-ionophore and TPA, permitting the simultaneous detection of a broad range of cytokines.
We conclude that it is possible to accurately detect differential cytokine gene expression in T cells, isolated from blood of healthy controls and asthmatic children. This can be achieved by using a RT-PCR with differential cycle times for different cytokines and analysing the results with a scanner and specially designed computer software. Cytokine gene expression can thus be studied in a very limited amount of material.

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CHAPTER 3

ANALYSIS OF IMMUNOGLOBULIN PRODUCTION, GERMLINE-ε mRNA EXPRESSION AND mRNA EXPRESSION AND PROTEIN PRODUCTION OF T CELL DERIVED CYTOKINES IN ALLERGIC CHILDREN

3.1 The analysis of immunoglobulin production and germline-ε mRNA expression in allergic children
Submitted for publication

3.2 T cell subsets and cytokines in allergic and non-allergic children. I. Analysis of IL-4, IFN-γ and IL-13 mRNA expression and protein production
Submitted for publication

3.3 T cell subsets and cytokines in allergic and non-allergic children. II. Analysis of IL-5 and IL-10 mRNA expression and protein production
Submitted for publication

CHAPTER 3.1

THE ANALYSIS OF IMMUNOGLOBULIN PRODUCTION AND GERMLINE-ε mRNA EXPRESSION IN ALLERGIC CHILDREN

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ABSTRACT

A variety of parameters involved in the pathogenesis of allergic diseases were analyzed in a cross-sectional study in young children. Possible intrinsic B cell differences in allergic children were investigated by analyzing total serum immunoglobulin E (IgE) levels, germline-ε mRNA expression and IgE synthesis after B cell stimulation. Possible intrinsic differences in the T cell compartment were analyzed by fluorescence activated cell scanner (FACScan)-analysis and by measuring spontaneous IL-4 mRNA expression. To investigate this, B and T cells were purified from peripheral blood mononuclear cells (PBMC) of healthy children, allergic children (allergic asthma and atopic dermatitis) and children with non-allergic asthma. The relationship between the laboratory parameters and clinical features was studied.

Total serum IgE levels were elevated in allergic children suffering from atopic dermatitis and allergic asthma. Significantly increased levels of IgE *in vitro* after cognate stimulation of B cells induced by B21 cells (T_h0 clone) and IL-4 were restricted to B cells from children with allergic asthma. No significant differences in the *in vitro* production of IgA, IgM, total IgG and IgG4 were observed between the various groups of patients. Several differences were detected in the cellular distribution of children with non-allergic asthma as compared to the other groups: an increased CD4/CD8 ratio; an increased percentage of $CD4^+CD45RA^+$ T cells and a decreased percentage of natural killer (NK) cells. In children with atopic dermatitis the percentage of $CD4^+CD45RO^+$ T cells and IL-4 mRNA expression by purified T cells were increased as compared to healthy controls. In children with atopic dermatitis we also found an inverse correlation between the percentage of $CD4^+CD45RA^+$ T cells and the severity scoring of atopic dermatitis (SCORAD) index ($r_s = -0.52$, $p = 0.02$). Spontaneous germline-ε mRNA expression by purified B cells showed

no significant differences between the patient groups and the healthy controls. The combination of measurement of serum IgE levels, germline-ε and IL-4 mRNA expression and $CD45RA^+$ and $CD45RO^+$ T cells demonstrated differences between the various patient groups. Therefore, this combination may give information about developmental mechanisms of allergic disease and can be of discriminative or predictive value for development of allergic disease. Longitudinal studies are needed to further establish its importance in predicting allergy development in childhood.

INTRODUCTION

Elevated serum immunoglobulin E (IgE) levels are generally considered to be a typical factor in the expression of allergic diseases. Cord blood total IgE levels and total serum IgE levels in infants have been found of low prediction for developing allergic diseases in childhood. Allergen-specific IgE levels, on the other hand, are much more important, when discerning between allergic- and non-allergic asthma, eczema and healthy subjects. In recent years the insight into the immunoregulation of IgE antibodies is increasing, although still few information exists with regard to such processes in early childhood with developing allergy.

A number of research groups have reported that spontaneous IgE synthesis by peripheral blood mononuclear cells (PBMC) is increased in atopic in comparison with non-atopic subjects (1-3). IgE synthesis, although more pronounced during times of allergen challenge, may persist in periods of non-challenge (4). These findings suggest that either B lymphocytes from atopic donors are intrinsically different from those of non-atopics, or alternatively that T lymphocytes from atopics are different from those of non-atopic donors in their capacity to regulate IgE synthesis. Claassen *et al.* demonstrated that unfractioned PBMC from normal and atopic subjects synthesize comparable amounts of IgE in response to recombinant IL-4 (rIL-4) (5). Furthermore, rIL-4-induced IgE synthesis by enriched B cells from normal and atopic subjects, when co-cultured with T cells from either source, did not demonstrate differences in IgE production (5). In contrast, Poulsen *et al.* found a significantly increased spontaneous and IL-4 inducible IgE synthesis in patients with atopic dermatitis (6).

Immunoglobulin class switching is generally considered a two step process. In the first step, the downstream target C_H gene locus becomes transcriptionally active, resulting in the synthesis of C_H germline transcripts. This transcriptional activity has been proposed to reflect the opening up of the C_H locus, rendering it accessible to proteins that mediate switch recombination (7,8). In particular, IL-4 and IL-13 have been shown to be involved in the induction of germline-ε transcripts (9-12). Recently, the existence of an IL-4 Response Region of the

germline-ε promotor was demonstrated (13). To our knowledge, only one study (in healthy and immunodeficient adults) has analyzed germline-ε mRNA expression in PBMC (14). Germline-ε mRNA expression has not been studied previously in allergic children.

The second step in the isotype switching process encompasses the actual recombination event induced through an additional signal delivered to the B cell. For switching to IgE, this signal can be provided by activated $CD40^+$ T cells via triggering of the CD40 molecule, infection with Epstein-Barr virus or the presence of hydrocortisone (15-17). Some, but not all, of these IgE switch inducing signals can be inhibited by interferons, suggesting the existence of multiple signalling pathways.

The membrane antigen B7.1, expressed on activated B cells, monocytes and dendritic cells, interacts with CD28 on T cells. This interaction leads to higher IL-4 production, resulting in enhanced IgE synthesis (18,19).

A large body of evidence suggests that allergen-specific T helper 2 (T_h2)-like cells are preferentially expanded in atopic subjects, particularly at the level of target tissues, like skin and lungs (20). Apart from IL-4, also other genes of the IL-4 gene family, like IL-3, IL-5 and granulocyte macrophage colony stimulating factor are overexpressed in atopic individuals (21). Several genetic alterations could be active at the level of signal transduction pathways or complexes formed by transcription factors and their corresponding promotor regulatory elements (22). Alternatively, deficiencies in the production or regulatory activity of the cytokines normally responsible for the inhibition of T_h2 cell development (e.g. interferon-γ (IFN-γ)), might induce such overexpression of T_h2 subsets (23).

The $CD4^+$ subset can be further subdivided by the differential expression of CD45 isoforms, reflecting different stages of maturation and cytokine production (24). CD45RA is expressed on unprimed or naive cells, while CD45RO is expressed on a reciprocal and non-overlapping subset of activated/memory cells which preferentially respond to recall antigens (24,25) and is superior in delivering help for IgE synthesis.

In this study we analyzed the role of several parameters involved in the pathogenesis of allergic diseases. In order to investigate whether intrinsic B cell differences were present in allergic children, total IgE, germline-ε mRNA expression directly after isolation of B cells and IgE synthesis after stimulation of B cell cultures were analyzed. We additionally analyzed whether differences existed between allergic and non-allergic children in T cell subsets (CD4, CD8, CD45RA, CD45RO and CD25) and in IL-4 mRNA expression immediately after isolation of T cells. Furthermore, the interrelationship between these B- and T-cell parameters was investigated, as well as the association with expression of allergy in asthma and eczema.

MATERIALS AND METHODS

Patient groups

Children with allergic or non-allergic asthma, atopic dermatitis and healthy controls were included in the study. All subjects were between 0 and 4 years (Table I). The age and sex distribution in the four groups was comparable.

Table I. Characteristics of the patient groups studied.

	age in months median (ranges)	sex distr.	family history median (ranges)	total IgE median (ranges)	RAST median (ranges)	SCORAD median (ranges)
Healthy controls	28 (5-59)	33 ♂ 11 ♀	0 (0-0)	16 (0-952)	0	N.D.
Allergic asthma	43 (12-54)	8 ♂ 2 ♀	3 (2-7)	246 $^{p<0.001}$ (45-12134)	3 (2-5)	N.D.
Non-allergic asthma	21 (7-56)	7 ♂ 5 ♀	4 (2-6)	13 (2-168)	0	N.D.
Atopic dermatitis	27 (7-50)	14 ♂ 11 ♀	2.5 (1-6)	196 $^{p<0.001}$ (6-4000)	3 (2-6)	35 (12-69)

Ages are expressed in months. Family history was calculated by taking 2 points for each affected family member of the first grade and 1 point for each affected family member of the second grade. Total IgE levels were determined by IgE CAP RAST and are expressed as kU/l. P-values were calculated using regression analysis. RAST data represent median values of the RAST-classes as determined by Phadiatop® and/or food mix CAP RAST and ranges. SCORAD index scores are expressed according to the European Task Force on Atopic Dermatitis (ETFAD). N.D. represents not determined.

Children with asthma were selected using the following criteria of the American Thoracic Society: coughing and/or shortness of breath, at least 3 times a year, for a period of at least 14 days (26), and a positive family history of asthma and/or atopy, as well as absence of treatment with (inhalation-) corticosteroid. The group of children with allergic asthma was further defined by the presence of specific IgE (>0.35 kU/l) in their serum for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The children with non-allergic asthma were selected according to the same criteria as the allergic asthmatic children, but at the time of the study they had no measurable specific IgE to inhalation and/or food allergens. The children with atopic dermatitis were included according to the diagnostic criteria of Hanifin and Rajka (27). The main diagnostic criteria were pruritis, typical skin lesions and localization, a chronical recurrent course and the presence of other atopic diseases or atopic relatives. Children with atopic dermatitis had specific IgE (>0.35 kU/l) for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The severity scoring of atopic dermatitis (SCORAD) index was used as a measure for disease severity (28). Healthy control children were included by completing a questionnaire by their parents, indicating the absence of allergic symptoms and of a family history of atopy. Healthy children had no specific IgE (<0.35 kU/l) for inhalation and/or food allergens in their serum as tested by Phadiatop® and food mix and no allergic symptoms.

The Medical Ethical Committee of the Erasmus University Medical School and University Hospital Rotterdam approved this study. Informed consent was given by all the parents of the patients and healthy control children included in the study.

Analysis of total serum IgE and specific IgE levels

Determination of total serum IgE levels was performed using the Pharmacia CAP radio-allergo-sorbent test (RAST) (Kabi Pharmacia, Uppsala, Sweden). Analysis of specific IgE antibodies directed towards the most common inhalation allergens (house dust mite, grass and birch pollen, cat, dog and moulds) was performed using the Pharmacia CAP system Phadiatop® RAST.

Analysis of specific IgE antibodies directed towards the common food allergens (chicken egg white, milk, fish, wheat, peanut, soy) was performed using the Pharmacia CAP RAST. All tests were performed according to the manufacturers instructions. The results of the specific IgE determinations were classified in the following RAST classes according to the amount of specific IgE expressed in kU/l: class 1: 0.35-0.7; class 2: 0.7-3.5; class 3: 3.5-17.5; class 4: 17.5-50; class 5: 50-100; class 6: >100.

B cell separation and purification

PBMC were purified from 4 ml heparinized blood by density centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) (29). These PBMC were incubated (15 min, 6-8°C) with 20 µl CD19 microbeads (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands) per 10^7 cells and 80 µl magnetic activated cell sorter (MACS) washing solution (10 mM phosphate-buffered saline (PBS, pH 7.2) with 1% bovine serum albumin (BSA), 5 mM EDTA and 0.01% sodium azide), while shaking occasionally. After washing twice in FACS washing solution (PBS with 0.5% BSA and 0.1% sodium azide), cells were resuspended in 500 µl MACS buffer and separated on the MACS (CLB), according to the method described by Miltenyi et al. (30). Briefly, the negative fraction, containing monocytes, T cells and natural killer (NK) cells, was eluted from the MACS column with a 23Gx1¼" needle (Braun, Melslungen, Germany). Subsequently, the column was washed twice to remove possible contaminating cells, by removing the column out of the magnetic field and reflushing and subseqently putting the column back into the magnetic field, using a 22Gx1¼" needle (Braun). Finally, the B cell fraction was collected by removing the column out of the magnetic field and flushing it 3 or 4 times with a 10 ml syringe filled with MACS solution.

The purity of the collected B cells was > 95%, as determined by CD19 staining and subsequent fluorescence activated cell scanner (FACScan)-analysis. Briefly, 50 µl (2.5 µg/ml) IgG_2-FITC and 50 µl (2.5 µg/ml) IgG_1-PE were used as negative control antibodies and 50 µl (4 µg/ml) B4-PE (Coulter Cytometry, Hialeah, FL), 50 µl (2.5 µg/ml) Leu-4-FITC (Becton Dickinson, San Jose, CA), 50 µl (2.5 µg/ml) CD16-PE and CD56-PE (Becton Dickinson) and 50 µl (2.5 µg/ml) MY4-FITC (Coulter Cytometry) per 1-5x10^5 pelleted cells to stain B, T and NK cells and monocytes, respectively.

T cell separation and purification

T cells were isolated from the negative fraction of the PBMC after B cell purification. This fraction was incubated (30 min, on ice) with monoclonal antibodies (mAb) specific for monocytes (My4, 100 µl (40 µg/ml) per 10^7 pelleted cells, Coulter Cytometry) and NK

cells (CD16 and CD56PE, both 50 µl (100 µg/ml) per 10^7 pelleted cells, Becton Dickinson). Subsequently, the samples were incubated (30 min, 4°C on a roller bank) with sheep-anti mouse Dynabeads (IgG M450, Dynal, Oslo, Norway). T cells were negatively selected by using a magnet (Dynal). The purity of the T cells fraction varied between 85-95%, as determined by FACScan-analysis, after staining with CD3-specific antibodies (Leu-4-FITC, 50 µl (2.5 µg/ml) per 1-5x10^5 pelleted cells, Becton Dickinson).

Cell culture conditions
The purified B cells were washed twice in RPMI 1640 medium + 25 mM HEPES with 5% fetal calf serum (FCS) to remove the remaining sodium azide. The B cells were resuspended in Yssels medium (31) containing 10% heat-inactivated FCS to a concentration of 5x10^3 cells/well and cultured at 37°C and 5% CO_2 in a flat-bottom 96 wells plate. B cells were also cocultured with 5x10^4 B21 cells/well (a T_h0 clone)(32) and 200 U/ml IL-4. After 12 days the supernatants were harvested and stored at -70°C until determination of isotypes.

Determination of immunoglobulins
Ig secretion was determined by enzyme-linked immuno sorbent assay (ELISA) as described (33). Briefly, 96-well plates (flat-bottom, Falcon 3912, Becton Dickinson) were coated overnight at 4°C with 100 µl/well of 0.2 µg/ml GAH (goat anti human)-IgA (Tago Inc., Burlingame, CA), 0.2 µg/ml GAH-IgM (Tago), 1.0 µg/ml GAH-IgG (Tago), 1.0 µg/ml MAH (mouse anti human)-IgG4 (Zymed Laboratories, San Francisco, CA) or 1.0 µg/ml MAH-IgE (IgG2a fraction, clone E4/3, the Binding Site, Birmingham, England) and 1.0 µg/ml MAH-IgE (IgG1 fraction, clone E3, the Binding Site). All mAb were diluted in 125 mM carbonate buffer pH 9.6. The plates were washed three times in PBS containing 0.01 % Tween 20 and a blocking step was performed for 1 hour at RT in RPMI 1640 medium + 25 mM HEPES with 10% FCS. 100 µl of the samples (1:5 diluted in RPMI 1640 medium + 25 mM HEPES with 10% FCS) was added. 100 µl standard was added and 1:2 diluted for 6 times. For IgA, IgM and total IgG: human reference serum H00-4-T was used (100 ng/ml CLB, Amsterdam, the Netherlands), for IgG4: human reference serum H00-3-T (100 ng/ml, CLB) and for IgE: human myeloma IgE (200 ng/ml, Calbiochem, La Jolla, CA). After washing 3 times 100 µl/well of 0.1 µg/ml anti-IgA (Tago), 0.1 µg/ml anti-IgM (Tago), 0.2 µg/ml anti-IgG (Tago), 0.1 µg/ml anti-IgG4 (GAM (goat anti mouse)-Kappa and GAM-Lambda, Tago) and 1.0 µg/ml anti-IgE (anti-human Kappa and anti-human Lambda, the Binding Site), conjugated to peroxidase were added overnight at optimally titrated concentrations. After washing 5 times, bound enzyme activity was measured with 1 mg/ml 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) (Sigma, St. Louis, MO) in a 1:1 mixture of 0.1 M $C_6H_8O_7.H_2O$ and 0.2 M $Na_2HPO_4.2H_2O$, containing 0.003% H_2O_2 as substrate. The optical density (OD) was read in an ELISA reader (Titertek Multiskan, MCC) at 414 nm.

Analysis of germline-ε and IL-4 mRNA expression
After spinning down the B cells or the T cells, RNA was isolated from the cells by the RNAzol B method (Cinna/-Biotec Laboratories Inc., Houston, Texas)(34). 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 phenol:chloroform (1:1) was added. 20 µg glycogen (Boehringer

Mannheim, Germany) was added during the phenol:chloroform extraction as a carrier. 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. After incubation at 4°C for at least 2 hrs, the RNA was pelleted by centrifugation and washed with 20 µl 70% ethanol per 10^6 cells. The RNA was resuspended in 10 µl water and the OD260/OD280 ratio was determined by spectrophotometry (Ultrospec III, Pharmacia-LKB).
Copy DNA (cDNA) synthesis (35) was performed starting with 1 µg RNA, after heating for 10 min at 65°C. Random hexamer primers were used to ensure all RNA to be represented equally in the cDNA pool (36). 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), 1 mM spermine HCl (Sigma), 40 units of RNAsin (Promega, Madison, WI), 2.5 OD $(dN)_6$ (Pharmacia), 0.2 µg oligo$(dT)_{15}$ (Boehringer) and 5 units reverse transcriptase (from avian myeloblastosis virus; Promega). The total reaction volume was 20 µl. Incubation was performed for 1 hour at 41°C. Afterwards the cDNA was diluted to 200 µl and stored at -70°C.
For the polymerase chain reaction (PCR), a mixture was prepared containing 50 ng cDNA (in 10 µl) and 10 µl of 10x Taq polymerase buffer (100 mM Tris-HCl containing 500 mM KCl, 15 mM $MgCl_2$ and 1 % gelatin; pH 8.3), 0.03 mM of each dNTP and 1 unit of Taq polymerase (Ampli Taq, Perkin Elmer Cetus, Norwalk CT). 1 µl of the following sense- and anti-sense primers (10 OD/ml) were used:

HPRT	sense:	5'-GTGATGATGAACCAGGTTTATGACCTT-3' (exon2)
	antisense:	5'-CTTGCGACCTTGACCATCTTTGGA-3' (exon 6)
	probe:	5'-GAAGAGCTATTGTAATGACCAGTCA-3' (exon 3-4)
	(product size 454 bp) (37)	
IL-4	sense:	5'-ACTCTGTGCACCGAGTTGACCGTAA-3' (exon2)
	antisense:	5'-TCTCATGATCTGCTTTAGCCTTTCC-3' (exon 4)
	probe:	5'-CAGTTCCACAGGCACAAGCAG-3' (exon 3)
	(product size 300 bp) (38)	
Germline-ε	sense:	5'-AGGCTCCACTGCCCGGCACAGAAAT-3' (germline-ε exon)
	antisense:	5'-ACGGAGGTGGCATTGGAGGGAATGT-3' (C_ε region bp 289-265)
	probe:	5'-AGCTGTCCAGGAACCCGACAGGGAG-3' (germline-ε exon)
	(product size 196 bp) (39)	

We developed the HPRT (hypoxanthine phosphoribosyl transferase) primerset from the genomic structure, by selecting primers for exons 2 and 6. In this way the product including the intron sequences will be too large (3.5 kb) to be amplified by PCR. The IL-4 primerset was kindly donated by Dr. R. de Waal Malefijt (DNAX Research Institute, Palo Alto, CA). It was verified by Southern blot analysis that the three primersets did not amplify genomic DNA (data not shown).
The total reaction volume was 100 µl. As a negative control 50 ng RNA in 10 µl water,

which had not been transcribed into cDNA, was used from 6 patient samples per PCR. The cDNA mixture alone was also used as a negative control.
The PCR protocol started with an adaptation of the hot-start (40), to prevent aspecific primer annealing. After preparing the PCR mixture on ice, the reaction tubes were placed for 3 minutes in the PCR-block (DNA Thermal Cycler, Perkin Elmer Cetus), which was preheated at 94°C. Subsequently, the first 25 PCR cycles were started (30 for IL-4): denaturation at 94°C for 0.5 minute, annealing at 55°C for 0.5 minute and extension at 72°C for 1 minute. After 25 PCR cycles the block was cooled to 4°C, 10 µl of PCR-product was removed from the reaction tubes and the tubes were taken out of the machine. Before re-introducing the reaction tubes, again a hot-start was performed, but now only during 1.5 minutes. After 5 additional PCR cycles, again 10 µl was collected. This was repeated 3 times. A batch of B21 cDNA (a T_h0 clone) was used as a positive control standard in the HPRT and IL-4 PCR reactions. The B21 T cell clone was kindly provided by Dr. H. Yssel (DNAX Research Institute, Palo Alto, CA). A batch of JY cDNA (a B cell line) was used as a positive control standard for the germline-ε PCR reactions. After the PCR amplification, the 5 time-point samples were loaded on a 1.2% agarose gel (Sea Kem LE agarose, FMC Bio Products, Rockland, ME), stained with 75 µg/500 ml ethidium bromide (Boehringer). 1 µl PhiX174 (HaeIII digest, 0.5 µg/µl, New England Biolabs, Beverly, MA) was used as a molecular weight marker.

Southern blotting and hybridization
Subsequently, the gel was put into an acid solution (0.25 M HCl) for 10 min, a denaturing solution (1 M NaCl, 0.5 M NaOH) twice for a period of 15 min, and a neutralizing solution (0.5 M Tris-HCl, 1.5 M NaCl pH 7.0) twice for 20 min. After this a Southern transfer was performed, by which the PCR products were transferred overnight to a Nytran filter (0.45 µm, Schleicher and Schuell, Dassel, Germany). DNA was crosslinked to the filter in a UV crosslinker (120 mJ) (UV Stratalinker 2400, Stratagene, La Jolla, CA).
Probes to the inner region of the HPRT, IL-4 and germline-ε amplification targets mentioned above were end-labelled with ^{32}P-gamma ATP using T_4-polynucleotide kinase (Boehringer) (41). After 1 hour of prehybridisation at 55°C, the end-labelled oligonucleotide was added and hybridization was carried out o/n at 55°C (42).

Analysis of PCR-products
The bands of the PCR products on the gel were visualized with UV light. A life-size photograph was taken and used for scanning. Each row (30 mm x 200 mm) of the photograph was scanned separately with a handscanner (Colorscanner 2^{24}, Highscreen, Würselen, Germany) at a resolution of 100 dots per inch. Computer software was developed to analyze the intensity of the bands. A rectangle was drawn automatically around each band of a row. In each rectangle the intensity was measured on all 20 spots, from which the mean value was calculated by integrating the values. Two rectangles of the same size were drawn automatically directly next to the first row of rectangles. In this way the background was measured on two subsequent positions of the gel. After extrapolation, a correction could be made for a possible drift in the background staining of the gel. The mean background value was subtracted from the mean band value. The resulting value will be subsequently called "scan value".
After hybridization of the filter with germline-ε PCR product, a film was exposed to the

radioactive filter. After development, these films were scanned with the handscanner. A similar program was developed to analyze the intensity of the bands.

Analysis of scan values

Firstly, the mean scan values were calculated at two different cycle numbers, in the linear phase of the PCR reaction. The PCR cycle numbers were 30-35 cycles for HPRT and germline-ε and 35-40 cycles for IL-4. In order to correct for inter-experimental variation a batch of a B21 cDNA mix was used for standardization of HPRT and IL-4. JY (lymphoblastoid B cell line) cDNA was used for standardization of germline-ε expression. The inter-experimental variation was analyzed by adding to each gel containing patient samples one B21 or JY sample from a cDNA batch that was subjected to the same PCR. The mean scan value of the B21 and JY samples of all PCR runs was calculated for HPRT, IL-4 and germline-ε. With this mean scan value a correction factor could be calculated for each individual gel, by dividing the mean scan value of B21 or JY by the scan value of B21 or JY on that gel. Next, the expression of HPRT, IL-4 and germline-ε mRNA expression in patient samples were corrected by this factor for inter-experimental variation, by calculating their expression relative to that in B21 or JY cells analyzed in the same PCR.

Finally, in order to correct for differences in the quality of isolated RNA and efficacy of the cDNA reaction, the end scan values of IL-4 or germline-ε were corrected for the housekeeping gene expression (HPRT) in the same sample.

$$\frac{\text{scan value of IL-4 or } \varepsilon \text{ corrected by B21 or JY}}{\text{scan value of HPRT corrected by B21}} = \text{end scan value of IL-4 or germline-}\varepsilon$$

Flow cytometry

1-5 x 10^5 PBMC were stained (30 min, on ice) with mAb specific for B cells (B4-PE, Coulter Cytometry), T cells (Leu4-FITC, Becton Dickinson), monocytes (My4-FITC, Coulter Cytometry) and NK cells (CD16-PE and CD56-PE, Becton Dickinson). For the analysis of CD4/CD8 ratios PBMC were double stained with Leu4-FITC (CD3), Leu3-PE (CD4, Becton Dickinson) and Leu2-PE (CD8, Becton Dickinson). For the analysis of CD45RA and CD45RO expression PBMC were double stained with Leu3-FITC (Becton Dickinson), Leu2-FITC (Becton Dickinson), 2H4-PE (CD45RA, Coulter Cytometry) and UCHL1-PE (CD45RO, Dako, Glostrup, Denmark). Finally, PBMC were double stained with Leu3-PE, Leu2-PE and CD25-FITC (IL-2 receptor, Becton Dickinson). After staining, cells were washed again for two times and resuspended in 100 µl FACSflow solution (Becton Dickinson). Flow cytometry was performed on a FACScan (Becton Dickinson).

Statistics

Statistical analysis was 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. The n-value depicts the number of patients from which two separate parameters were correlated. In either test, P-values smaller than 0.05 were considered significant.

RESULTS

Characteristics of the patient groups

Table I shows the characteristics of the patient groups. Age ranges and sex were not significantly different among the various groups. Allergen-specific IgE, determined by RAST analysis, was positive in all children included in the allergic groups, while negative in children in the non-allergic groups. Total serum IgE levels were significantly elevated in children with allergic asthma ($p<0.001$), as well as in children with atopic dermatitis ($p<0.001$) in comparison with healthy controls (Table I). In children with non-allergic asthma IgE levels were similar to those in healthy control children. In the study population as a whole a positive correlation was found between total serum IgE levels and the age of the individual children ($r_s=0.31$, $p=0.0053$, $n=82$).

In children with atopic dermatitis a significant positive correlation was observed between total serum IgE levels and the height of the RAST class ($r_s=0.75$, $p<0.001$, $n=22$). This correlation was not due to age related differences. In children with allergic asthma such a correlation between total serum IgE levels and the height of the RAST class was not found ($r_s=0.49$, $p=0.18$, $n=9$).

Immunoglobulin isotype production from cultured B cells

The elevation of IgE could be based on a higher frequency of IgE-secreting B cells or more IgE synthesis per IgE-positive B cell. In order to investigate whether an intrinsic B cell difference was present in allergic children, cognate stimulation conditions were used that were previously shown to induce maximum isotype switching to IgE *in vitro* (43). Purified B cells were cultured with or without the addition of a T_h0 clone (B21) delivering B-cell help independent of the donor B cell haplotype (32), in the presence of a sufficiently high dose IL-4. Under the latter culture conditions the IgE production observed has become independent of aberrances in T-cell derived IL-4 production. As shown in Table II, only B cells from children suffering from allergic asthma produced significantly enhanced levels of IgE relatively to healthy controls (ratio 20.9 *vs* 1.5, $p<0.03$). Stimulated B cells of children with atopic dermatitis and non-allergic asthma showed a non-significantly enhanced IgE production as compared to healthy controls. In B cell cultures from children with allergic asthma and atopic dermatitis the supernatant levels of IgA, IgM, total IgG and IgG4 were comparable to the levels obtained with B cells from healthy controls. In children with non-allergic asthma, the total IgG ratio was lower ($p=0.052$) and the IgM ratio tended to be lower than those in healthy controls, whereas the IgA and IgG4 ratios were comparable. The supernatant levels of all isotypes in B cell cultures without B21 cells were low and comparable between all studied patient groups (data not shown).

Table II. Isotype production by *in vitro* cultured B cells stimulated by T cells and IL-4

	IgA ratio	IgM ratio	IgG ratio	IgG4 ratio	IgE ratio
Healthy controls *n* = 12	1.4 (0.001-5.1)	98.5 (1.8-820)	3.3 (2.2-15.2)	1.0 (0.8-16.3)	1.5 (1-9.3)
Allergic asthma *n* = 5	1.9 (1.0-16.0)	52.4 (10.3-788)	3.3 (0.1-19.1)	1.2 (0.8-14.0)	$20.9^{p<0.03}$ (1.1-298)
Non-allergic asthma *n* = 7	1.4 (1.0-6.3)	14.5 (5.9-244)	1.5 (1.3-16.0)	0.9 (1.3-16.0)	1.1 (0.6-1010)
Atopic dermatitis *n* = 12	1.3 (1.0-9.7)	43 (3.4-476)	3.2 (1.4-14.1)	1.1 (0.9-55.9)	7.6 (1-60.0)

Purified peripheral blood B cells ($5x10^3$/well) were cultured in YM + 10% heat-inactivated FCS (unstimulated) and cocultured with B21 cells ($5x10^4$/well) and IL-4 (200U/ml) (stimulated). After 10-12 days the supernatants were harvested and isotype ELISAs for IgA, IgM, IgG, IgG4 and IgE were performed. The ratios were calculated by dividing the values of isotype production observed in stimulated cultures by the values of unstimulated cultures. Data represent the median values of these ratios and ranges. P-values were calculated using the Mann-Whitney test.

Distribution of cell populations and T cell subsets in PBMC

When analyzing the cellular distribution in the PBMC fraction purified from peripheral blood of healthy children and children with asthma (allergic and non-allergic) or atopic dermatitis, no significant differences were found between the various groups in the percentages of $CD19^+$ B cells, $CD3^+$ T cells and $CD14^+$ monocytes (Table IIIA).. The percentage of $CD16^+/CD56^+$ NK cells was significantly decreased in children with non-allergic asthma ($p = 0.049$) compared to healthy controls.

The CD4/CD8 ratio in children with non-allergic asthma was significantly increased ($p = 0.0026$) as compared to healthy controls. In children with allergic asthma and atopic dermatitis the CD4/CD8 ratio was comparable with that in healthy controls.

Differences in the proportions of $CD25^+$ activated (IL-2 receptor positive) $CD4^+$ and $CD8^+$ T cells in the various patient groups failed to reach significance (Table IIIB).

Furthermore, the percentage of $CD4^+$ T cells expressing CD45RA was significantly increased in children with non-allergic asthma ($p = 0.036$) as compared to healthy controls. The percentage of $CD4^+$ T cells expressing CD45RO was significantly elevated in the children with atopic dermatitis ($p = 0.038$). No differences were found between the various groups in the percentages of $CD8^+$ T cells expressing CD45RA and CD45RO.

Table IIIA. Flow cytometric analysis of the cell distribution in the PBMC fraction (mean ± SEM)

	B cells	T cells	monocytes	NK cells	CD4/CD8 ratio
Healthy controls *n=40*	23.1 ± 1.2	48.7 ± 1.6	10.7 ± 1.0	10.6 ± 1.1	2.0 ± 0.1
Allergic asthma *n=9*	23.8 ± 2.5	47.8 ± 3.0	14.3 ± 2.1	10.1 ± 1.1	1.7 ± 0.2
Non-allergic asthma *n=11*	25.1 ± 2.2	52.3 ± 2.7	11.3 ± 1.9	7.4 ± 1.0$^{p<0.05}$	2.9 ± 0.3$^{p<0.003}$
Atopic dermatitis *n=23*	23.4 ± 1.1	52.1 ± 1.7	9.6 ± 0.9	8.7 ± 0.9	2.0 ± 0.1

Table IIIB. Flow cytometric analysis of T cell subsets in the PBMC fraction (mean ± SEM)

	CD45RA		CD45RO		CD25	
	CD4	CD8	CD4	CD8	CD4	CD8
Healthy controls *n=32*	23.2 ± 1.4	13.1 ± 0.8	5.5 ± 0.7	4.5 ± 1.0	1.9 ± 0.2	0.6 ± 0.1
Allergic asthma *n=9*	21.5 ± 1.9	14.5 ± 1.4	6.3 ± 0.7	3.7 ± 1.8	1.8 ± 0.5	0.7 ± 0.3
Non-allergic asthma *n=10*	31.2 ± 3.3$^{p<0.05}$	12.0 ± 0.8	6.1 ± 1.1	2.5 ± 0.7	2.5 ± 0.5	0.5 ± 0.2
Atopic dermatitis *n=21*	23.9 ± 1.6	12.8 ± 0.6	6.6 ± 0.6$^{p<0.05}$	2.4 ± 0.4	2.4 ± 0.3	0.5 ± 0.2

PBMC were stained with monoclonal antibodies specific for B cells, $CD4^+$ and $CD8^+$ T cells, monocytes and NK cells (Table IIIa) and double stained with monoclonal antibodies specific for CD4, CD8, 45RA, CD45RO and CD25 (Table IIIb), after which flow cytometry was performed. Data represent mean percentages of a cell population from the PBMC fraction ± SEM. P-values were calculated using the Mann-Whitney test.

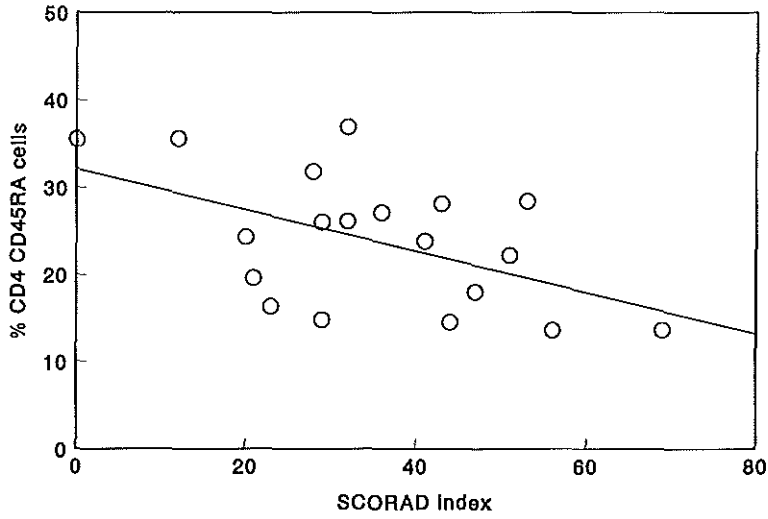

Figure 1. Relationship between the SCORAD index and the percentage of $CD4^+CD45RA^+$ T cells in children with atopic dermatitis ($r=-0.52$, $p=0.02$).

In children with atopic dermatitis a significant inverse correlation was observed between the disease severity (height of the SCORAD index) and the percentage of $CD4^+CD45RA^+$ T cells ($r=-0.52$, $p=0.02$) as shown in Figure 1.

IL-4 mRNA expression in purified T cells

An intrinsic dysregulation of the T cell at the level of IL-4 expression could explain the increased total serum IgE level observed in allergic children. We therefore measured the IL-4 mRNA production *ex vivo* in T cells directly purified from PBMC of allergic and non-allergic children.

IL-4 mRNA expression in T cells was significantly elevated in children with atopic dermatitis as compared to healthy controls ($p=0.038$) (Figure 2A). In children with non-allergic asthma a consistent tendency was observed of elevated IL-4 mRNA expression levels. In children with allergic asthma IL-4 mRNA expression levels were comparable to healthy control levels. Within all groups of patients a positive correlation was found between IL-4 mRNA expression and the age of the individual children ($r_s=0.30$, $p=0.0017$), while the age distribution of patients was comparable in all groups (Table I).

Between the percentages of $CD4^+CD45RA^+$ T cells and the levels of IL-4 mRNA expression in freshly isolated T cells a significant inverse correlation was observed in children with non-allergic asthma ($r_s=-0.82$, $p=0.0038$), as illustrated in Figure 2B.

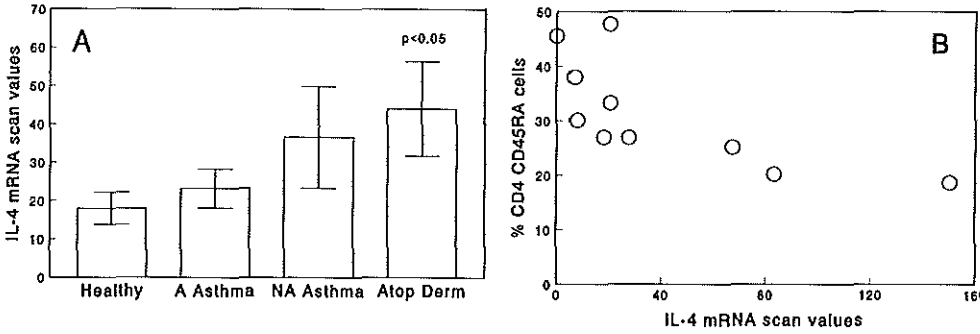

Figure 2. (A) IL-4 mRNA expression in freshly isolated T-cells. 10^6 T cells were purified from PBMC of 17 healthy children, 10 allergic asthmatic children, 12 non-allergic asthmatic children and 23 children with atopic dermatits. After RNA isolation and cDNA synthesis, semi-quantitative PCR analysis for HPRT and IL-4 was performed. After densitometric analysis, scan values were calculated in the linear phase of the reaction. IL-4 scan values were corrected for HPRT and a positive control (a B21 cDNA batch). Data present mean values ± SEM. P-values were calculated using regression analysis.
(B) Correlation between the level of IL-4 mRNA production in freshly isolated T cells and the percentage of $CD4^+CD45RA^+$ T cells in children with non-allergic asthma ($r_s = -0.82$, $p = 0.0038$).

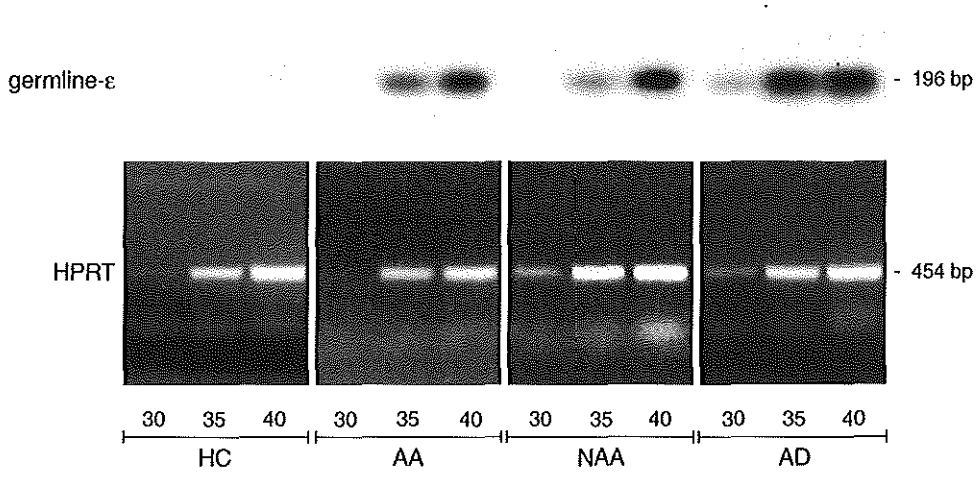

Figure 3. Germline-ε mRNA expression in purified B cells. RNA was isolated from purified B cells of healthy children (HC), children with allergic asthma (AA), children with non-allergic ashtma (NAA) and children with atopic dermatitis (AD). Reverse transcriptase-PCR (RT-PCR) was performed for germline-ε and HPRT at 30, 35 and 40 cycles. HPRT mRNA was analyzed after gel electrophoresis, whereas for germline-ε subsequent Southern blotting and radioactive hybridization was performed.

Germline-ε mRNA expression in B cells

Germline-ε mRNA expression appears shortly after contact of a B cell with T cells in the presence of IL-4. The appearance of germline-ε mRNA is generally

considered to be indicative for isotype switching to productive IgE mRNA expression.

To investigate *ex vivo* germline-ε mRNA expression in B-cells of allergic and healthy children, B cells were purified from the peripheral blood of healthy and allergic children and subsequently RNA was isolated. The results of ε-germline transcription were calculated relative to the expression of the HPRT house keeping gene. Figure 3 shows a representative example of germline-ε mRNA expression in the 4 patient groups. The germline-ε transcript expression tended to be higher in allergic ($p=0.08$) and non-allergic asthmatic patients ($p=0.10$) compared to healthy controls, but this did not reach significance (Figure 4A). In children with allergic asthma (7/7), all B cells had positive germline-ε mRNA expression, whereas in B cells of 4 out of 12 healthy children no germline-ε mRNA expression was detected. The levels of germline-ε mRNA expression in B cells of children with atopic dermatitis were comparable to those in healthy children.

After stimulation of the B cells with a T_h0 clone (B21), germline-ε expression was positive in all individual samples (data not shown), indicating that no intrinsic differences did occur in the capacity of B cells of allergic versus control children to switch to the expression of productive IgE transcripts and subsequent IgE production.

A significant positive correlation was observed ($r_s=0.83$, $p=0.042$) between germline-ε mRNA expression and the percentage of $CD4^+CD45RA^+$ T cells in children with non-allergic asthma, as shown in Figure 4B. Such a correlation was not observed in other groups.

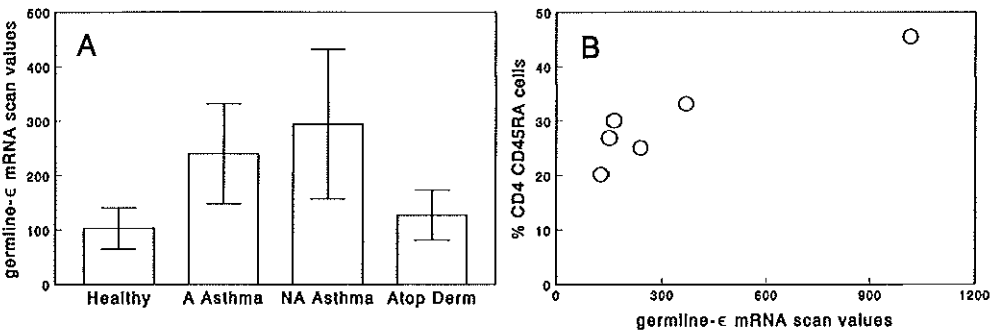

Figure 4. (A) Germline-ε mRNA expression in purified B cells. B cells were purified by magnetic activated cells sorting from PBMC of 12 healthy children, 7 allergic asthmatic children, 7 non-allergic asthmatic children and 14 children with atopic dermatitis. After RNA isolation and cDNA synthesis, semi-quantitative PCR-analysis for germline-ε and HPRT was performed. After densitometric analysis, scan values were calculated in the linear phase of the reaction. Values were corrected for HPRT and a positive control (a JY cDNA batch). Data represent mean values ± SEM.
(B) Correlation between the level of germline-ε mRNA expression and the percentage of $CD4^+CD45RA^+$ T cells in children with non allergic asthma ($r_s=0.83$, $p=0.042$).

DISCUSSION

In this study we showed that in young children with asthma (allergic and non-allergic) and atopic dermatitis total serum IgE levels, IL-4 and germline-ε mRNA expression and the distribution of $CD45RA^+$ and $CD45RO^+$ T cell subsets showed distinct patterns. Combination of these parameters may give information about development of allergic disease, as well as discriminative or predictive variables.
Children with atopic dermatitis had strongly elevated serum IgE levels, IL-4 mRNA expression and percentages of $CD4^+CD45RO^+$ T cells, while germline-ε mRNA expression, CD4/CD8 ratios and the percentage of $CD4^+CD45RA^+$T cells were comparable to healthy controls. Elevated IL-4 mRNA expression in purified T cells, as found in children with atopic dermatitis, is generally considered indicative of a preferentially selected T_h2 phenotype. The rise in the percentage of $CD4^+CD25^+$ T cells is consistent with this, since it reflects T cell activation, possibly by T_h2 cells. IL-4 mRNA expression from purified T cells reflects the *ex vivo* situation and may therefore be dependent on disease severity at the time of blood sampling. The correlation found between IL-4 mRNA expression and age of the children is in line with the data of Tang *et al.* (44), who demonstrated an age-related increase in IL-4 production in PHA-stimulated PBMC cultures. Recently, Borres *et al.* (45) demonstrated that elevated serum IL-4 levels were recorded before onset of clinical symptoms of allergic disease in infancy and of increased serum IgE levels, suggesting that atopic disease is associated with a primary disorder in T-cell function.
The discrepancy between elevated IL-4 mRNA and normal germline-ε mRNA expression, may result from B cells having already switched to IgE *in vivo* and becoming independent from IL-4, as has been shown in an *in vitro* model (46).
Total serum IgE levels differed strongly between allergic and non-allergic children. It has, however, been shown that analysis of total serum IgE levels alone at 6 months has a low predictive value for development of allergic disease (47,48). In children with atopic dermatitis we did show a positive correlation between serum IgE levels and a clinical parameter (heigth of the RAST).
The elevation of $CD4^+CD45RO^+$ T cells, which *in vitro* are capable of giving enhanced help for switching to IgE (25), may contribute to enhanced IgE levels. The raised percentage of $CD4^+CD45RO^+$ T cells in children with atopic dermatitis is consistent with the results from Corrigan *et al.* (49), who demonstrated an increased population of $CD4^+CD45RO^+$ T cells in adult patients with asthma. We could, however, not demonstrate this in children with asthma. This might be due to differences in disease characteristics and age.
In children with atopic dermatitis an inverse relationship was demonstrated between the percentage of $CD4^+CD45RA^+$ T cells and the SCORAD index, indicating that along with disease severity less naive T cells were present. This may be due to either less $CD4^+CD45RA^+$ or more $CD4^+45RO^+$ cells, inducing

increased switching to IgE in B cells by their cytokines.
Such a relation between the percentage of $CD4^+CD45RA^+$ T cells and disease severity could apply for the children with non-allergic asthma. However, this could not be verified since this group was heterogeneous in disease expression and its severity was very difficult to assess in this study.
In the non-allergic asthma group normal levels of serum IgE were found, combined with intermediate germline-ε and IL-4 mRNA expression, an elevated percentage of $CD4^+CD45RA^+$ naive T cells and an increased CD4/CD8 ratio, together with a decreased percentage of NK cells.
Our finding of elevated percentages of $CD4^+CD45RA^+$ T cells in non-allergic asthmatic children differs from a study which found elevated percentages of naive T cells in atopic children (0-17 years) (50). The increase in the CD4/CD8 ratio in non-allergic asthmatics, relative to healthy controls, is most likely due to the elevated percentage of $CD4^+CD45RA^+$ cells. In a study which compared atopic asthmatic children (7-16 years) with age-matched non-asthmatic children, no differences were found in the percentages of $CD4^+$ and $CD8^+$ T lymphocytes (51). However, in both studies a large part of the children were older than the children we studied. Interestingly, in the same study elevated percentages of peripheral blood $CD4^+$ T lymphocytes expressing the activation markers CD25 and HLA-DR, and of $CD8^+$ T lymphocytes expressing CD25 were observed in allergic asthmatic patients as compared with non-asthmatic but atopic controls (50), suggesting that T lymphocyte activation may be physiologically associated with asthma and not simply with the atopic state itself. An increase in $CD25^+$ T cells in children with non-allergic asthma and atopic dermatitis in our study did not reach significance. This might be due to differences in age and disease severity of the patient groups, since we studied children with intermittent to mild asthma, whereas others studied children with moderate to severe chronic asthma (50).
The relative decrease of NK cells in children with non-allergic asthma was interesting since the role of NK cells in allergic diseases has not been elucidated yet. Since NK cells are able to produce IFN-γ (52), this decreased ability to produce IFN-γ could contribute to enhanced IgE synthesis.
The expression of IL-4 mRNA in the children with non-allergic asthma results in germline-ε mRNA expression. Because of the relatively high percentage of naive T cells, which are not capable of providing efficient help for switching to IgE, limited IgE production may take place. Due to ethical reasons, we had to limit our study to the analysis of peripheral blood B and T cells. Since IgE producing plasma cells are normally not present in the peripheral blood, our analysis of isotype production does not fully reflect the *in vivo* situation. After stimulation, however, analysis of isotype production reveals possible intrinsic differences among B cells of the various patient groups. Principally, we used our techniques for comparison between the various patient groups. The isotype analysis after B cell culturing demonstrated that B cells of children with non-allergic asthma

have the potency to switch to IgE under proper stimulatory conditions. A significantly lower IgG ratio (IgG synthesis of stimulated/unstimulated B cells) in this patient group might be associated with a lower, although not significantly, IgM ratio, and reflect an overall lower degree of activation of the B cells in this group.
We hypothesize, taking these findings into account, that increasing age and repeated allergen exposures will result in more $CD4^+CD45RO^+$ T cells, able to provide efficient, T_h2-like B cell help, resulting in higher IgE levels and subsequently in allergic symptoms. In non-allergic asthmatic children IL-4 mRNA expression was negatively correlated with the percentage of $CD4^+CD45RA^+$ T cells, indicating that the more naive T cells are present, the less the IL-4 mRNA expression. Therefore, the non-allergic group may be a pre-stage of allergic group. To establish this we currently perform a longitudinal study of children with high risk on the development of allergic diseases to analyze this. The allergic asthmatic group has aspects of both the non-allergic asthmatic and the atopic dermatitis group. Total serum IgE levels were elevated, whereas IL-4 mRNA expression was normal, while germline-ε mRNA expression was slightly elevated. Islam *et al.* (14) found positive germline-ε mRNA expression in PBMC of 11 out of 12 studied healthy adult donors, whereas the purified B cells in 8 out of 12 healthy children had positive germline-ε mRNA expression. On the other hand, all children with allergic asthma had positive germline-ε mRNA expression. Thus, no significant differences in the number of patients with positive germline-ε mRNA expression were found in this study, however, the level of germline-ε mRNA expression was not analyzed.
In vitro after the appropriate stimulation, IgE production was elevated in children with allergic asthma. This reflects that at the moment of the study, *in vivo* the proper stimuli (e.g., IL-4 and activated T cells) were not sufficiently present in children with allergic asthma. Thus either the precursor frequency of ε-positive B cells *in vivo* may be high in children with allergic asthma, as compared to healthy controls, or their B cells possess an enhanced switching potential to IgE. To discriminate between these possibilities it would be necessary to perform limiting dilution analysis of B cells. The small amount of blood available from these children did not allow such an analysis in the present study. In the literature two studies were reported on the precursor frequency of IgE producing B cells, yielding conflicting results. One study found no differences between atopic and non-atopic subjects (53), whereas the other did show a higher precursor frequency in atopic subjects (54). A close correlation was demonstrated between the IgE producing B cell frequency and levels of circulating IgE (54,55). This implies that elevated serum IgE levels result, in a large part, from clonal expansion of B cells able to produce IgE, rather than from increased amounts of IgE actually made and released per individual IgE-committed B cell. In our study IgG4 production was not elevated in B cell cultures of allergic children compared to healthy controls. As reviewed by

Aalberse *et al.* (56) changes in IgE and IgG4 serum levels are normally found to be closely linked, presumably because the production of both isotypes is IL-4 dependent and thus requires the stimulation of T_h2 cells. However, a lack of correlation between allergen-specific IgE and IgG4 levels was also recently demonstrated by Tame *et al.* (57).
In conclusion we postulate that the ratio between the percentages of naive and activated/memory T cells plays an important role in the quantitative differences in the parameters analyzed in the patient groups studied here. In atopic dermatitis more activated/memory T cells are present, B cells have already switched to IgE and have become relatively independent of IL-4, whereas in non-allergic asthma naive T cells dominate and little switching to IgE takes place. Based on these criteria children with allergic asthma have characteristics both of children with non-allergic asthma and of children with atopic dermatitis, with normal levels of naive as well as activated/memory T cells. Larger patient cohorts need to be studied longitudinally to investigate whether the combination of these parameters has predictive value.

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CHAPTER 3.2

T CELL SUBSETS AND CYTOKINES IN ALLERGIC AND NON-ALLERGIC CHILDREN. I. ANALYSIS OF IL-4, IFN-γ AND IL-13 mRNA EXPRESSION AND PROTEIN PRODUCTION

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ABSTRACT

Interleukin-4 (IL-4) and IL-13 are key cytokines inducing switching to immunoglobulin E (IgE), whereas interferon-γ (IFN-γ) acts inhibitory on this process. We analyzed whether differences existed in IL-4, IFN-γ and IL-13 mRNA expression and protein production between T cells of children with allergic and non-allergic asthma, atopic dermatitis and healthy control children. IL-4 mRNA expression was increased in stimulated T cells of children with allergic asthma and atopic dermatitis, but not in those with non-allergic asthma as compared with healthy controls. Thus the increase in IL-4 expression can be considered as an underlying mechanism of the allergic disease process and not so much of the asthmatic state of the children. In unstimulated T cells of children with atopic dermatitis increased IFN-γ mRNA expression with a reduced IFN-γ protein production was found, indicating a post-translational defect in IFN-γ. Differences in IL-13 expression between the groups were not significant, but IL-13 was significantly correlated with the height of the radio-allergo-sorbent test (RAST) class and with the severity scoring of atopic dermatitis (SCORAD) index. This indicates the clinical relevance of IL-13 for the degree of allergen-specific sensitization and severity of atopic dermatitis. In conclusion, the imbalance in IL-4 and IFN-γ secretion in patients with atopic dermatitis may reflect general T cell activation in the presence of an intrinsic defect of IFN-γ secretion.

INTRODUCTION

Cytokines play a crucial role in the regulation of immunoglobin E (IgE) synthesis in normal conditions and in diseased states like allergy. Interleukin-4 (IL-4) is a

key cytokine in the production of IgE by naive B cells (1). This is based on its ability to induce expression of ε-germline transcripts (2). Another T-cell derived cytokine, IL-13, can also induce germline-ε expression in B cells, but it is two- to five-fold less potent than IL-4 in inducing IgE synthesis (3). For this activation by IL-4, physical contact between activated $CD4^+$ T cells and B cells is required, called cognate interaction (4). The activating effect of IL-4 is promoted by IL-5 and IL-6, but inhibited by interferons (IFN)-α and -γ, transforming growth factor-β and prostaglandin E_2 (PGE_2) (4-7).
Based on their cytokine production profile, $CD4^+$ T helper (T_h) cells can be divided into at least a T helper 1 (T_h1)-like (producing a.o. IL-2 and IFN-γ) and a T helper 2 (T_h2)-like subset (producing a.o. IL-4, IL-5 and IL-10) (8-9). Several factors, most of them cytokines themselves, may specifically promote T_h1 or T_h2 outgrowth. IFN-γ and IL-12 strongly promote the generation of T_h1-like cells, whereas IL-4, IL-10 and PGE_2 promote the generation of T_h2-like cells (10-12).
In neonatal T cells, IFN-γ production is strongly decreased to as low as 10% of the adult IFN-γ production, while the production of IL-2 and the expression of IL-2 receptor are comparable to those in adults (13). This reduced capacity to produce IFN-γ coincides with the period that the baby is first exposed to environmental antigens in the gastro-intestinal and respiratory tract. This may be important in the development of specific allergic disease (14).
A large body of evidence suggests that allergen-specific T_h2-like cells are expanded in atopic subjects, particularly at the level of target tissues, like skin and lungs (15). Already at birth, allergen-specific T_h2 cells can be expanded, suggesting that aero-allergen sensitization can occur during fetal life (intrauterine sensitization) (16). Therefore, it might be expected that in young children developing allergic diseases a T_h2-like phenotype is already present. There is some evidence that atopic diseases are associated with a primary disorder in T-cell function (8,9,17).
In order to study a preferential T_h2-like outgrowth in children with allergic diseases and to investigate whether a primary T cell disorder can be demonstrated, we analyzed whether differences existed in IL-4, IFN-γ and IL-13 mRNA expression and protein production between allergic (allergic asthma and atopic dermatitis) and non-allergic children (healthy and with non-allergic asthma) in a cross-sectional study. The mRNA expression was analyzed in freshly isolated peripheral blood mononuclear cells (PBMC) and purified T cells, the latter in order to investigate the *ex vivo* situation. After polyclonal stimulation of PBMC and T cells, in order to abolish influences of other cell populations, mRNA expression as well as protein production were analyzed. Furthermore, the relationship between the presence of these cytokines and clinical parameters was investigated.

MATERIALS AND METHODS

Patient groups

Children with allergic or non-allergic asthma, atopic dermatitis and healthy controls were included in the study. All subjects were between 0 and 4 years. The age distribution in the four groups was comparable.

Children with asthma were selected using the following criteria of the American Thoracic Society: coughing and/or shortness of breath, at least 3 times a year, for a period of at least 14 days (18) and a positive family history of asthma and/or atopy, as well as absence of treatment with (inhalation-) corticosteroid. The exclusion of patients who used steroids was based on the demonstration of modulatory effects of *in vitro* steroid treatment on IL-4 expression (19,20) and of *in vivo* steroid treatment on IL-4, IL-5 and IFN-γ cytokine gene expression (21). The group of children with allergic asthma was further defined by the presence of specific IgE (>0.35 kU/l) in their serum for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The children with non-allergic asthma were selected according to the same criteria as the allergic asthmatic children, but at the time of the study they did not have measurable specific IgE to inhalation and/or food allergens. The children with atopic dermatitis were included according to the diagnostic criteria of Hanifin and Rajka (22). The main diagnostic criteria were pruritis, typical skin lesions and localization, a chronical recurrent course and the presence of other atopic diseases or atopic relatives. Children with atopic dermatitis had specific IgE (>0.35 kU/l) for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The severity scoring of atopic dermatitis (SCORAD) index (23) was used as a measure for disease severity. Healthy control children were included after completing a questionnaire by their parents, indicating absence of allergic symptoms and of a family history of atopy. Healthy children had no specific IgE (<0.35 kU/l) for inhalation and/or food allergens in their serum as tested by (Phadiatop®) and food mix and no allergic symptoms.

The Medical Ethical Committee of the Erasmus University Medical School and University Hospital Rotterdam approved this study. Informed consent was given by all the parents of the different patients and healthy control subjects included in the study.

Analysis of total serum IgE and specific IgE levels

Determination of total serum IgE levels was performed using the Pharmacia CAP radio-allergo-sorbent test (RAST) (Kabi Pharmacia, Uppsala, Sweden). Analysis of specific IgE antibodies directed towards the most common inhalation allergens (house dust mite, grass and birch pollen, cat, dog and moulds) was performed using the Pharmacia CAP system Phadiatop® RAST.

Analysis of specific IgE antibodies directed towards the common food allergens (chicken egg white, milk, fish, wheat, peanut, soy) was performed using the Pharmacia CAP RAST. All tests were performed according to the manufacturers instructions. The results of the specific IgE determinations were classified in the following RAST classes according to the amount of specific IgE expressed in kU/l: class 1: 0.35-0.7; class 2: 0.7-3.5; class 3: 3.5-17.5; class 4: 17.5-50; class 5: 50-100; class 6: >100.

Isolation of PBMC and T cells

PBMC were isolated from a maximum of 4 ml heparinized blood of children as described

previously (24). T cells were purified from PBMC by negative selection using Dynabeads (IgG M450, Dynal, Oslo, Norway) in order to prevent stimulation of the cells (24). T cells ($1x10^6$ cells/well) were cultured for 16-18h in Yssel's medium (25) containing 1% human serum with and without the addition of 4-bromo-calcium-ionophore (A23187, final concentration 500 ng/ml, Sigma, St. Louis, MO) and TPA (12-O-tetradecanoylphorbol-13-acetate), final concentration 1 ng/ml, Sigma) at 37°C, 5% CO_2. PBMC ($1x10^6$ cells/well) were cultured under the same conditions. After culturing, the cells were pelletted, resuspended in 200 µl/$1x10^6$ cells RNAzol B (Cinna-Biotecx Laboratories Inc., Houston, TX) and stored at -20°C. The cell supernatants were collected and stored at -70°C.
For flow cytometry 1-5 x 10^5 PBMC were stained (30 min, on ice) with monoclonal antibodies specific for T cells (Leu4-FITC, Becton Dickinson, San Jose, CA) and natural killer (NK) cells (CD16-PE and CD56-PE, Becton Dickinson). For the analysis of CD45RA and CD45RO expression PBMC were double stained with Leu3-FITC (Becton Dickinson), 2H4-PE (CD45RA, Coulter Cytometry, Hialeah, FL) and UCHL1-PE (CD45RO, Dako, Glostrup, Denmark). After staining, the cells were washed again for two times and resuspended in 100 µl FACSflow solution (Becton Dickinson). Flow cytometry was performed on a fluorescence activated cell scanner (FACScan) (Becton Dickinson). Numbers of eosinophils were counted in peripheral blood by leucocyte differentiation.

Analysis of cytokine production
IL-4, IFN-γ and IL-13 in the cell culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits. IL-4 with an IL-4 enzyme-amplified sensitivity immuno assay (EASIA) kit (Medgenix Diagnostics SA, Fleurus, Belgium). The detection level was 2 pg/ml. IFN-γ with an IFN-γ EASIA kit (Medgenix Diagnostics SA). The detection level was 1 pg/ml. IL-13 with a Pelikan Compact™ human IL-13 kit (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). The detection level was 1.5 pg/ml. All samples were tested in duplicate.

Analysis of cytokine mRNA epxression
RNA was isolated from the PBMC and T cells by the RNAzol B method (26) as described previously (24). Subsequently, a copy DNA (cDNA) synthesis reaction was performed, followed by a polymerase chain reaction (PCR) (24), using the following primersets:

HPRT sense: 5'-GTGATGATGAACCAGGTTTATGACCTT-3' (exon2)
antisense: 5'-CTTGCGACCTTGACCATCTTTGGA-3' (exon 6)
probe: 5'-GAAGAGCTATTGTAATGACCAGTCA-3' (exon 3-4)
(product size 454 bp) (27)

IL-4 sense: 5'-ACTCTGTGCACCGAGTTGACCGTAA-3' (exon2)
antisense: 5'-TCTCATGATCTGCTTTAGCCTTTCC-3' (exon 4)
probe: 5'-CAGTTCCACAGGCACAAGCAG-3' (exon 3)
(product size 300 bp) (28)

IFN-γ sense: 5'-TTTAATGCAGGTCATTCAGATG-3' (exon 1-2)
antisense: 5'-CAGGGATGCTTCTTCGACCTCGAAAC-3' (exon 4)
probe: 5'-TGACTAATTATTCGGTAACTGACTTGAATG-3' (exon 3-4)
(product size 388 bp) (28)

IL-13 sense: 5'-CCCAGAACCAGAAGGCTCCGC-3' (exon 1-2)
antisense: 5'-GCTGGAAAACTGCCCAGCTGAG-3' (exon 3-4)
probe: 5'-GCTGGCATGTACTGTGCAGCC-3' (exon 2-3)
(product size 187 bp) (29)

HPRT (hypoxanthine phosphoribasyl-transferase, house-keeping gene), IL-4 and IFN-γ mRNA expression were analyzed after agarose gel electrophoresis and subsequent densitometric analysis of a photograph of the gel (24). HPRT and IFN-γ were calculated by taking the average scan value at 30 and 35 cycles, whereas for IL-4 35 and 40 cycles were used. IL-13 mRNA expression was analyzed on film after gel electrophoresis and Southern blotting and subsequent hybridization with a radioactive probe (24). The elaborate calculation method was described previously (30).

Statistics

Statistical analysis was performed with STATA™ (Computing Resource Center, Los Angeles, CA). Parameters were tested using the Mann-Whitney test. Correlation coefficients (r_s) were calculated by the Spearman sign-rank correlation. The n-value depicts the number of patients from which two separate parameters were correlated. P-values smaller than 0.05 were considered significant.

RESULTS

Characteristics of the patient groups

Table I shows the characteristics of the patient groups studied. Total serum IgE levels were significantly elevated in children with allergic asthma ($p=0.0027$) and atopic dermatitis ($p<0.001$) as compared with healthy control children, whereas children with non-allergic asthma had IgE levels comparable with healthy control levels. In children with non-allergic asthma percentages of NK cells ($p=0.049$) were significantly decreased and percentages of $CD4^+CD45RA^+$ naive T cells ($p=0.036$) were significantly increased as compared with healthy controls. $CD4^+CD45RO^+$ activated/memory T cells ($p=0.038$) were significantly increased in children with atopic dermatitis as compared with healthy controls.

IL-4 mRNA expression and protein production in allergic children

The IL-4 mRNA expression *ex vivo* was measured directly after isolation of PBMC and in the purified T cell fraction. IL-4 mRNA expression and protein production were analyzed after overnight culture of polyclonally activated PBMC and T cells, to establish the maximum cytokine mRNA and protein production capacity of these cells.

Table I. Characteristics of the patient groups.

	age (months)	total IgE (kU/l)	RAST classes	T cells (%)	NK cells (%)	CD4 CD45RA (%)	CD4 CD45RO (%)	eosinophils
Healthy controls *n=42*	28 (5-59)	16 (0-952)	0	49 (29-70)	9 (4-44)	23 (7-36)	5 (2-23)	0,12 (0.075-0.386) *n=5*
Allergic asthma *n=10*	43 (12-54)	246$^{p<0.005}$ (45-12134)	3 (3-5)	50 (34-57)	10 (6-17)	21 (14-33)	6 (4-11)	0.578 (0.042-1.048) *n=9*
Non-allergic asthma *n=12*	21 (7-56)	13 (2-168)	0	53 (34-67)	7$^{p<0.05}$ (4-16)	29$^{p<0.05}$ (19-48)	5 (3-15)	0.368 (0.118-1.33)$^{p<0.05}$ *n=11*
Atopic dermatitis *n=25*	27 (7-50)	196$^{p<0.001}$ (6-4000)	3 (2-6)	52 (39-68)	9 (2-19)	24 (14-37)	6$^{p<0.05}$ (2-13)	0.465 (0.12-1.37)$^{p<0.05}$ *n=22*

Ages are expressed in months. Total IgE levels were determined by IgE CAP RAST and are expressed as kU/l. RAST data represent median values of the RAST-classes as determined by Phadiatop® and/or food mix CAP RAST with ranges.
PBMC were stained with monoclonal antibodies specific for T cells and NK-cells and double stained with monoclonal antibodies specific for CD4, CD45RA and CD45RO. Flow cytometric results are expressed as median percentages (ranges). Numbers of eosinophils were counted in peripheral blood by leucocyte differentiation. Data represent median numbers of eosinophils x 10^9/l and ranges. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

Table IIA. RT-PCR analysis of IL-4 mRNA expression

	PBMC	T purified	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	26.7 (1.0-97.3) *n=35*	11.6 (1.0-50.3) *n=17*	16.2 (1.0-111.8) *n=23*	9.5 (1.0-58.7) *n=22*	0.4 (0.01-12.9) *n=20*
Allergic asthma	23.4 (1.0-78.6) *n=9*	24.2 (1.0-53.2) *n=10*	6.0 (1.0-62.0) *n=8*	28.9 (1.0-85.6) *n=7*	1 (0.6-31.5)$^{p<0.05}$ *n=7*
Non-allergic asthma	30.1 (12.1-61.7) *n=9*	20.4 (1.0-150.3) *n=12*	19.9 (1.0-60.7) *n=8*	10.5 (1.0-53.0) *n=9*	0.7 (0.2-17.0) *n=8*
Atopic dermatitis	31.5 (1.0-122.2) *n=22*	18.7 (1.0-222.1) *n=23*	23.4 (1.0-310.5) *n=22*	34.8 (1.0-108.4) *n=23*	1.1 (0.1-58.1)$^{p<0.01}$ *n=22*

From PBMC, purified T cells and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children, children with allergic and non-allergic asthma and children with atopic dermatitis, RNA was isolated and reverse transcriptase-PCR (RT-PCR) was performed for IL-4. Data represent median scan values and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

Table IIB. Analysis of IL-4 protein production by ELISA

	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	1.0 (1.0-11.0) *n=28*	1.0 (1.0-14.0) *n=28*	1.0 (0.3-14.0) *n=28*
Allergic asthma	1.0 (1.0-8.2) *n=8*	2.5 (1.0-133.0) *n=8*	1.0 (0.5-133.0) *n=8*
Non-allergic asthma	1.0 (1.0-14.0) *n=9*	1.0 (1.0-7.1) *n=10*	1.0 (0.07-7.1) *n=9*
Atopic dermatitis	1.0 (1.0-4.9) *n=25*	2.1 (1.0-11.0)$^{p<0.05}$ *n=25*	1.9 (0.3-11.0)$^{p<0.05}$ *n=25*

T cells of healthy children, children with allergic and non-allergic asthma and children with atopic dermatitis were cultured and IL-4 protein production was measured by ELISA. Data represent median IL-4 production in pg/ml and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

As shown in Table IIA IL-4 mRNA expression by the PBMC fraction was comparable in all four groups of patients. Although no significant differences were found in IL-4 mRNA expression by purified T cells between the groups, the expression was consistently increased in T cells of children with non-allergic asthma and atopic dermatitis as compared with healthy controls. IL-4 mRNA expression by the unstimulated T cells was again comparable between the groups. After stimulation of the T cells, the IL-4 mRNA ratio (stimulated/unstimulated IL-4 mRNA expression) was significantly increased in children with allergic asthma ($p=0.036$) and atopic dermatitis ($p=0.0054$).
The IL-4 protein production by the unstimulated T cells was comparable between the four groups (Table IIB). The IL-4 protein ratio (stimulated/unstimulated IL-4 protein production) was, in contrast to the IL-4 mRNA expression, only elevated in children with atopic dermatitis ($p=0.016$) as compared with healthy controls, but not in children with allergic asthma.
In the PBMC fraction of all four patient groups, IL-4 mRNA and protein production could be detected, but none of the differences in IL-4 mRNA expression and protein production as observed in the purified T cell cultures could be detected in these PBMC cultures.

IFN-γ mRNA expression and protein production
After stimulation of T cells, IFN-γ mRNA expression was significantly elevated in stimulated T cells of children with atopic dermatitis ($p=0.029$), but was comparable between children with asthma and healthy controls (Table IIIA). In different cell fractions (purified PBMC, purified T cells and unstimulated cultured T cells) of allergic asthmatic children a non-significant increase was observed in IFN-γ mRNA expression as compared to the expression level in healthy

Table IIIA. RT-PCR analysis of IFN-γ mRNA expression

	PBMC	T purified	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	17.0 (1.0-96.0) *n=33*	10.7 (1.0-45.1) *n=17*	2.0 (1.0-167.8) *n=24*	63.0 (5.7-161.0) *n=23*	19.1 (0.4-161.0) *n=22*
Allergic asthma	32.4 (1.0-77.9) *n=10*	20.1 (1.0-102.4) *n=10*	26.0 (1.0-58.0) *n=8*	76.8 (24.4-137.4) *n=7*	3.3 (0.8-51.1) *n=7*
Non-allergic asthma	25.4 (1.0-40.8) *n=9*	14.3 (1.0-179.8) *n=12*	9.1 (0-27.4) *n=8*	47.6 (7.5-188.8) *n=9*	6.4 (0.8-48.4) *n=8*
Atopic dermatitis	13.6 (1.0-50.7) *n=21*	6.1 (1.0-47.0) *n=23*	6.9 (1.0-63.4) *n=22*	85.2 (39.8-304.9)$^{p<0.05}$ *n=22*	14.2 (1.0-175.4) *n=22*

From PBMC, purified T cells and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children, children with allergic and non-allergic asthma and children with atopic dermatitis, RNA was isolated and RT-PCR was performed for IFN-γ. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. Data represent median scan values and ranges. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

Table IIIB. Analysis of IFN-γ protein production by ELISA

	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	4.6 (1.0-42.2) *n=28*	82.5 (1.0-9108) *n=28*	19.6 (0.02-690) *n=28*
Allergic asthma	1.0 (1.0-10.6) *n=7*	213.8 (1.0-5676) *n=8*	43.7 (1.0-5676) *n=7*
Non-allergic asthma	1.0 (1.0-5.7)$^{p<0.05}$ *n=9*	21.7 (1.0-91.1)$^{p<0.05}$ *n=10*	27.7 (1.0-91.1) *n=9*
Atopic dermatitis	1.0 (1.0-6.6)$^{p<0.05}$ *n=25*	117.5 (1.0-2376) *n=25*	81.8 (1.0-1716)$^{p<0.05}$ *n=25*

T cells of healthy children, children with allergic and non-allergic asthma and children with atopic dermatitis were cultured and IFN-γ protein production was measured by ELISA. Data represent median IFN-γ production in pg/ml and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

controls.
IFN-γ protein production was significantly reduced in unstimulated T cell cultures of children with non-allergic asthma ($p=0.012$) and atopic dermatitis ($p=0.015$) as compared with healthy controls (Table IIIB). After polyclonal stimulation of T cells only in the group of children with non-allergic asthma a significant reduction in IFN-γ protein production ($p=0.028$) was detectable as compared with healthy controls. The IFN-γ protein ratio was only significantly elevated in children with atopic dermatitis ($p=0.017$) as compared with healthy control children.
The stimulated PBMC cultures did not show any significant differences with regard to the IFN-γ mRNA expression and protein production (data not shown).

IL-13 mRNA expression and protein production

Although some consistent differences were observed in IL-13 expression between the various groups, overall no significant differences between any of the patient groups and the healthy controls in IL-13 mRNA expression or protein production were observed (Table IVA and IVB).

Relationships between the analyzed parameters

In order to exclude age-related influences on the correlations between the parameters, we tested for age-related effects. These were all negative (data not shown). A positive correlation was found between IL-4 and IFN-γ expression both at the mRNA and the protein level in some, but not in all patient groups.

Table IVA. RT-PCR analysis of IL-13 mRNA expression

	PBMC	T purified	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	1.0 (1.0-141.2) *n=30*	1.0 (1.0-176.7) *n=17*	2.1 (1.0-429.0) *n=25*	106.1 (1.0-346.0) *n=23*	14.8 (0.1-159.7) *n=22*
Allergic asthma	1.0 (1.0-12.3) *n=10*	1.0 (1.0-180.1) *n=9*	9.7 (1.0-32.8) *n=8*	128.6 (1.0-381.6) *n=6*	5.4 (0.6-149.8) *n=6*
Non-allergic asthma	1.0 (1.0-8.4) *n=8*	1.0 (1.0-59.5) *n=11*	12.9 (1.0-61.1) *n=8*	219.8 (45.0-259.8) *n=9*	15.4 (1.4-235.8) *n=8*
Atopic dermatitis	2.4 (1.0-206.0) *n=21*	1.0 (1.0-252.7) *n=23*	1.0 (1.0-445.9) *n=22*	67.1 (1.0-594.2) *n=23*	16.2 (0.01-594.2) *n=22*

From PBMC, purified T cells and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children, children with allergic and non-allergic asthma and children with atopic dermatitis, RNA was isolated, and RT-PCR for IL-13 and subsequent radio-active hybridization was performed. Data represent median scan values and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

Table IVB. Analysis of IL-13 protein production by ELISA

	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	1.0 (1.0-4.0) *n = 16*	12.2 (2.7-31.0) *n = 16*	11.1 (1.2-28.0) *n = 16*
Allergic asthma	1.0 (1.0-5.3) *n = 7*	5.3 (2.2-62.0) *n = 7*	3.9 (0.6-15.0) *n = 7*
Non-alergic asthma	1.0 (1.0-6.2) *n = 8*	7.6 (1.0-29.7) *n = 7*	3.5 (1.0-29.7) *n = 17*
Atopic dermatitis	1.0 (1.0-8.9) *n = 18*	20.7 (4.0-66.0) *n = 18*	7.7 (1.5-66.0) *n = 18*

T cells of healthy children, children with allergic and non-allergic asthma and children with atopic dermatitis were cultured and IL-13 protein production was measured by ELISA. Data represent median IL-13 production in pg/ml and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

Figure 1 shows a representative example of the correlation between IL-4 and IFN-γ mRNA expression by stimulated PBMC of healthy children ($r_s = 1.0$, $p < 0.001$, $n = 5$). Such a positive correlation between IL-4 and IFN-γ mRNA expression was also observed in purified T cells of children with non-allergic asthma ($r_s = 0.77$, $p = 0.0035$, $n = 12$). The comparison of IL-4 and IFN-γ protein production of stimulated PBMC resulted in a positive correlation in healthy children ($r_s = 0.89$, $p = 0.041$, $n = 5$) and in children with atopic dermatitis ($r_s = 0.80$, $p = 0.003$, $n = 7$). Several relationships between the percentages of $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cells and expression of cytokines were observed. IFN-γ mRNA expression by purified T cells of children with allergic asthma was positively correlated with the percentage of $CD4^+CD45RA^+$ T cells (Figure 2, $r_s = 0.70$, $p = 0.036$, $n = 9$), whereas IFN-γ mRNA expression by isolated PBMC of children with atopic dermatitis was inversely correlated to the percentage of $CD4^+CD45RO^+$ T cells (Figure 3, $r_s = -0.53$, $p = 0.025$, $n = 18$). The latter correlation was also observed when comparing IFN-γ protein production by unstimulated PBMC of children with atopic dermatitis with the percentage of $CD4^+CD45RO^+$ T cells ($r_s = -0.89$, $p = 0.041$, $n = 5$). A positive correlation was found when comparing IL-13 mRNA expression by stimulated PBMC of children with atopic dermatitis with the percentage of $CD4^+CD45RO^+$ T cells ($r_s = 1.0$, $p < 0.001$, $n = 5$).

In children with allergic asthma a significant positive correlation was observed between the IL-4 mRNA expression of stimulated T cells and the number of eosinophils in the peripheral blood (Table I) ($r_s = 0.86$, $p = 0.007$, $n = 8$). In healthy children a positive correlation existed between IFN-γ mRNA expression by PBMC and the percentage of natural killer cells in the PBMC fraction (Table I) ($r_s = 0.47$, $p = 0.0074$, $n = 32$).

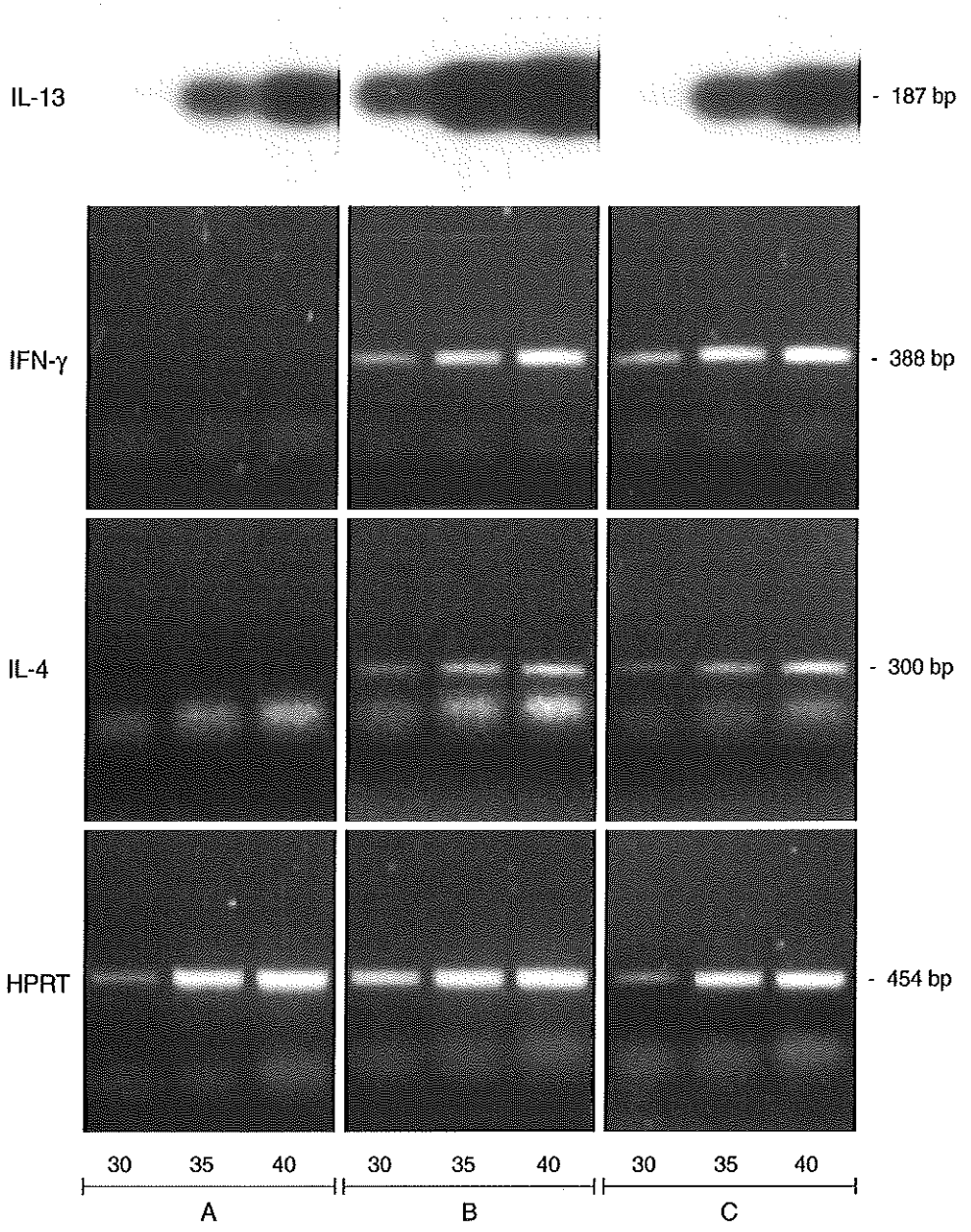

Figure 1. Illustration of the positive correlation between IL-4 and IFN-γ mRNA expression. After stimulation of 10^6 PBMC of healthy control children (A, B and C) with TPA and Ca-Ionophore for 16 hrs, RNA was isolated and RT-PCR was performed for HPRT, IL-4, IFN-γ and IL-13 at 30, 35 and 40 cycles. HPRT, IL-4 and IFN-γ were analyzed after gel electrophoresis, whereas for IL-13 subsequent Southern blotting and radioactive hybridization was performed.

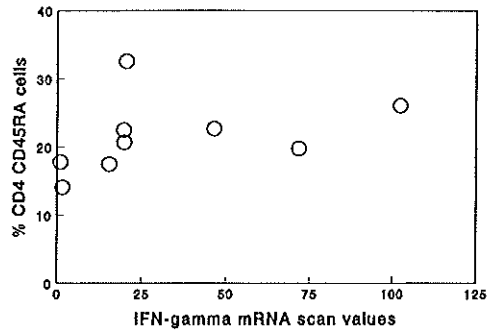

Figure 2. Positive correlation between IFN-γ mRNA expression by purified T cells and the percentage of $CD4^+CD45RA^+$ T cells in children with allergic asthma. IFN-γ mRNA expression was analyzed in purified T cells of children with non-allergic asthma by RT-PCR. Percentages of $CD4^+CD45RA^+$ T cells were determined by flow-cytometry after double-staining the cells with Leu3-FITC and 2H4-PE. Correlation between the two parameters was calculated by the Spearman sign-rank correlation ($r_s = 0.70$, $p = 0.036$).

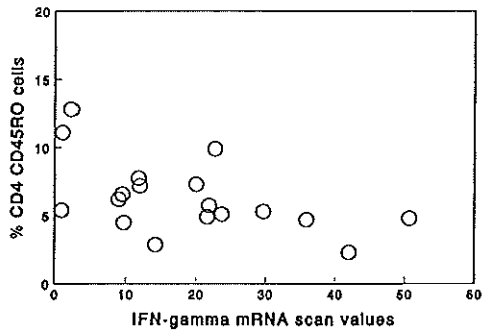

Figure 3. Negative correlation between IFN-γ mRNA expression by isolated PBMC and the percentage of $CD4^+CD45RO^+$ T cells in children with atopic dermatitis. IFN-γ mRNA expression was analyzed in isolated PBMC of children with atopic dermatitis by RT-PCR. Percentages of $CD4^+CD45RO^+$ T cells were determined by flow-cytometry after double-staining the cells with Leu3-FITC and UCHL1-PE. Correlation between the two parameters was calculated by the Spearman sign-rank correlation ($r_s = -0.53$, $p = 0.025$).

Although no significant differences were detected in IL-13 mRNA expression and protein production, we did observe significant correlations between IL-13 expression and clinical parameters. In children with atopic dermatitis a positive correlation was found between IL-13 protein production by unstimulated PBMC and the SCORAD index ($r_s = 0.98$, $p = 0.0048$, $n = 5$). IL-13 protein production by unstimulated T cells was positively correlated with the height of the RAST class in children with allergic asthma ($r_s = 0.94$, $p = 0.0057$, $n = 6$).

DISCUSSION

This study provides further evidence that development of allergy occurs against a background of maturational $CD4^+$ T cell dysfunction and supports the notion of a T cell dysfunction in both T_h1 and T_h2 cell subsets in children with allergic disease.

Since we studied cytokine expression in young children, only limited volumes of peripheral blood were available, thereby necessitating direct analysis in freshly isolated and cultured PBMC. No significant differences were found in IL-4, IFN-γ or IL-13 mRNA expression and protein production in these fractions. However, as will be discussed later, we did detect statistical significant relationships between cytokine expression in PBMC and several clinical features in these patients.

This study differs from many others in that it studied cytokine mRNA expression

and protein production in freshly isolated and short-term cultured T cells. In this way the intrinsic capacity of T cells to produce cytokines could be studied precisely, since possible influences, like cytokine production by other cell populations, were prevented (24). A positive correlation was found between IFN-γ mRNA expression and the percentage of natural killer cells in the PBMC fraction of healthy children. Similar results were found by others demonstrating that non-T cells have a significant effect on the IFN-γ and IL-4 levels in supernatants of polyclonally activated PBMC (31). Such results support the necessity to study both isolated T cells and PBMC for determining possible differences among patients or healthy individuals in the ability of T cells to secrete cytokines. Analysis of cytokine mRNA expression by the purified T cell fraction rather than PBMC did show a more profound differential expression between the various patient groups. These differences, however, did not reach significance. These differences would, most likely, reach significance when larger groups of patients would be studied.

Interestingly, most of the significant differences in cytokine mRNA expression and protein production were found after culturing the purified T cell fraction. Polyclonal stimulation of T cells thus augmented the differences already present between the purified T cell fractions. It should be noted that polyclonal stimulation in some of the patients did not result in stimulation of mRNA expression or protein production, in particular of IL-4. This could be due to a concomitant increase in the expression level of the HPRT house-keeping gene as has been found by our own group (unpublished data). Alternatively, the differential kinetics of expression of the cytokines studied (16 hrs) could be suboptimal in the case of IL-4 (31). Also a high base-line expression could result in difficulties to further stimulate the cells (31).

IL-4 mRNA expression was found to be significantly elevated after stimulation of T cells of children with allergic asthma and atopic dermatitis as compared with healthy controls. Significantly increased IL-4 protein production could only be determined in children with atopic dermatitis, whereas the increase in children with allergic asthma did not reach significance, probably due to a lower sensitivity of protein analysis versus PCR. Since IL-4 was elevated in allergic asthmatic children as well as in children with atopic dermatitis but not in children with non-allergic asthma, this may be considered to be related to the allergic disease process, more than to the asthmatic state of the children. Similar findings were described by others in cells obtained by different procedures (bronchoalveolar lavage cells and bronchial biopsies) of asthmatic patients (32-34) and in PBMC of children with atopic dermatitis (35). Recently, an association between serum IL-4 levels with development of allergic disease in infancy was demonstrated. Elevated levels were even recorded before onset of clinical symptoms (17), suggesting that atopic disease is associated with a primary disorder in T cell function.

An increased IFN-γ protein ratio in stimulated over unstimulated T cells, as

observed in children with atopic dermatitis, was to a large extent caused by the decreased spontaneous IFN-γ production by unstimulated T cells. In comparable studies in children (36) and adults (37-39) with atopic dermatitis a reduced IFN-γ production was reported in polyclonally stimulated PBMC. The decreased IFN-γ production in unstimulated cultures of children with atopic dermatitis, was not reflected in diminished IFN-γ mRNA expression, which was comparable to healthy control levels. Interestingly, in children with atopic dermatitis also IFN-γ mRNA expression by stimulated T cells was found to be increased as compared to healthy controls. This might be explained by the demonstration in children with atopic dermatitis of an earlier and more prolonged expression of IFN-γ mRNA following polyclonal stimulation, compared with healthy controls (40). These findings further support the hypothesis of a post-translational defect in IFN-γ production (41). Not only decreased, but also increased spontaneous IFN-γ mRNA expression has been reported in children with atopic dermatitis (40). Similarly, a recent study on active atopic dermatitis skin lesions reported more frequent expression of IFN-γ mRNA (in 13 out of 15 patients) compared with IL-4 mRNA (in 4 out of 15 patients), as well as greater expression of IFN-γ mRNA than IL-4 mRNA in patients expressing both cytokines (42).
Together these findings do not sustain a selective activation of T_h2-like cell subsets in allergic disease, and instead suggest activation of both T_h1- and T_h2-like T cells. As has been demonstrated, T cells from patients with atopic dermatitis have an intrinsic defect in IFN-γ secretion (37,38). The imbalance of IL-4 and IFN-γ secretion as described in patients with atopic dermatitis (7,36-39) could therefore reflect general activation of T cells. In a study using stimulated $CD4^+$ T cell clones from babies born within allergic families, both the IFN-γ- and the IL-4-producing capacity were significantly reduced relative to clones from normal infants (43). This suggests that in children who develop allergy the immune responses to environmental allergens in early childhood occur against a background of maturational $CD4^+$ T cell dysfunction, resulting in abnormal immunological responsiveness.
Next to a post-translational defect in IFN-γ production in children with atopic dermatitis, an increased sensitivity to stimulation might be intrinsically present in their T cells. This was suggested by the positive correlation we demonstrated between IL-4 and IFN-γ expression, although we only detected this association in healthy children and children with non-allergic asthma. This finding was not due to age-related differences in these groups of patients as the age distribution of the healthy children and the children with atopic dermatitis was similar. Furthermore, neither IL-4 mRNA expression ($r_s = 0.15$, $p = 0.50$, $n = 22$), nor IFN-γ mRNA expression ($r_s = 0.20$, $p = 0.34$, $n = 23$) of stimulated T cells from children with atopic dermatitis showed any correlation with the age of the children.
In children with allergic asthma we found, comparable to children with atopic dermatitis, also a smaller spontaneous IFN-γ production by unstimulated T cells,

although the difference with the healthy controls was not significant. This might be due to the smaller number of patients we analyzed in this group, since we excluded all children who used oral- or inhalation corticosteroids, based on its modulatory effect on cytokine gene expression (19-21).
No correlation was found between either IL-4 or IFN-γ expression and total serum IgE levels in any of the groups of children studied. Controversial results have been reported in this respect (32,44). Between the various patient groups studied no differences were observed either in IL-13 mRNA expression or in IL-13 protein production. This might be explained by the different kinetics of IL-13 from that of IL-4 among others. Unlike IL-4, IL-13 mRNA expression in T cells is rapid and is sustained for 72 hours (45). Furthermore, IL-13 is produced by T cells with T_h0, T_h1 and T_h2 phenotypes (45), and both $CD4^+$ and $CD8^+$ T cells (45,46). We did, however, demonstrate a positive correlation between IL-13 protein production by unstimulated PBMC and the SCORAD index in children with atopic dermatitis, suggesting a clinical relevance of IL-13 expression, most likely via IgE. The positive correlation between IL-13 protein production by unstimulated T cells of allergic asthmatic children with the height of the RAST class, gives further support for the role of IL-13 in allergic disease, as suggested before (47,48). Evaluation of the role of other cytokines which may be relevant for the development of allergic diseases, such as IL-5 and IL-10 has been performed (see accompanying paper).
Children with non-allergic asthma had comparable IL-4 mRNA expression and protein production to healthy controls, which corroborates with the study by Walker *et al.* in adult patients with non-allergic asthma at the IL-4 protein level (49). On the other hand, spontaneous IFN-γ production by unstimulated T cells and IFN-γ production by stimulated T cells of non-allergic children were found to be decreased as compared to healthy controls. This decreased IFN-γ secretion might be another indication that this patient group may be in the process of developing allergic asthma, as we suggested previously (24).
In conclusion, we have demonstrated differences in T cell function in allergic diseases, which point to T_h2 upregulation by increased IL-4 expression and in addition a T_h1 dysfunction probably caused by a post-translational defect in IFN-γ secretion. Although our data on IL-13 suggest a clinical relevance of this cytokine, the precise role of IL-13 in allergy remains to be established.

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CHAPTER 3.3

T CELL SUBSETS AND CYTOKINES IN ALLERGIC AND NON-ALLERGIC CHILDREN. II. ANALYSIS OF IL-5 AND IL-10 mRNA EXPRESSION AND PROTEIN PRODUCTION

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ABSTRACT

Interleukin-5 (IL-5) has an enhancing effect on IL-4 induced immunoglobulin E (IgE) synthesis. Furthermore, IL-5 plays an important role in the differentiation, recruitment, activation and survival of eosinophils. IL-10 has a down-modulating effect on interferon-γ (IFN-γ) production and can exert strong anti-inflammatory activities. Therefore, we analyzed whether differences were present in IL-5 and IL-10 mRNA expression and protein production between T cells of children with allergic and non-allergic asthma, atopic dermatitis and healthy control children. We demonstrated significant increases in IL-5 mRNA expression and protein production in different T cell fractions of children with allergic and non-allergic asthma and children with atopic dermatitis as compared to healthy controls. This indicates that IL-5 is not only involved in allergy, but also plays a role in the inflammatory process of non-allergic asthma. Interestingly, IL-10 mRNA expression by purified T cells of children with allergic and non-allergic asthma and children with atopic dermatitis was strongly decreased as compared with that of healthy controls. In the peripheral blood mononuclear cell (PBMC) fraction, IL-10 mRNA expression was comparable between the four groups. We hypothesize that this decreased T cell derived IL-10 expression results in a lack of immunosuppression of the inflammatory process in these diseases. However, a role of monocyte derived IL-10 cannot be ruled out.

INTRODUCTION

The activating effect of interleukin-4 (IL-4) on switching to immunoglobulin E (IgE) is promoted by IL-5 and IL-6, but inhibited by interferons (IFN)-α and -γ, transforming growth factor-β and prostaglandin E_2 (PGE_2) (1-4). Next to the

enhancing effect of IL-5 on IL-4 induced IgE synthesis, IL-5 promotes the differentiation (5), recruitment (6), activation and survival of eosinophils (7). Therefore, IL-5 is considered a pivotal cytokine in allergen-mediated eosinophilic responses.

Based on their cytokine production profile, $CD4^+$ T_h cells can be divided into a T helper 1 (T_h1)-like (e.g., IL-2 and IFN-γ), a T helper 2 (T_h2)-like subset (e.g., IL-4 and IL-5) and a naive T helper 0 (T_h0) subset (producing all of these cytokines) (8,9). Apart from IL-4, overexpression of other genes of the IL-4 gene family, like IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF) also may be important in atopics.

T cells can be divided in subpopulations based on cell surface expression of isoforms of the tyrosine phosphatase CD45 (10). $CD45RA^+$ T cells represent naive T cells that have not yet encountered antigen, whereas the $CD45RO^+$ subset contains memory T cells (11). Expression of these isoforms correlates with functional differences. $CD45RA^+$ T cells produce IL-2 following activation but no IL-4, IL-5 or IFN-γ, while $CD45RO^+$ cells contain IL-4, IL-5 and IFN-γ producing T cells (12-14). $CD45RA^+$ T cells produce significant amounts of IL-10 (15). However, $CD4^+CD45RO^+$ memory T cells produce 10-fold higher levels of IL-10 than $CD45RA^+$ T cells (15). IL-10 can be produced by T_h0-, T_h1- and T_h2-like $CD4^+$ T cell clones (15). Production of IL-10 is not restricted to T cells, since also B cells (16) and lipopolysaccharide (LPS) stimulated monocytes (17) are able to produce IL-10. Interestingly, following activation, IL-10 is synthesized later than other cytokines, both by T cells (15) and monocytes (17), suggesting that IL-10 is active during later phases of the immune response. From several studies it appears that IL-10 is a general suppressor of immune responses. IL-10 inhibits strongly the production of IFN-γ, GM-CSF, tumor necrosis factor-α (TNF-α) and TNF-β of peripheral blood mononuclear cells (PBMC) following activation with anti-CD3, phytohemagglutinin (PHA) or IL-2 (18-20). Furthermore, IL-10 inhibits not only the activation of T_h1 and T_h0 but also of T_h2 clones (20). IL-10 has a down-modulating effect on the production of IFN-γ (21). The production of cytokines (IL-1α, IL-1ß, IL-6, IL-8, IFN-γ, TNF-α, GM-CSF and granulocyte colony stimulating factor) by activated monocytes/macrophages is also strongly inhibited by IL-10 (22-24). Finally, IL-10 strongly reduces antigen-specific T cell proliferation by diminishing the antigen-presenting capacity of monocytes, via down-regulation of major histocompatibility complex-class-II expression (20). Together this indicates that IL-10 has strong anti-inflammatory properties.

In this study we analyzed whether differences existed in IL-5 and IL-10 mRNA expression and protein production between allergic (allergic asthma and atopic dermatitis) and non-allergic children (healthy and non-allergic asthma). To our knowledge IL-5 mRNA expression and protein production has not been studied in young children with allergic diseases before, whereas IL-10 has only been studied in adult patients with atopic dermatitis (25). The mRNA expression was

analyzed in freshly isolated PBMC and purified T cells in order to investigate the *ex vivo* situation. In polyclonally stimulated PBMC and T cell cultures (to abolish the influences of other cell populations) mRNA expression as well as protein production were analyzed. Furthermore, the relationship between these cytokines and relevant clinical parameters was investigated. The correlation with cytokines (IL-4, IL-13, IFN-γ) studied previously was also analyzed.

MATERIALS AND METHODS

Patient groups

A description of the patient groups and characteristics was given elsewhere (accompanying paper).

Analysis of cytokine production and mRNA expression

PBMC, and purified and cultured T cells were obtained from a limited amount of heparinized blood of children and flow cytometric analysis of the various subpopulations was performed as described previously (26, accompanying paper). Numbers of eosinophils were counted in peripheral blood by leucocyte differentiation.

IL-5 and IL-10 in the culture supernatants were measured with enzyme-linked immuno sorbent assay (ELISA) kits: Quantikine™ human IL-5 Immunoassay (R&D Systems Inc., Minneapolis, MN) with a detection level of 1 pg/ml and IL-10 enzyme-amplified sensitivity immuno assay (EASIA) kit (Medgenix Diagnostics SA, Fleurus, Belgium) with a detection level of 1 pg/ml.

RNA was isolated from the PBMC and T cells by the RNAzol B method (27) as described previously (26). Afterwards a copy DNA (cDNA) synthesis reaction was performed, followed by a polymerase chain reaction (PCR) (26), using the following primersets:

HPRT sense: 5'-GTGATGATGAACCAGGTTTATGACCTT-3'
antisense: 5'-CTTGCGACCTTGACCATCTTTGGA-3'
probe: 5'-GAAGAGCTATTGTAATGACCAGTCA-3'
(product size 454 bp) (28)

IL-5 sense: 5'-AGCCAATGAGACTCTGAGGA-3'
antisense: 5'-TAAAATGTCCTTCTCCTCCAAAATC-3'
probe: 5'-GACGGCCAAAAAAAAAAGTGTGAGAAGAAA-3'
(product size 322 bp) (29)

IL-10 sense: 5'-ATGCCCCAAGCTGAGAACCAAGACCCA-3'
antisense: 5'-TCTCAAGGGGCTGGGTCAGCTATCCCA-3'
probe: 5'-CAGGTGAAGAATGCCTTTAATAAGCTCC-3'
(product size 352 bp) (16)

For HPRT (hypoxanthine phosphoribosyl-transferase) and IL-10 the PCR conditions were: denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 1 min. The same conditions applied for IL-5. Only the annealing was performed at 50°C instead of 55°C.

Table IA. RT-PCR analysis of IL-5 mRNA expression

	PBMC	T purified	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	1.0 (1.0-33.6) *n=35*	1.0 (1.0-8.3) *n=17*	1.0 (1.0-38.7) *n=25*	120.6 (1.0-293.1) *n=22*	116.4 (1.0-293.1) *n=21*
Allergic asthma	1.0 (1.0-10.1) *n=8*	1.0 (1.0-12.7) *n=8*	1.0 (1.0-14.6) *n=7*	236.0 (103.9-434.0)$^{p<0.01}$ *n=6*	161.8 (10.6-434.0) *n=6*
Non-allergic asthma	1.0 (1.0-6.0) *n=8*	1.0 (1.0-19.6) *n=10*	1.0 (1.0-1.0) *n=8*	10.8 (1.0-222.4) *n=19*	21.3 (1.0-222.4) *n=8*
Atopic dermatitis	1.0 (1.0-50.4) *n=21*	1.0 (1.0-44.0) *n=22*	1.0 (1.0-48.2) *n=20*	171.0 (45.3-655.8)$^{p<0.01}$ *n=22*	167.3 (3.7-655.8)$^{p<0.05}$ *n=7*

From PBMC, purified T cells and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children, children with allergic asthma, children with non-allergic asthma and children with atopic dermatitis, RNA was isolated, and reverse transcriptase-PCR (RT-PCR) for IL-5 and subsequent radio-active hybridization was performed. Data represent median scan values and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

HPRT mRNA expression was analyzed after agarose gel electrophoresis and subsequent densitometric analysis of the photograph of the gel (26). IL-5 and IL-10 mRNA expression were analyzed on film after gel electrophoresis, Southern blotting and subsequent hybridization with a radioactive probe (26). Mean scan values were calculated by densitometric analysis of the autoradiograph of the bands after 30 and 35 cycles. The elaborate calculation method was described previously (30).

Statistics

Statistical analysis was performed with STATA^{TM} (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 n-value depicts the number of patients from which two separate parameters were correlated. P-values smaller than 0.05 were considered significant.

Table IB. Analysis of IL-5 protein production by ELISA and numbers of eosinophils

	T unstimulated	T stimulated	T stim/T unstim	eosinophils
Healthy controls	1.0 (1.0-10.7) *n=19*	1.0 (1.0-27.0) *n=20*	1.0 (0.4-27.0) *n=18*	0.12 (0.075-0.386) *n=5*
Allergic asthma	1.0 (1.0-9.1) *n=8*	3.4 (1.0-103.0) *n=8*	2.9 (0.7-19.5) *n=8*	0.578 (0.042-1.048) *n=9*
Non-allergic asthma	6.1 (1.0-28.4)$^{p<0.005}$ *n=8*	7.0 (1.0-63.9) *n=8*	0.4 (0.2-63.9) *n=8*	0.368(0.118-1.33)$^{p<0.05}$ *n=11*
Atopic dermatitis	5.0 (1.0-68.0)$^{p<0.005}$ *n=24*	4.7 (1.0-36.0)$^{p<0.05}$ *n=24*	1.0 (0.3-23.0) *n=23*	0.465(0.12-1.37)$^{p<0.05}$ *n=22*

T cells of healthy children, children with allergic asthma and children with non-allergic asthma and children with atopic dermatitis were cultured and IL-5 protein production was measured by ELISA. Data represent median IL-5 production in pg/ml and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. Numbers of eosinophils were counted in peripheral blood by leucocyte differentiation. Data represent median numbers of eosinophils x 10^9/l and ranges. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

RESULTS

IL-5 mRNA expression and protein production and eosinophils

IL-5 mRNA expression was measured directly after isolation of PBMC and subsequent purification of T cells, in order to analyze the *ex vivo* mRNA expression. IL-5 mRNA expression and protein production were analyzed after overnight culture of polyclonally activated PBMC and purified T cells.

Only in children with atopic dermatitis, IL-5 mRNA expression reached higher levels in the PBMC and purified T cell fraction than in healthy controls, although these differences did not reach significance (Table IA). Unstimulated T cell cultures showed comparable IL-5 mRNA expression in healthy and allergic children, but in unstimulated T cells of children with non-allergic asthma IL-5 mRNA expression was negligible. A significant increase was observed in IL-5

mRNA expression after stimulation of purified T cells of allergic asthmatic children ($p = 0.006$) and children with atopic dermatitis ($p = 0.005$) as compared with healthy controls. IL-5 mRNA expression by stimulated T cells of children with non-allergic asthma and healthy children was comparable. A representative example of IL-5 mRNA expression by stimulated T cells of the four patient groups is shown in Figure 1. The IL-5 mRNA ratio (stimulated/unstimulated IL-5 mRNA expression) was significantly elevated in children with atopic dermatitis ($p = 0.023$) and was increased, though it did not reach significance, in children with allergic asthma.

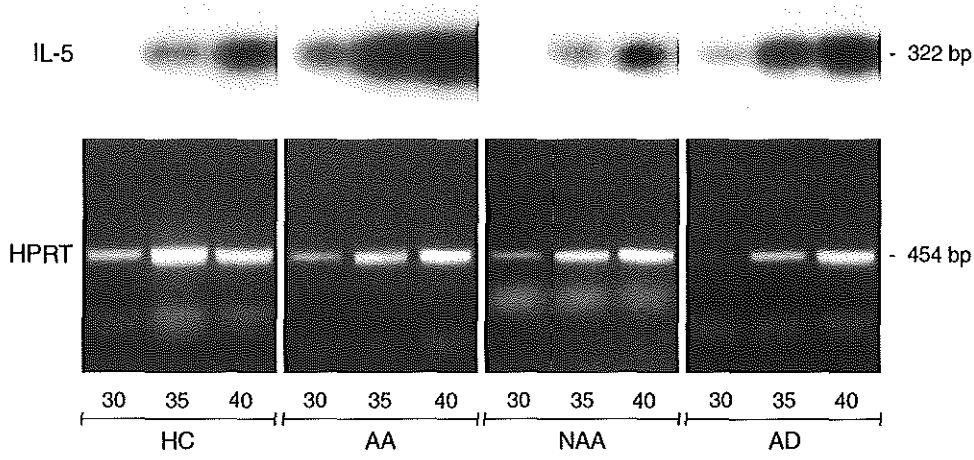

Figure 1. IL-5 mRNA expression by stimulated T cells. After stimulation of 10^6 T cells of healthy children (HC), children with allergic asthma (AA), children with non-allergic asthma (NAA) and children with atopic dermatitis (AD) with TPA and Ca-Ionophore for 16 hrs, RNA was isolated and RT-PCR was performed for HPRT and IL-5 at 30, 35 and 40 cycles. HPRT mRNA expression was analyzed after gel electrophoresis, whereas for IL-5 subsequent Southern blotting and radioactive hybridization was performed.

IL-5 protein production by unstimulated T cells of both children with non-allergic asthma ($p = 0.0014$) and atopic dermatitis ($p = 0.0041$) was significantly elevated, whereas that of children with allergic asthma was comparable to healthy control levels (Table IB). After stimulation of the T cells, IL-5 protein production was elevated in all 3 patient groups as compared with healthy controls. However, it reached significance only in children with atopic dermatitis ($p = 0.036$). The IL-5 protein ratio in the T cell cultures (stimulated/unstimulated IL-5 protein production) did not show significant differences between the studied groups of patients.

None of the differences in IL-5 mRNA expression and protein production as observed in the T cell cultures could be detected in PBMC cultures (data not shown).

Numbers of eosinophils were significantly elevated in children with atopic

Table IIA. RT-PCR analysis of IL-10 mRNA expression

	PBMC	T purified	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	125.6 (1.0-214.3) *n=34*	118.0 (1.0-316.0) *n=17*	1.0 (1.0-131.6) *n=25*	43.1 (1.0-164.5) *n=23*	3.5 (0.008-69.9) *n=22*
Allergic asthma	29.5 (1.0-160.9)$^{p<0.05}$ *n=8*	1.0 (1.0-128.0)$^{p<0.05}$ *n=8*	33.4 (1.0-339.2) *n=7*	29.9 (1.0-159.2) *n=6*	0.8 (0.5-30.6) *n=6*
Non-allergic asthma	82.1 (3.2-130.8) *n=9*	8.0 (1.0-175.6)$^{p<0.05}$ *n=12*	4.2 (1.0-41.9) *n=8*	53.3 (6.0-185.7) *n=9*	27.7 (0.5-105.0) *n=8*
Atopic dermatitis	96.9 (1.0-201.3) *n=21*	14.2 (1.0-327.1)$^{p<0.01}$ *n=23*	9.7 (1.0-174.8) *n=22*	84.8 (1.0-611.0) *n=23*	3.5 (0.7-172.4) *n=22*

From PBMC, purified T cells and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children with allergic asthma and children with non-allergic asthma and children with atopic dermatitis, RNA was isolated, and RT-PCR for IL-10 and subsequent radio-active hybridization was performed. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. Data represent median scan values and ranges. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

dermatitis (p=0.014) and non-allergic asthma (p=0.04) as compared with numbers in healthy controls (Table IB). In children with allergic asthma the increase in the numbers of eosinophils just failed to reach significance (p=0.07).

IL-10 mRNA expression and protein production

IL-10 mRNA expression and protein production RT-PCR analysis of IL-10 mRNA expression in freshly isolated PBMC revealed a significant decrease in children with allergic asthma (p=0.014) as compared with levels in healthy subjects, whereas this expression in children with non-allergic asthma and atopic dermatitis was comparable with healthy control levels (Table IIA). Strikingly, IL-10 mRNA expression was significantly diminished in purified T cells of children with allergic (p=0.012) and non-allergic (p=0.018) asthma and in children with atopic dermatitis (p=0.008) as compared to purified T cells of healthy control children. A representative example of this decreased IL-10 mRNA expression is shown in Figure 2. IL-10 mRNA expression in the unstimulated as well as in the stimulated T cell cultures did not show significant differences between the 3 patient groups and the healthy control group (Table IIA).

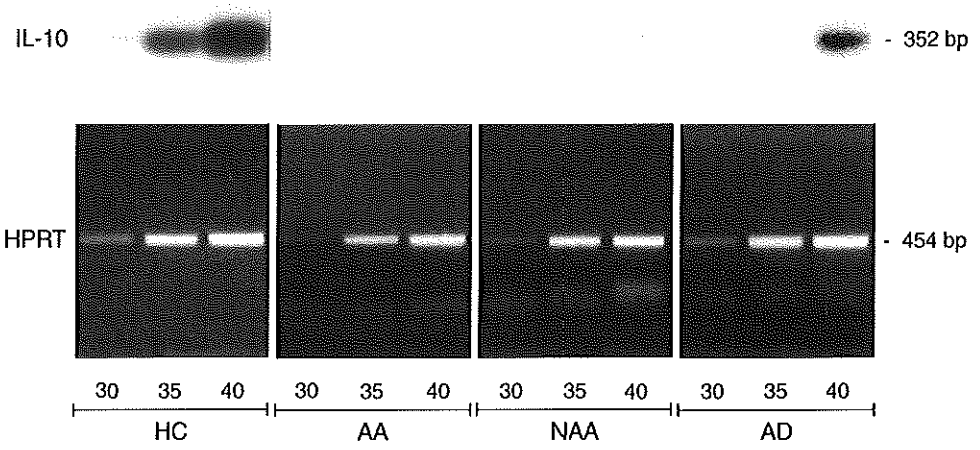

Figure 2. IL-10 mRNA expression by purified T cells. RNA was isolated from purified T cells of healthy children (HC), children with allergic asthma (AA), children with non-allergic asthma (NAA) and children with atopic dermatitis (AD) and RT-PCR was performed for HPRT and IL-10 at 30, 35 and 40 cycles. HPRT mRNA expression was analyzed after gel electrophoresis, whereas for IL-10 subsequent Southern blotting and radioactive hybridization was performed.

Analysis of IL-10 protein expression in the unstimulated and stimulated T cell cultures also did not show significant differences between the groups, although in the unstimulated T cell culture of children with allergic asthma a reduction

was observed (Table IIB). The IL-10 protein ratio was, however, significantly reduced in T cells of children with non-allergic asthma ($p = 0.019$) and atopic dermatitis ($p = 0.02$), whereas the ratio was comparable between children with allergic asthma and healthy controls.

Table IIB. Analysis of IL-10 protein production by ELISA

	T unstimulated	T stimulated	T stim/T unstim
Healthy controls	5.3 (1.0-20.9) *n = 19*	10.3 (1.0-42.0) *n = 19*	2.2 (0.2-19.2) *n = 19*
Allergic asthma	14.4 (1.0-114.0) *n = 8*	22.0 (1.0-48.0) *n = 8*	1.0 (0.02-20.0) *n = 8*
Non-allergic asthma	7.5 (1.0-40.0) *n = 10*	5.3 (1.0-26.8) *n = 10*	1.0 (0.4-3.3)$^{p<0.05}$ *n = 10*
Atopic dermatitis	6.6 (1.0-78.0) *n = 24*	6.5 (1.0-74.0) *n = 24*	1.0 (0.03-6.9)$^{p<0.05}$ *n = 24*

T cells of healthy children, children with allergic asthma and children with non-allergic asthma and children with atopic dermatitis were cultured and IL-10 protein production was measured by ELISA. Data represent median IL-10 production in pg/ml and ranges. T stim/T unstim represent the stimulated T cell values divided by the unstimulated. P-values were calculated by the Mann-Whitney test relative to the healthy controls.

None of the differences in IL-10 mRNA expression and protein production as observed in the T cell cultures could be detected in PBMC cultures, whether or not stimulated with Ca-ionophore and TPA. However, as shown in Table III, IL-10 mRNA expression in unstimulated PBMC cultures was significantly higher

Table III. Analysis of IL-10 mRNA expression and protein production in cultured PBMC

	mRNA		protein	
	PBMC unstimulated	PBMC stimulated	PBMC unstimulated	PBMC stimulated
Healthy controls	280.5 (243.0-412.8) *n = 3*	85.8 (1.0-125.2) *n = 4*	58.0 (32.0-278.0) *n = 5*	26.0 (20.0-62.0) *n = 5*
Atopic dermatitis	369.5 (254.9-656.7)$^{p<0.001}$ *n = 7*	72.5 (1.0-138.1) *n = 7*	32.0 (1.0-524.0) *n = 6*	30.6 (5.1-72.0) *n = 6*

Table III. From unstimulated and stimulated (with Ca-ionophore and TPA) PBMC of healthy children and children with atopic dermatitis, RNA was isolated, and RT-PCR for IL-10 and subsequent radio-active hybridization was performed. IL-10 protein production was measured by ELISA. Data represent median scan values and median IL-10 production in pg/ml and ranges. P-values were calculated by the Mann-Whitney test by comparing the unstimulated with the stimulated PBMC fractions of both groups of children.

than in stimulated PBMC cultures of children with atopic dermatitis ($p < 0.001$).

A similar tendency was observed when comparing IL-10 mRNA expression in the unstimulated and stimulated PBMC cultures from healthy children ($p = 0.057$). Also, when analyzing IL-10 protein production a non-significant increase was found in PBMC cultures of healthy children ($p = 0.095$), but not in PBMC cultures from children with atopic dermatitis.

Relationship between the analyzed parameters

In order to exclude age-related influences on the correlations between the parameters, we tested for age-related effects. These were all negative (data not shown). As shown in Figure 3, an inverse correlation was observed between IL-10 protein production by unstimulated T cells and the percentage of $CD4^+CD45RA^+$ T cells of children with atopic dermatitis ($r_s = -0.48$, $p = 0.028$, $n = 21$). Similarly, an inverse correlation was found when comparing IL-10 production by stimulated T cells of children with atopic dermatitis with the percentage of $CD4^+CD45RA^+$ T cells ($r_s = -0.49$, $p = 0.029$, $n = 20$). On the other hand, a positive correlation existed between IL-10 mRNA expression by unstimulated T cells and the percentage of $CD4^+CD45RO^+$ T cells of children with allergic asthma ($r_s = 0.94$, $p = 0.005$, $n = 6$).

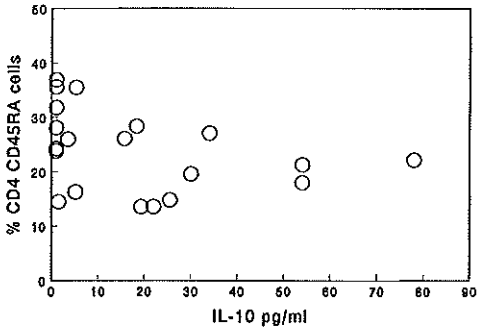

Figure 3. Correlation between IL-10 protein production and the percentage of $CD4^+CD45RA^+$ T cells. IL-10 protein expression of 10^6 unstimulated T cells (16 hrs culture) of children with atopic dermatitis was determined by ELISA. Percentages of $CD4^+CD45RA^+$ T cells were determined by flow-cytometry after double-staining the cells with Leu3-FITC and 2H4-PE. Correlation between the two parameters was calculated by the Spearman sign-rank correlation ($r_s = -0.48$, $p = 0.028$).

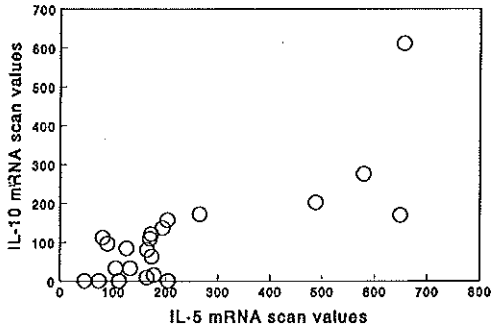

Figure 4. Correlation between IL-5 and IL-10 mRNA expression by stimulated T cells. After stimulation of 10^6 T cells of children with atopic dermatitis with TPA and Calonophore for 16 hrs, RNA was isolated and RT-PCR was performed for IL-5 and IL-10. Scan values were calculated at 30 and 35 PCR cycles after gel electrophoreses and subsequent Southern blotting and radioactive hybridization. Correlation between the two parameters was calculated by the Spearman sign-rank correlation ($r_s = 0.68$, $p < 0.001$).

A positive correlation was also found when comparing the IL-5 and IL-10 protein ratio by T cells of healthy children ($r_s = 0.76$, $p < 0.001$, $n = 18$). Such a positive correlation was also observed between IL-5 and IL-10 mRNA expression by stimulated T cells of children with atopic dermatitis (Figure 4, $r_s = 0.68$, $p < 0.001$, $n = 22$). No significant correlations were found between IL-

5 expression and percentages of $CD4^+CD45RO^+$ cells.
IL-5 as well as IL-10 expression did show some associations with characteristics of the children. The ratio of IL-5 mRNA expression (stimulated/unstimulated) by T cells was positively correlated with serum IgE levels, both in children with non-allergic asthma ($r_s = 0.78$, $p = 0.024$, $n = 8$) and in healthy children ($r_s = 0.53$, $p = 0.035$, $n = 22$). Furthermore, the IL-10 mRNA expression by purified T cells showed an inverse correlation with the severity scoring of atopic dermatitis (SCORAD) index in children with atopic dermatitis (Figure 5, $r_s = -0.46$, $p = 0.034$, $n = 21$). A similar relationship was observed in healthy children between IL-10 mRNA expression in PBMC and the number of eosinophils in the peripheral blood ($r_s = -0.9$, $p = 0.0037$, $n = 5$).
In different patient groups under certain conditions a positive correlation was observed between IL-5 and IL-4 mRNA expression. In children with allergic asthma a positive correlation existed between IL-4 and IL-5 mRNA expression by unstimulated T cells ($r_s = 0.76$, $p = 0.046$, $n = 7$). The same was found for stimulated T cells of healthy children ($r_s = 0.53$, $p = 0.014$, $n = 7$) and children with atopic dermatitis ($r_s = 0.49$, $p = 0.022$, $n = 22$). On the other hand, an inverse correlation was found between IL-5 and IFN-γ protein production by unstimulated PBMC of children with atopic dermatitis ($r_s = -0.91$, $p = 0.0049$, $n = 7$).
When comparing the IL-10 and IL-13 protein production in unstimulated (Figure 6, $r_s = 0.9$, $p = 0.015$, $n = 6$) and in stimulated PBMC ($r_s = 0.99$, $p < 0.001$, $n = 6$) of children with atopic dermatitis, a positive correlation was found.

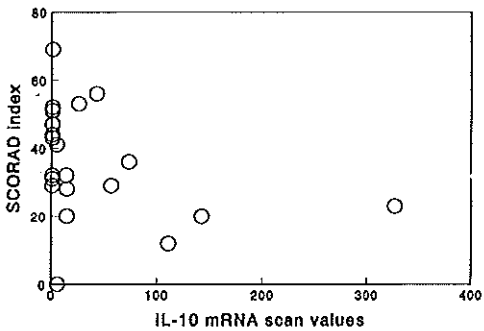

Figure 5. Correlation between IL-10 mRNA expression and the SCORAD index. RNA was isolated from purified T cells of children with atopic dermatitis and RT-PCR was performed for IL-10. Scan values were calculated at 30 and 35 PCR cycles after gel electrophoreses and subsequent Southern blotting and radioactive hybridization. The SCORAD index was calculated according to the guidelines (European Task Force on Atopic Dermatitis). Correlation between the two parameters was calculated by the Spearman sign-rank correlation ($r_s = -0.46$, $p = 0.034$).

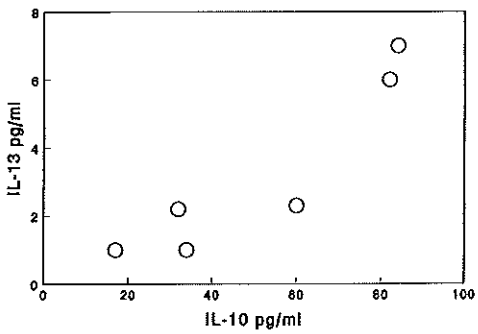

Figure 6. Correlation between IL-10 and IL-13 protein production. IL-10 and IL-13 protein production by 10^6 unstimulated PBMC (16 hrs culture) were measured by ELISA. Correlation between the two parameters was calculated by the Spearman sign-rank correlation ($r_s = 0.9$, $p = 0.015$).

DISCUSSION

In this study we show strongly elevated IL-5 and markedly diminished IL-10 mRNA expression and protein production by T cells of young children with allergic and non-allergic asthma and atopic dermatitis, as compared to healthy controls.

In young children with allergic asthma we found IL-5 mRNA expression by stimulated T cells to be strongly increased as compared to healthy control children. IL-5 protein expression by stimulated T cells of children with allergic asthma showed a similar tendency. Also in studies describing adult atopic asthmatic patients an upregulation of IL-5 mRNA expression was found in bronchoalveolar lavage T cells (31), in bronchial mucosal biopsies (32) and in bronchial biopsies after allergen inhalation challenge (33) as compared to healthy controls. In adult patients with asthma, the presence of IL-5, IL-3 and GM-CSF have been demonstrated in the serum. Furthermore, *in vivo* activated T cells spontaneously release these cytokines into supernatants (34). It has been demonstrated that at local sites of inflammation IL-5 mRNA can also be expressed by eosinophils and mast cells, although over 70% of IL-5 mRNA positive cells were $CD3^+$ T cells (35). IL-5 immunoreactive mast cells have also been demonstrated (36).

So far, many data have been published about the presence of IL-5 in adult atopic asthma, but less about the possible role of IL-5 in atopic dermatitis. We show that the increase of IL-5 mRNA expression and protein production as observed in children with asthma, was even stronger in children with atopic dermatitis. This indicates that IL-5 also plays a role in the inflammatory process and enhanced IgE levels in atopic dermatitis.

Remarkably, IL-5 mRNA levels in unstimulated T cells of children with non-allergic asthma were negligible, whereas protein levels were significantly increased as compared to healthy controls. Comparably, IL-5 mRNA expression by stimulated T cells of children with non-allergic asthma was similar to healthy control levels, whereas the protein secretion in this fraction was elevated. The demonstration of elevated IL-5 protein expression in children with allergic and non-allergic asthma are in agreement with earlier findings in adult patients with non-allergic asthma. In the peripheral blood and bronchoalveolar lavage fluid of adult allergic asthmatics, IL-4 and IL-5 protein production was predominating in allergic asthma, whereas in non-allergic asthma only IL-5 was found to be increased (37). In allergic asthmatic children and children with atopic dermatitis we found increased IL-4 (accompanying paper) and IL-5 mRNA expression and protein production. In children with non-allergic asthma IL-4 mRNA expression and protein production were comparable to healthy control levels (accompanying paper). We found a co-expression between IL-4 (accompanying study) and IL-5 mRNA expression in children with allergic asthma, atopic dermatitis and healthy controls. This was reported previously in atopic diseases (38). This

co-expression can be explained by the joined localization of the IL-4 and the IL-5 gene in the cytokine gene cluster on chromosome 5q31 (39). This co-expression between IL-4 and IL-5 was, however, not found in children with non-allergic asthma. This is in favour of a differential expression of these cytokines or differences in the kinetics of expression. A recent study demonstrated that IL-4 and IL-5 are rarely co-expressed by human T cells at the single cell level (40), providing evidence for independent regulation of these cytokines. This finding may also explain the differential IL-4 and IL-5 expression we observed in children with non-allergic asthma. IL-10 was also not found to be co-expressed with IL-4 and IL-5 (40).

Recent studies have shown that IL-4 also enhances expression of VCAM-1 (vascular cell adhesion molecule-1) on the surface of endothelial cells (41,42). Because VCAM-1 participates in selective eosinophil adhesion via a VLA-4-dependent (very late activation antigen-4) mechanism, IL-4 may account, at least in part, for selective eosinophil recruitment at sites of allergic tissue reactions. The close correlation with eosinophilia suggests that IL-5 has a large impact on the characteristic eosinophilia in asthma and that eosinophilia is regulated through similar mechanisms in allergic and non-allergic asthmatics (37). We could demonstrate an increased number of eosinophils in children with atopic dermatitis and non-allergic asthma as compared with healthy controls, whereas the increase in children with allergic asthma just failed to reach significance, probably due to the small number of patients. The degree of the eosinophilia has been shown to correlate with the severity of airways hyperresponsiveness (43,44). Thus, whereas IL-5 is probably responsible for the observed eosinophilia in both allergic and non-allergic asthma, our data of differential IL-4 expression (accompanying paper) and differences in percentages of $CD4^+CD45RA^+$ cells and NK cells (30) suggest that there are basic immunologic differences between allergic and non-allergic asthma. Recently, *in vitro* IL-5 secretion by NK cells stimulated with IL-2 was demonstrated and its production was found to be regulated by IL-4, IL-10 and IL-12 (45).

The fact that both in allergic and in non-allergic asthma the increase in IL-5 production by the stimulated T cell fraction failed to reach significance could be due to the relatively small number of asthmatic patients we studied and the mild disease severity of the children. Both were mainly caused by the exclusion of patients using corticosteroids, which concerns the large majority of young asthmatics. This decision was validated by the finding that IL-5 levels are reduced in the serum of asthmatic patients who are receiving oral glucocorticoids (46,47). These results are consistent with *in vitro* studies that show a potent inhibitory effect of corticosteroids on gene expression and on the release of proinflammatory cytokines, including IL-5, from inflammatory cells (48).

We found a striking decrease in IL-10 mRNA expression by purified T cells in children with allergic asthma children with non-allergic asthma as well as in

children with atopic dermatitis. In children with allergic asthma this decrease in IL-10 mRNA expression was already detectable in the PBMC fraction, suggesting an even stronger decrease of IL-10 mRNA expression in this patient group. After stimulation of T cells, the IL-10 mRNA expression of the patient groups was comparable to healthy control levels, suggesting the absence of an intrinsic T cell defect. However, the IL-10 protein ratio after stimulation of T cells was significantly decreased in non-allergic asthmatic children and children with atopic dermatitis as compared to healthy controls. The positive correlation we found in children with allergic asthma between IL-10 mRNA expression and $CD45RO^+$ T cells and the inverse correlation we observed in children with atopic dermatitis between IL-10 protein production and $CD45RA^+$ T cells, extend the finding of a preferential IL-10 expression by $CD45RO^+$ T cells in adults to children (15).

In our study, the IL-10 mRNA expression by the unstimulated PBMC fraction of children with atopic dermatitis was significantly higher than by the corresponding stimulated PBMC fraction. This IL-10 mRNA expression by unstimulated PBMC of children with atopic dermatitis was also higher, although not significantly, than by unstimulated PBMC from healthy controls, suggesting that the high IL-10 mRNA expression in the PBMC fraction is monocyte-derived. Also in adult patients suffering from atopic dermatitis an overexpression of IL-10 mRNA was found in monocytes (25). Thus, in our experiments either the monocytes were already activated upon isolation and could not be further activated, or the *in vitro* stimulation conditions were not optimal for stimulation of IL-10 expression by monocytes resulting in activation of other cell types producing less IL-10.

The question remains whether the decreased IL-10 expression by T cells we found in children with asthma and atopic dermatitis results in a lack of immunosuppresion of the inflammatory process, or whether this decreased IL-10 expression by T cells is counterbalanced by the IL-10 expression by monocytes.

We did show th ; the IL-10 mRNA expression by purified T cells from children with atopic dermatitis was inversely correlated with the SCORAD index. This implies that the decreased IL-10 expression by T cells might have a clinical relevance. Furthermore, it has been shown that high IL-10 producing cells may have a general inhibitory function, since they will possibly inhibit both T_h1 and T_h2 cytokine production (49), antigen-specific proliferation of T cells, as well as monocyte activation. Therefore, we hypothesize that the decreased IL-10 expression in children with asthma and atopic dermatitis may result in a diminished immunosuppressive function of T cells (18-20), leading to a lack of downregulation of the inflammatory process. On the other hand, IFN-γ promotes the release of IL-10 which downregulates allergic eosinophilia and IL-5 production (50). The decreased IFN-γ production we described previously in children with asthma and atopic dermatitis (accompanying paper), would further

support this hypothesis.
Monocyte-derived IL-10 could play a role in the suppression of inflammation as suggested by a decreased cognate interaction between monocytes and regulatory T cells (51), and the inhibitory effect of IL-10 on IL-4 (52) and allergen-induced IgE synthesis (53).
It was demonstrated that the inhibitory effect of recombinant IL-10 (rIL-10) on the production of IL-5 by T_h2 clones was indirect and related to functional inhibition of APC (49). The lack of a direct inhibitory effect of rIL-10 on the production of IL-5 by T_h0 and T_h2 clones was also observed by de Waal Malefyt *et al.* (54). Recently, it was demonstrated that both rIL-10 and endogenous IL-10 were able to inhibit IL-5 production by T cells costimulated by B7/CD28 signalling (55). If *in vivo* production of IL-5 would prove to depend on B7/CD28-CTLA-4 interactions, possible therapeutic applications of IL-10 might include diseases related to IL-5 hyperproduction such as hypereosinophilic syndromes (56) or bronchial asthma.
Taken together, our findings of elevated IL-5 expression and reduced IL-10 expression in purified T cells of children with asthma and atopic dermatitis indicates that treatment of patients with IL-10 needs to be further investigated. Recently, the safety and immunomodulatory effects of human IL-10 administration in humans has been studied. This study showed that a single intravenous injection of IL-10 is safe in humans, has inhibitory effects on T cells, and suppresses the production of the pro-inflammatory cytokines TNF-α and IL-1ß (57).

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CHAPTER 4

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

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CHAPTER 4

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

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ABSTRACT

During inflammation not only membrane expression of adhesion molecules (intercellular adhesion molecule-1 (ICAM-1), E-selectin), and tumor necrosis factor (TNF)-receptors (TNF-R55 and TNF-R75) are increased, but also soluble forms of these adhesion molecules and TNF-R are released.
This study analyzed whether plasma levels of sICAM-1, sE-selectin as well as TNF-α, sTNF-R55 and sTNF-R75 could be used for discrimation between atopic and non-atopic children and whether these levels were related to the severity of disease.
We measured plasma levels of these markers in 9 non-allergic asthma (NAA) and 9 allergic asthma patients (AA), 10 atopic dermatitis patients (AD) and 10 healthy children (HC) by enzyme-linked immunosorbent assay (ELISA).
Plasma levels of sICAM-1, sE-selectin, sTNF-R55 and sTNF-R75, 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 severity scoring of atopic dermatitis (SCORAD) index ($r = 0.73$, $p = 0.038$).
Our data demonstrate that the presence of plasma levels of sICAM-1, sE-selectin, TNF-α, sTNF-R55 and sTNF-R75 could not be used as discriminative markers between atopic and non-atopic children, during a stable phase of the disease. Levels of sE-selectin could be clinically relevant in AD patients.

INTRODUCTION

Several cytokines, including tumor necrosis factor-α (TNF-α), promote the induction and upregulation of adhesion molecules, both on the endothelium and on leucocyte surfaces (1). At the 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 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 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. Allergy represents a state of immunological activation. Therefore, we studied TNF-α and sTNF-R levels in several groups of atopic patients.
This study analyzed whether the above mentioned markers could be used for discrimination 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

Patient groups

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, soy 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). For evaluating the severity of AD the severity scoring of atopic dermatitis (SCORAD) system was developed by the European Task Force on Atopic Dermatitis (27). The total SCORAD-index was not used in the comparison with the studied markers, because subjective opinions of parents differ from case to case. Objective SCORAD gives a global objective opinion of the severity and extent of atopic dermatitis. Therefore, we used only the objective evaluation of the SCORAD in this study.
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 (28). All patients had a positive family history of asthma and/or atopy and none of the patients had used (inhalation-) corticosteroids. 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.
The healthy controls had no history of allergy and a negative family history as judged by a questionnaire. All healthy children lacked specific immunoglobulin E (IgE) for inhalation and/or food allergens as measured by RAST (Phadiatop® and food mix).

Collection of plasma samples

Five ml of peripheral blood was collected by vena puncture of each subject. Plasma samples were obtained and stored at -70°C until analysis. The analysis 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 (29-31). 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, 5µg/ml) or sTNF-R75 (MR2.2, 5µg/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 TNF-R75.

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 (32). 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-ethylbenzthiazolinesulfonicacid) (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 analysis
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 could be used as discriminative markers between atopic and non-atopic children. Table I 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 allergic asthmatic patients (AA), non-allergic asthmatic patients (NAA), atopic dermatitis patients (AD) and healthy controls (HC) for any of the molecules analyzed. TNF-α levels could not be detected in any of the samples.

Table I. 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 *n = 10*	65.4 (43.2-101.8)	5.1 (2.4-15.5)	385 (230-870)	1620 (930-2930)
Non-allergic asthma *n = 9*	60.4 (49.4-85.7)	5.2 (3.4-8.5)	390 (350-520)	1370 (350-1820)
Allergic asthma *n = 9*	65.8 (50.6-126.4)	4.5 (2.4-15.5)	390 (270-1290)	860 (290-3150)
Atopic dermatitis *n = 10*	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.

Relationship between plasma levels of sICAM-1 and sE-selectin or sTNF-R55 or sTNF-R75 and the 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 evaluate whether the regression lines in each group showed a same trend with age. An age-related effect was observed for sTNF-R55 ($p = 0.003$, Figure 1A) and for sTNF-R75 ($p < 0.001$, Figure 1B). No significant age-related effects were observed for sICAM-1 (Figure 1C) and sE-selectin (Figure 1D).

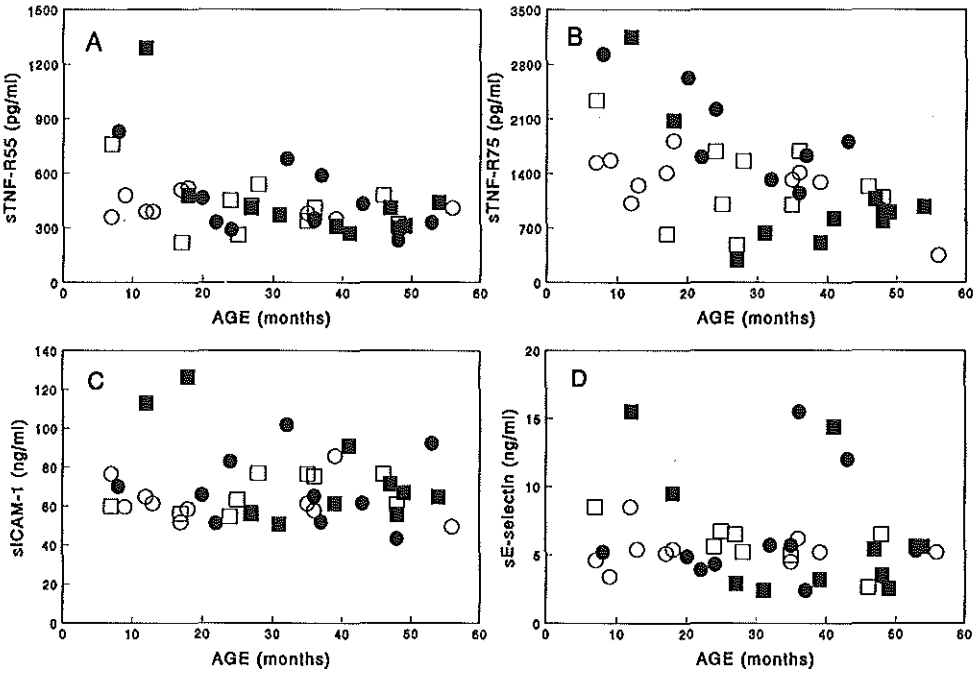

Figure 1. Plasma levels of sTNF-R55 (pg/ml; A), sTNF-R75 (pg/ml; B), sICAM-1 (ng/ml; C), sE-selectin (ng/ml; D), in relation to the age of the children in months. Healthy children are represented by closed circles, children with non-allergic asthma by open circels, children with allergic asthma by closed squares and children with atopic dermatitis by open squares. 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 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 ($r_s=-0.56$, $p=0.152$) (Figure 2C), but was significant in the AA group ($r_s=0.78$, $p=0.008$) (Figure 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 (Figure 3). No correlation could be detected between sICAM-1, sTNF-R55 or sTNF-R75 and the SCORAD (data not shown).

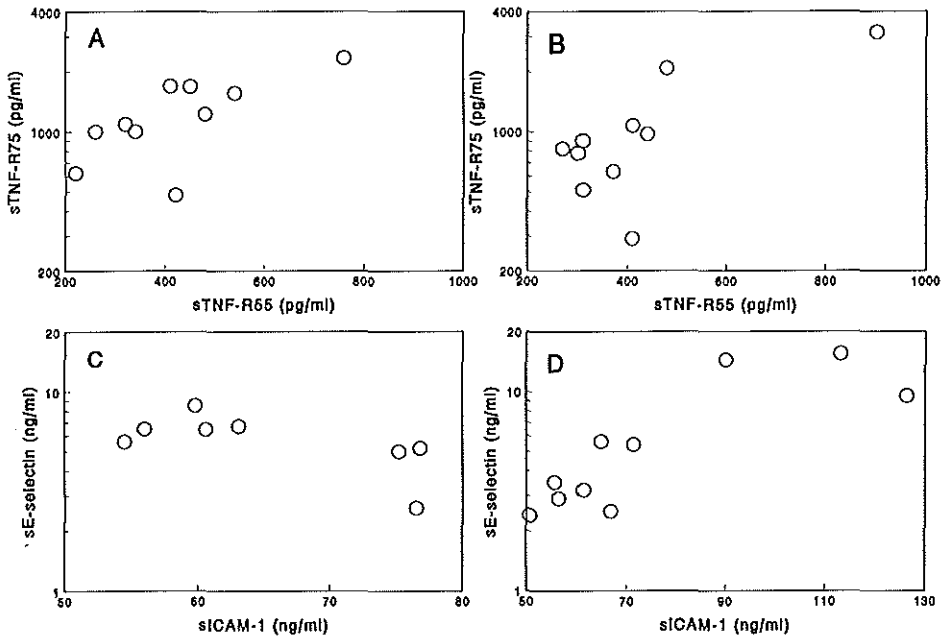

Figure 2. Correlation between sTNF-R55 (pg/ml) and sTNF-R75 (pg/ml) or sICAM-1 (ng/ml) and sE-selectin (ng/ml) in AD and AA. (A) Correlation between sTNF-R55 and sTNF-R75 in AD ($r_s = 0.69$, $p = 0.027$). (B) Correlation between sTNF-R55 and sTNF-R75 in AA ($r_s = 0.70$, $p = 0.025$). (C) Correlation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

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 group of AD patients a significant correlation was found between the sE-selectin level and an index of disease severity (SCORAD).
Inflammatory cytokines, like TNF-α and 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

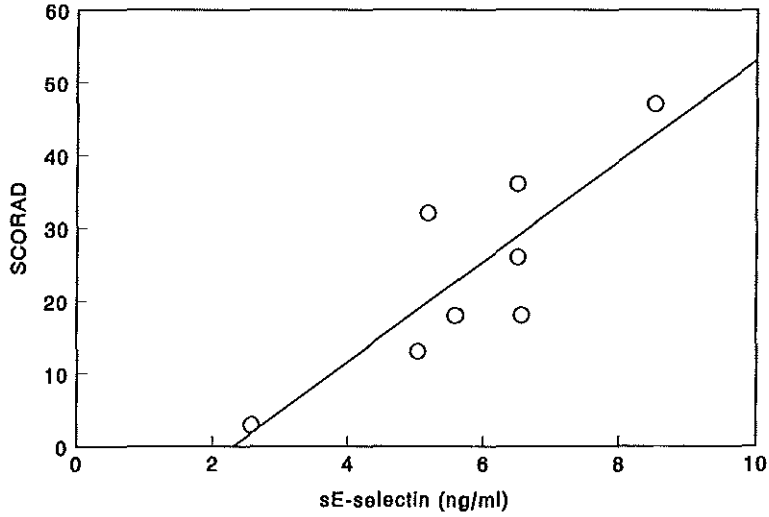

Figure 3. Correlation between sE-selectin (ng/ml) and SCORAD in AD, with a correlation coefficient of $r = 0.73$ and $p = 0.038$.

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. The results indicate that in young children the levels of sICAM-1 or sE-selectin in plasma could not be used to discriminate between atopic and non-atopic children. 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.

The cytokines (TNF-α, IL-1) released due to the inflammation in asthmatic patients (both AA and NAA) play a pivotal role in the increased expression and subsequent release of adhesion molecules. In our study, dealing with allergic children we could not find differences in the levels of soluble adhesion molecules among the various patient groups. This might be due to the relative immaturity of the immune system.

A second explanation for 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 (33). 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 (34). Soluble TNF-R in the circulation are considered to be more stable (19). Therefore, it was of interest to study the levels of sTNF-R as well. We were able to detect sTNF-R55 and sTNF-R75 levels in healthy children similar as observed in healthy adults (17,35). For all patient groups, a similar age-related decrease in the levels of sTNF-R was found. For sTNF-R also no differences were 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 with 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 (36-38). Thus, variation in soluble adhesion molecules and sTNF-R levels could be a reflection of disease activity. Further study is necessary to establish such a relation.
Between plasma sE-selectin levels and the SCORAD in AD 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 (27). Czech *et al.* (39) described a correlation between sE-selectin and the severity of the dermatitis in adult AD patients, using another 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 on 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 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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CHAPTER 5

ANALYSIS OF CYTOKINES IN TWO CHILDREN WITH COMÈL-NETHERTON SYNDROME

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ANALYSIS OF CYTOKINES IN TWO CHILDREN WITH COMÈL-NETHERTON SYNDROME

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ABSTRACT

Comèl-Netherton syndrome is a disease characterized by atopic dermatitis-like skin eruptions, hair abnormalities, elevated immunoglobulin E (IgE) levels and food allergy. Interleukin-4 (IL-4) and IL-13 are cytokines inducing IgE synthesis, that can be further enhanced by IL-5 and suppressed by interferon-γ (IFN-γ). IL-5 is generally considered to play a role in the blood eosinophilia often observed in Comèl-Netherton patients. IL-10 is a cytokine with strong anti-inflammatory activities. We analyzed cytokine mRNA expression and protein production of these cytokines in peripheral blood mononuclear cells (PBMC) and cultures of purified T cells of two young children (patient 1: 19 months, patient 2: 9 months) with Comèl-Netherton syndrome, and compared it with that of age-matched healthy children and children with atopic dermatitits. Patient 1 had low IFN-γ expression, whereas patient 2 had high IL-4 and IL-5 expression as compared to both control groups. This suggests that the high IgE level in patient 1 is due to diminished IFN-γ production, whereas in patient 2 overactivation of T_h2-like cells seems to be responsible. The level of IL-5 expression correlated positively in both patients with that of serum IgE levels and the number of blood eosinophils, suggesting a role for IL-5 in both processes. IL-10 mRNA expression in the PBMC fraction of both Comèl-Netherton patients was low as compared to both control groups, possibly resulting in diminished anti-inflammatory activities. No differences were found in IL-13 expression between the 3 groups of children. The combination of high IL-5 expression, low IL-10 expression, high numbers of eosinophils and high IgE levels as compared with healthy controls (both patients) and atopic dermatitis (patient 2) were characteristic for patients with Comèl-Netherton syndrome in this study. Analysis of IL-4, IFN-γ and IL-13 expression is also useful, however, no correlation with clinical symptoms was found.

INTRODUCTION

Netherton syndrome is a rare genetic disease characterized by an atopic dermatitis-like skin eruption, hair abnormalities such as trichorrhexis invaginata (bamboo hairs), elevated immunoglobulin E (IgE) levels and food allergy (1-4). The disease has been described first by Comèl and later by Netherton (1,2). Therefore Comèl-Netherton syndrome and Netherton's syndrome both refer to the same disease. Often the affected children are erythrodermic at or shortly (1 or 2 days) after birth (3,4). After 1-2 years the erythroderma evolves towards linear circumflex erythematous lesions with lamellar collerette desquamation (3). The skin eruption is incorrectly named ichthyosis circumflexa. The histology is not ichtyoform but psoriatiform (3). For the typical hair abnormality often more than one sample should be taken, because not all the hairs are affected (2). Food allergy is present in particular to peanut, egg and fish (4).
IgE levels are extremely high (>1000 IU/ml) in Comèl-Netherton syndrome. It is therefore tempting to investigate whether cytokines regulating IgE synthesis are involved in this syndrome.
Cytokines play a crucial role in the regulation of IgE synthesis under normal conditions and in diseased states like allergy. Interleukin-4 (IL-4) is a key cytokine in the production of IgE by naive B cells (5). This is based on its ability to induce expression of ε-germline transcripts (6). Another T cell derived cytokine, IL-13, can also induce germline-ε expression in B cells, but it is two- to five-fold less potent than IL-4 in inducing IgE synthesis (7). To our knowledge only one study analyzed serum IL-4 levels in a patient with Comèl-Netherton syndrome (8).
The activating effect of IL-4 on switching to IgE is promoted by IL-5 and IL-6, but inhibited by interferons (IFN)-α and -γ, transforming growth factor-β and prostaglandin E_2 (PGE_2) (9-12). Next to the enhancing effect of IL-5 on IL-4 induced IgE synthesis, IL-5 promotes differentiation (13), recruitment (14), activation and survival of eosinophils (15). Therefore, IL-5 is considered a pivotal cytokine in allergen-mediated eosinophilic responses. IL-5 may thus be responsible for the blood eosinophilia which is often observed in patients with Comèl-Netherton syndrome (8).
IL-10 can be produced by T helper 0 (T_h0)-, T helper 1 (T_h1)- and T helper 2 (T_h2)-like $CD4^+$ T cell clones (16-18), but the production of IL-10 is not restricted to T cells alone. IL-10 is also produced by B cells (17) and by lipopolysaccharide (LPS) stimulated monocytes (18). Interestingly, IL-10 is synthesized later than other cytokines both by T cells (16) and monocytes (18), following activation, suggesting that IL-10 regulates later phases of the immune response. IL-10 inhibited strongly the production of pro-inflammatory cytokines (amongst others IFN-γ and tumor necrosis factor-α) following activation of peripheral blood mononuclear cells (PBMC) and monocytes/macrophages (19-24). Moreover, IL-10 inhibited not only activation of T_h1 and T_h0 but also of

T_h2 clones (21) and IL-10 had a down-modulating effect on the production of IFN-γ (25) with a simultaneous strong reduction in antigen-specific T-cell proliferation (21). Together, this indicates that IL-10 can exert strong anti-inflammatory activities.
Since several allergic phenomena, such as allergic rhinitis, food allergy, elevated IgE levels, positive radio-allergo-sorbent test (RAST) results and eczematoid changes are often observed as a part of Comèl-Netherton syndrome (8), we investigated whether an altered cytokine gene expression and production was present in two children with Comèl-Netherton syndrome, compared with healthy age matched control children and children with atopic dermatitis.

PATIENTS AND METHODS

Patients

Comèl-Netherton patient 1:
A girl born in 1992 was observed at our outpatient clinic of the Pediatric Dermatology Unit for second opinion in 1993 (aged 19 months). From birth on she is suffering from a skin eruption over the whole body earlier diagnosed as atopic dermatitis. Her diet was cow's milk and egg free. The family history of father and mother was positive for atopy. She had an elevated serum IgE level (554 kU/l) compared to healthy controls and comparable to levels of children with atopic dermatitis.
At dermatological examination, generalized erythematous and squamous eczematous lesions were observed (Figure 1). In the face the area between mouth and nose was free. The severity scoring of atopic dermatitis (SCORAD) index was 57, indicating very severe eczema (26). Hairs were varying in length and easily broke off (Figure 2). Later, after some months, serpiginous collarette lamellar desquamation appeared (Figure 3). She is a small child with a length-age curve below the third percentile (P_3) and a weigth-length curve at the tenth percentile (P_{10}). Anti-eczematous therapy was not successful. Food allergy to cow's milk, egg, peanut, hazel nut, and wheat was diagnosed by skin tests and confirmed by RAST. The skin tests were performed by the methods of the skin application food tests (SAFT) developed by Oranje (27,28). Hair analysis revealed trichorrhexis invaginata (bamboo hairs). Aminoaciduria was not detected.

Comèl-Netherton patient 2:
A boy born in 1992 was referred by to the Sophia Children's Hospital at the age of 9 months. Before he was treated by a dermatologist and pediatrician because of neonatal erythroderma, diagnosed as staphylococcal scalded skin syndrome and hypertonic dehydration. He was already hospitalized elsewhere because of a refractory eczema. The family history revealed that father had been suffering from asthmatic bronchitis. The child's serum IgE level (7825 kU/l) was elevated compared to both healthy controls and children with atopic dermatitis.
At dermatological examination we observed a boy with an extended erythematosquamous disorder. The SCORAD index was 45, indicating very severe eczema (26). The hairs did not grow very well and broke easily. He was very short for his age, with length-age and weight-length curves both below the third percentile (P_3).

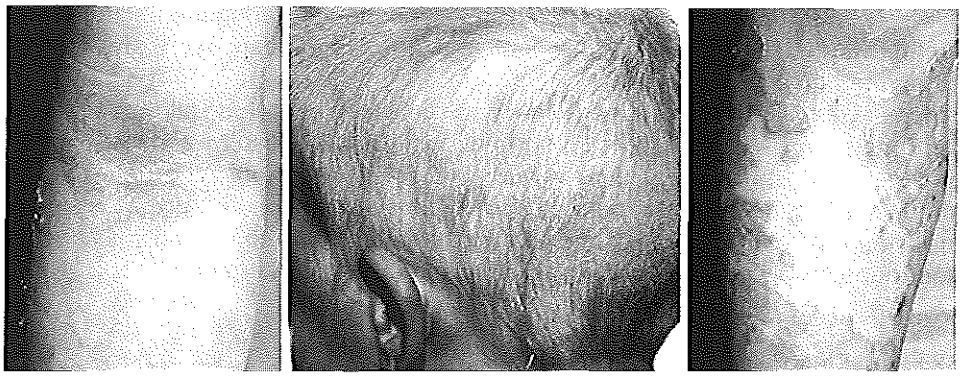

Figure 1. Erythematosquamous elements on the left arm of patient one mimicking atopic eczema (left).

Figure 2. Alopecia due to broken hairs of patient 1 (middle).

Figure 3. Typical serpiginous collarette lamellar desquamation on the right leg of patient 1 appearing at age 2 (right).

Anti-eczematous therapy was not successful. Food allergy to cow's milk, egg, peanut, carrots and wheat was diagnosed by SAFTs and confirmed by RASTs. Hair analysis revealed pili torti, but no trichorrhexis invaginata. Aminoaciduria was not detected.

Atopic dermatitis:
8 children between 0 and 2 years old with atopic dermatitis were included according to the diagnostic criteria of Hanifin and Rajka (29). The main diagnostic criteria were pruritis, typical skin lesions and localization, a chronical recurrent course and the presence of other atopic diseases or atopic relatives. Children with atopic dermatitis had specific IgE (>0.35 kU/l) for a mixture of inhalation allergens (Phadiatop®) and/or food allergens. The median SCORAD index of the children with atopic dermatitis was 32 (range 0-69).

Healthy controls:
14 healthy control children between 0 and 2 years old were included after a questionnaire was filled out by their parents, indicating absence of allergic symptoms and of a family history of atopy. Healthy children had no specific IgE (<0.35 kU/l) for inhalation and/or food allergens in their serum as tested by Phadiatop® and food mix and no allergic symptoms.
Numbers of eosinophils in the peripheral blood of the patients were determined by leucocyte differentiation.
The Medical Ethical Committee of the Erasmus University Medical School and University Hospital Rotterdam approved this study. Informed consent was given by all the parents of the different patient groups.

Isolation of PBMC and T cells

PBMC were isolated from a limited amount of heparinized blood of children as described previously (30). T cells were purified from the PBMC by negative selection using Dynabeads (IgG M450, Dynal, Oslo, Norway) in order to prevent stimulation of the cells (30). T cells (1 x 10^6 cells/well) were cultured for 16-18h in Yssel's medium (31) containing 1% human serum with and without the addition of 4-bromo-calcium-ionophore (A23187, final concentration 500 ng/ml, Sigma, St. Louis, MO) and TPA (12-O-tetradecanoylphorbol-13-acetate) final concentration 1 ng/ml, Sigma) at 37°C, 5% CO_2. PBMC (1x10^6 cells/well) were cultured under the same conditions. After culturing, the cells were pelletted, resuspended in 200 µl/1x10^6 cells RNAzol B (Cinna-Biotecx Laboratories Inc., Houston, TX) and stored at -20°C. The cell supernatants were collected and stored at -70°C.

Flow cytometry

1-5 x 10^5 PBMC were stained (30 min, on ice) with monoclonal antibodies specific for B cells (B4-PE, Coulter Cytometry, Hialeah, FL), T cells (Leu4-FITC, Becton Dickinson, San Jose, CA), monocytes (My4-FITC, Coulter Cytometry) and natural killer (NK) cells (CD16-PE and CD56-PE, Becton Dickinson). For the analysis of CD4/CD8 ratios PBMC were double stained with Leu4-FITC (CD3), Leu3-PE (CD4, Becton Dickinson) and Leu2-PE (CD8, Becton Dickinson). For the analysis of CD45RA and CD45RO expression PBMC were double stained with Leu3-FITC (Becton Dickinson), Leu2-FITC (Becton Dickinson), 2H4-PE (CD45RA, Coulter Cytometry) and UCHL1-PE (CD45RO, Dako, Glostrup, Denmark). Finally, PBMC were double stained with Leu3-PE, Leu2-PE and CD25-FITC (IL-2 receptor, Becton Dickinson). After staining, cells were washed again for two times and resuspended in 100 µl FACSflow solution (Becton Dickinson). Flow cytometry was performed on a fluorescence activated cell scanner (FACScan) (Becton Dickinson).

Analysis of cytokine production

IL-4, IL-5, IL-10, IL-13 and IFN-γ in the cell culture supernatants were measured with enzyme-linked immuno sorbent assay (ELISA) kits. IL-4 by an IL-4 enzyme-amplified sensitivity immuno assay (EASIA) kit (Medgenix Diagnostics SA, Fleurus, Belgium) with a detection level of 2 pg/ml. IFN-γ by an IFN-γ EASIA kit (Medgenix Diagnostics SA) with a detection level of 1 pg/ml. IL-13 by a Pelikan Compact™ human IL-13 kit (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) with a detection level of 1.5 pg/ml. IL-5 by a Quantikine™ human IL-5 Immunoassay (R&D Systems Inc., Minneapolis, MN) with a detection level was 1 pg/ml. IL-10 by an IL-10 EASIA kit (Medgenix Diagnostics SA, Fleurus, Belgium) with a detection level of 1 pg/ml.

Analysis of cytokine mRNA expression

RNA was isolated from the PBMC and T cells by the RNAzol B method (32) as described previously (33). Subsequently, a copy DNA (cDNA) synthesis reaction was performed, followed by a polymerase chain reaction (PCR) (30), using the following primersets:

HPRT sense: 5'-GTGATGATGAACCAGGTTTATGACCTT-3'
antisense: 5'-CTTGCGACCTTGACCATCTTTGGA-3'
(product size 454 bp) (34)

IL-4	sense:	5'-ACTCTGTGCACCGAGTTGACCGTAA-3'
	antisense:	5'-TCTCATGATCTGCTTTAGCCTTTCC-3'
	(product size 300 bp) (35)	
IFN-γ	sense:	5'-TTTAATGCAGGTCATTCAGATG-3'
	antisense:	5'-CAGGGATGCTTCTTCGACCTCGAAAC-3'
	(product size 388 bp) (35)	
IL-13	sense:	5'-CCCAGAACCAGAAGGCTCCGC-3'
	antisense:	5'-GCTGGAAAACTGCCCAGCTGAG-3'
	probe:	5'-GCTGGCATGTACTGTGCAGCC-3'
	(product size 187 bp) (36)	
IL-5	sense:	5'-AGCCAATGAGACTCTGAGGA-3'
	antisense:	5'-TAAAATGTCCTTCTCCTCCAAAATC-3'
	probe:	5'-GACGGCCAAAAAAAAAAGTGTGAGAAGAAA-3'
	(product size 322 bp) (37)	
IL-10	sense:	5'-ATGCCCCAAGCTGAGAACCAAGACCCA-3'
	antisense:	5'-TCTCAAGGGGCTGGGTCAGCTATCCCA-3'
	probe:	5'-CAGGTGAAGAATGCCTTTAATAAGCTCC-3'
	(product size 352 bp) (17)	

For all primer sets except IL-5 the PCR conditions were: denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 1 min. The same conditions applied for IL-5, except for annealing which was performed at 50°C.
HPRT (hypoxanthine phosphoribosyl-transferase, house-keeping gene), IL-4 and IFN-γ mRNA expression were analyzed after agarose gel electrophoresis and subsequent densitometric analysis of the photograph of the gel (30). IL-5, IL-10 and IL-13 mRNA expression were analyzed on film after gel electrophoresis, Southern blotting and subsequent hybridization with a radioactive probe (30). Mean scan values were calculated at 30 and 35 cycles for all primersets except IL-4, which was calculated at 35 and 40 cycles. The elaborate calculation method was described previously (33).

RESULTS

Cytokine mRNA expression and protein production

IL-4

IL-4 mRNA expression and protein production were different between the two Comèl-Netherton patients. Overall, patient 1 had IL-4 mRNA and protein levels in the same range as the healthy controls and the children with atopic dermatitis (Table I). Only after stimulation of purified T cells IL-4 mRNA expression was comparable with the highest healthy control value. As shown in Figure 4A, IL-4 protein production by stimulated T cells of patient 1 was not detectable, like in the majority of healthy control children.

Patient 2 had high IL-4 mRNA expression in the PBMC fraction as compared with healthy controls and children with atopic dermatitis, whereas IL-4 mRNA expression was comparable after T cell culturing. IL-4 protein production by unstimulated and stimulated T cells of patient 2 was higher than that of healthy controls and of children with atopic dermatitis (Table I and Figure 4A).

Table I. RT-PCR analysis of IL-4 mRNA expression

	PBMC	T unstimulated	T stimulated
HC	8.6 (1.0-70.5) *n = 14*	34.4 (1.0-111.8) *n = 10*	4.0 (1.0-35.8) *n = 10*
AD	16.0 (1.0-35.5) *n = 7*	14.7 (1.0-81.1) *n = 6*	18.1 (2.8-43.2) *n = 6*
CN patient 1	11.1	5.4	35.5
CN patient 2	81.2	33.4	22.1

From PBMC and cultured T cells (unstimulated and stimulated with Ca-Ionophore and TPA) of healthy children (HC), children with atopic dermatitis (AD) and with Comèl-Netherton syndrome (CN patient 1 and 2) RNA was isolated and reverse transcriptase-PCR (RT-PCR) was performed for IL-4. Data represent median scan values and ranges.

Table II. RT-PCR analysis of IFN-γ mRNA expression

	PBMC	T unstimulated	T stimulated
HC	10.0 (1.0-70.9) *n = 14*	1.4 (1.0-167.8) *n = 10*	66.2 (5.7-161.0) *n = 10*
AD	14.2 (1.0-23.7) *n = 7*	8.2 (1.0-63.4) *n = 6*	78.6 (60.4-95.6) *n = 6*
CN patient 1	8.6	1.0	56.5
CN patient 2	9.4	10.1	88.3

From PBMC and cultured T cells (unstimulated and stimulated with Ca-Ionophore and TPA) of healthy children (HC), children with atopic dermatitis (AD) and with Comèl-Netherton syndrome (CN patient 1 and 2) RNA was isolated and RT-PCR was performed for IFN-γ. Data represent median scan values and ranges.

IFN-γ

In contrast to the situation for IL-4, differences in IFN-γ mRNA expression and protein production were found in patient 1. IFN-γ mRNA expression was not detectable (Table II), like in some of the healthy controls and children with

atopic dermatitis. IFN-γ protein production was also below the detection limit of the assay and lower than most of the healthy controls and children with atopic dermatitis (Table II and Figure 4B).
No differences in IFN-γ mRNA expression and protein production with healthy controls and children with atopic dermatitis were found when analyzing PBMC and T cells of patient 2.

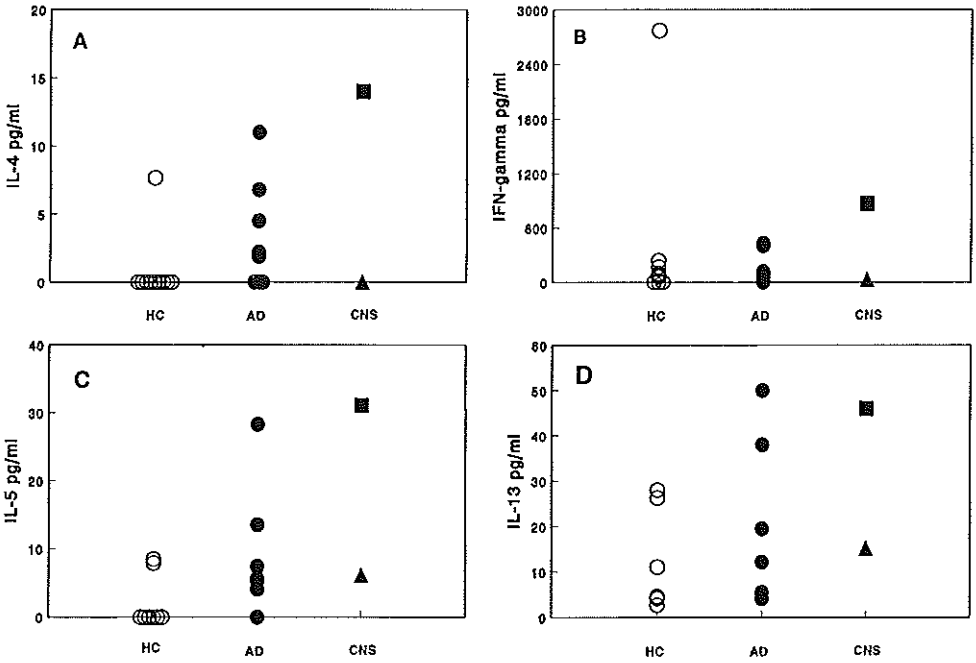

Figure 4. Protein production by stimulated T cells. T cells were purified from peripheral blood of healthy children (HC, open circles), children with atopic dermatitis (AD, closed circles) and two patients with Comèl-Netherton syndrome (CN): patient 1 (triangle) and patient 2 (square). 10^6 T cells were stimulated with Ca-ionophore and TPA and cultured for 16 hrs. (A) IL-4, (B) IFN-γ, (C) IL-5 and (D) IL-13 protein production were measured by ELISA.

IL-5

Patient 1 had IL-5 mRNA expression and protein levels by stimulated T cells comparable to levels of the highest healthy controls (Figure 4C).
Stimulated T cells of patient 2 had higher IL-5 mRNA expression and protein production than healthy controls and children with atopic dermatitis (Table III and Figure 4C).

IL-13

Patient 1 had low IL-13 mRNA expression in the PBMC fraction and the

unstimulated T cells as compared to both healthy controls and children with atopic dermatitis. In this patient the IL-13 protein production was in the same range as in the healthy controls and children with atopic dermatitis. IL-13 protein production was higher in stimulated T cells of patient 2 as compared to healthy controls. The IL-13 mRNA expression of patient 2 (Table IV) was in the normal range.

Table III. RT-PCR analysis of IL-5 mRNA expression

	PBMC	T unstimulated	T stimulated
HC	1.0 (1.0-1.0) *n = 12*	1.0 (1.0-38.7) *n = 10*	91.2 (8.6-136.3) *n = 10*
AD	1.0 (1.0-50.4) *n = 7*	1.0 (1.0-48.2) *n = 6*	144.7 (89.1-177.7) *n = 6*
CN patient 1	1.0	1.0	112.0
CN patient 2	1.0	1.0	175.0

From PBMC and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children (HC), children with atopic dermatitis (AD) and children with Comèl-Netherton syndrome (CN patient 1 and 2) RNA was isolated, and RT-PCR for IL-5 and subsequent radio-active hybridization was performed. Data represent median scan values and ranges.

Table IV. RT-PCR analysis of IL-13 mRNA expression

	PBMC	T unstimulated	T stimulated
HC	1.0 (1.0-141.2) *n = 14*	4.3 (1.0-429.2) *n = 10*	87.3 (1.0-346.0) *n = 10*
AD	16.9 (1.0-73.5) *n = 7*	16.5 (1.0-140.2) *n = 6*	92.5 (45.5-198.0) *n = 6*
CN patient 1	1.0	1.0	115.8
CN patient 2	98.8	1.9	90.4

From PBMC and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children (HC), children with atopic dermatitis (AD) and children with Comèl-Netherton syndrome (CN patient 1 and 2) RNA was isolated, and RT-PCR for IL-13 and subsequent radio-active hybridization was performed. Data represent median scan values and ranges.

IL-10

IL-10 mRNA expression was low in both patients as compared to healthy controls and children with atopic dermatitis (Table V). After stimulation of T

cells, IL-10 mRNA expression was heterogeneous being higher in patient 1 and lower in patient 2, both compared to healthy controls and children with atopic dermatitis.
Analysis of IL-10 protein production did not show differences between the two Comèl-Netherton patients and the 2 control groups (data not shown).

Table V. RT-PCR analysis of IL-10 mRNA expression

	PBMC	T unstimulated	T stimulated
HC	152.9 (4.1-240.0) *n = 14*	9.5 (1.0-131.6) *n = 10*	24.6 (1.0-86.9) *n = 10*
AD	80.4 (1.0-201.3) *n = 7*	1.0 (1.0-73.1) *n = 6*	39.8 (1.0-97.0) *n = 6*
CN patient 1	68.4	37.3	113.5
CN patient 2	57.0	25.9	15.9

From PBMC and cultured T cells (unstimulated and stimulated with Ca-ionophore and TPA) of healthy children (HC), children with atopic dermatitis (AD) and children with Comèl-Netherton syndrome (CN patient 1 and 2) RNA was isolated, and RT-PCR for IL-10 and subsequent radio-active hybridization was performed. Data represent median scan values and ranges.

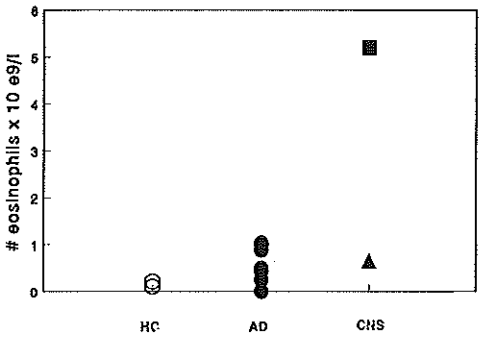

Figure 5. Numbers of eosinophil granulocytes in the peripheral blood of healthy children (HC, open circles), children with atopic dermatitis (AD, closed circles) and two patients with Comèl-Netherton syndrome (CN): patient 1 (triangle) and patient 2 (square).

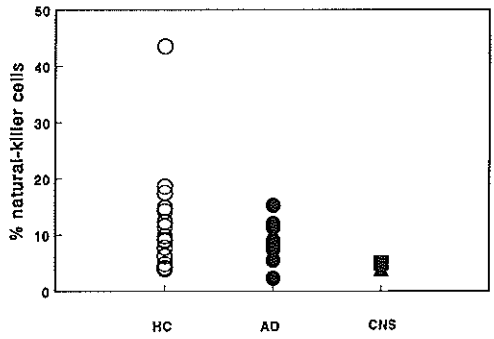

Figure 6. Percentages of natural-killer cells in the PBMC fraction. PBMC were isolated from peripheral blood of healthy children (HC, open circles), children with atopic dermatitis (AD, closed circles) and two patients with Comèl-Netherton syndrome (CN): patient 1 (triangle) and patient 2 (square). PBMC were stained with monoclonal antibodies specific for natural-killer cells (CD16, CD56) and flow-cytometric analysis was performed.

Distribution of cell populations

The number of eosinophils in the peripheral blood of patient 1 (0.65×10^9/l) was higher than in healthy controls, whereas the number of eosinophils of patient 2 (5.2×10^9/l) was much higher than in healthy controls and children with atopic dermatitis (Figure 5).

Both patients had low percentages of NK cells in the PBMC fraction compared to healthy controls and children with atopic dermatitis (Figure 6). Percentages of T cells, B cells and monocytes and CD4/CD8 ratios of the 2 patients were comparable to the 2 control groups (data not shown).

DISCUSSION

Two young children with Comèl-Netherton syndrome gave us the opportunity to analyze cytokine mRNA expression and protein production of IL-4, IL-5, IL-10, IL-13 and IFN-γ in comparison with healthy control and atopic dermatitis children. Few studies have analyzed cytokine production in Comèl-Netherton patients. One study failed to detect serum IL-4 levels in a patient with Comèl-Netherton syndrome (8). Because of the higher sensitivity of the PCR technique, especially in combination with stimulation of purified T cells (38) we applied this method to analyze cytokine mRNA expression.

Comèl-Netherton patient 2 had elevated expression of IL-4 and IL-5 as compared to healthy controls and children with atopic dermatitis, whereas IL-13 expression was only high as compared to the healthy control group. This cytokine pattern is indicative for the preferential activation of T_h2-like cells (39,40).

In Comèl-Netherton patient 1 high IL-5 mRNA expression and protein production was only present as compared with healthy control children, but was within the range of atopic dermatitis patients. In addition, IL-5 mRNA expression and protein production of patient 2 was also increased as compared with children with atopic dermatitis.

The increase in IL-5 expression of these patients was associated with their numbers of eosinophil granulocytes and the height of their serum IgE levels relative to the levels of healthy control and atopic dermatitis children. Since IL-5 plays a role in differentiation, recruitment, activation and survival of eosinophils (13-15), and also has an enhancing effect on IL-4 induced IgE synthesis (5), IL-5 is most likely responsible for the eosinophilia and the high IgE levels observed in Comèl-Netherton syndrome. However, the differential IL-5 expression and numbers of eosinophils were not reflected in differences in disease severity between the two patients. Hence, at least one other factor also influences the severity of Comèl-Netherton syndrome.

The low IFN-γ expression in T cells of Comèl-Netherton patient 1, as compared to both healthy controls and children with atopic dermatitis is the most striking

difference of this patient with the two other groups. The high serum IgE level of this patient may thus be due to diminished IFN-γ production by T_h1 cells, leading to a secondary stimulation of the T_h2-like pathway. The low percentages of natural killer cells, as observed in both Comèl-Netherton patients, however, could also contribute to the elevated IgE levels in these patients, because natural killer cells can be potential producers of IFN-γ (41).
Our results are thus suggestive for a crucial role of T_h2 cells in patients suffering from Comèl-Netherton syndrome. We could not discriminate whether this enhanced activation of T_h2 cells was due to a selective expansion of these cells or to an inhibition of T_h1 activation.
IL-10 mRNA expression was decreased in isolated PBMC of both Comèl-Netherton patients. We previously reported decreased IL-10 mRNA expression in purified T cells of children with atopic dermatitis and asthma (42). In children with atopic dermatitis we could not demonstrate this decrease in IL-10 mRNA expression in the PBMC fraction, suggesting that the decrease in IL-10 mRNA expression in patients with Comèl-Netherton syndrome is even stronger than in atopic dermatitis. Since IL-10 is a strong downregulator of inflammatory responses, the low expression of IL-10 could result in a less potent dampening of inflammatory responses in these patients.
On the basis of their symptoms both patients could be characterized as Comèl-Netherton patients, but immunopathologically the two patients showed marked differences in several parameters. Characteristic for our patients with Comèl-Netherton syndrome was the combination of high IL-5 expression, low IL-10 expression, high numbers of eosinophils and high IgE levels as compared with healthy controls, and with atopic dermatitis in the case of patient 2. For IL-4, IFN-γ and IL-13 expression no correlation with clinical symptoms was found. In both children with Comèl-Netherton syndrome the severity and extent of disease was comparable with an erythroderma in atopic dermatitis (generalized disease). There was no clear-cut relation between the differences in immunopathological parameters in the two patients and their disease severity.

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CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

As outlined in the aims of this thesis (chapter 1.3), the principal hypothesis of this thesis was:

> *"The production and the in vivo reactivity of cytokines known to play a role in the regulation of IgE synthesis is altered during the induction and development of allergic diseases in infancy".*

The first aim of the studies presented in this thesis was to develop techniques to enable the analysis of cytokine gene expression and production by T cells and the isotype switching potential and isotype production by B cells in small volumes of peripheral blood of atopic young children. We used these techniques to study whether the increased levels of serum immunoglobulin E (IgE) in allergic disease were caused by intrinsic properties of their B cells. Alternatively, B cells of allergic children are normal, but under the control of an altered regulation. Therefore, we also studied whether differences existed in T cell activation, resulting in differential cytokine mRNA expression and protein production, between children with allergic and non-allergic asthma, children with atopic dermatitis and healthy control children. Moreover, we investigated in these patient groups whether differences in concentrations of soluble adhesion molecules could be detected in their serum. Finally, we investigated whether the techniques developed to study immunopathological changes in T cells enabled us also to analyze related diseases like Comèl-Netherton syndrome, a disease often associated with elevated IgE levels, allergic symptoms and eczema.

Patient groups

In literature and among specialists there is no consensus on diagnostic criteria for childhood asthma. Thus, the diagnosis of asthma is difficult to establish at the age of 0-4 years at which we studied the children. The children with asthma were selected according to the diagnostic criteria for adult asthmatics of the American Thoracic Society: coughing (especially nocturnal) and/or shortness of breath, at least 3 times a year, for a period of at least 14 days (1) without fever and hypersecretion, and a positive family history of asthma and/or atopy. Retrospectively, the positive reaction to asthma medication was taken into consideration and the diagnosis of asthma was confirmed by the medical attendant of the child, based on the disease course. Children who did not react to asthma medication or with a disease course not characteristic for asthma were excluded from this study. We also excluded children who received treatment with (inhalation-) corticosteroids. Especially due to the latter criterium the size of the groups of asthmatic children was rather limited, since steroid treatment is presently applied frequently in

young asthmatic children. Probably, for this reason some of the differences observed in the asthmatic patient groups were consistently present, but just failed to reach significance. Moreover, due to the exclusion of patients using corticosteroids, most of the asthmatic children we studied had mild asthma. The differences we did observe thus most probably underestimate the situation in children with more severe asthma. Furthermore, we did not select for children having a recent asthma exacerbation.
We divided the children with asthma into two groups, one with allergic asthma and one with non-allergic asthma. The group of children with allergic asthma was defined by the presence of specific IgE in their serum for a mixture of inhalation allergens and/or food allergens, which was based on the allergens most frequently found to cause allergic reactions at this age. The children with non-allergic asthma had symptoms of asthma, but at the time of blood sampling they had no measurable specific IgE to inhalation and/or food allergens. The latter group of patients is heterogeneous, as it contains children with non-allergic asthma, that will presumably develop concomitant allergy in the future, while others will remain non-allergic. We did, however, choose to classify the children according to the actual situation at the time of the blood sampling. It would be all the more interesting to perform a follow-up study on such children.
Children with atopic dermatitis were included according to the well-accepted diagnostic criteria of Hanifin and Rajka (2). The main diagnostic criteria were pruritis, typical skin lesions and localization, a chronical recurrent course and the presence of other atopic diseases or atopic relatives. Children with atopic dermatitis had specific IgE for a mixture of inhalation allergens and/or food allergens. The severity scoring of atopic dermatitis (SCORAD) index was used as a measure for disease severity (3). The SCORAD index is a well accepted method for assessment of the severity of atopic dermatitis, combining extent, severity and subjective symptoms. The height of the SCORAD index among the children with atopic dermatitis ranged from 12 to 69 (median 35), thus from mild to very severe atopic dermatitis. Despite of this heterogeneity in disease severity in this group of children, we were able to demonstrate many significant differences in cytokine gene expression and protein production in this patient group as compared with healthy control children.
The healthy control children formed an adequate control group, since they were age-matched, had no specific IgE for inhalation and/or food allergens in their serum, had neither allergic symptoms, nor a family history of atopy. The latter two criteria were ensured by a suitable questionnaire which was answered by all the parents of the children included in this study.

Analysis methods

Cell separation

We optimized separation techniques from a limited amount of peripheral blood to obtain purified B and T cells. After Ficoll separation, a fraction of peripheral blood mononuclear cells (PBMC) was obtained. Subsequently, B cells were isolated by positive selection after labeling with CD19 monoclonal antibodies coupled to paramagnetic beads. Neither the monoclonal antibodies (4) nor the magnetic beads themselves (5) resulted in B cell activation or inhibition. T cells were purified by negative selection in order to avoid activation via antibodies during the isolation procedure, since T cells are very sensitive to stimulation. From both the purified B cells and the purified T cells aliquots were taken directly after the separation procedure for RNA isolation. This enabled us to analyze the *ex vivo* situation of germline-ε and cytokine mRNA expression, respectively. Despite the generally low numbers of PBMC available after Ficoll separation, the isolation procedures employed generally resulted in sufficient purified B cells and T cells, with a high degree of purity.

In order to study the isotype production potential of B cells and especially differences in switching potential to IgE, purified B cells had to be stimulated in a highly efficient manner using a T helper 0 (T_h0) clone, producing all the relevant cytokines (interleukin-4 (IL-4), IL-5, IL-6, IL-10, interferon-γ (IFN-γ))(6). To permit isotype switching to IgE, the activity of IFN-γ had to be counteracted by the addition of an excess of IL-4. B cells cultured in this way gave rise to substantial isotype production of all classes, including IgE when measured quantitatively by enzyme-linked immuno sorbent assay (ELISA).

The purified T cells were put in culture and stimulated polyclonally with Ca-ionophore and phorbol ester (TPA). This particular combination for stimulation was chosen based on optimal expression of all the relevant cytokines when compared to other modes of stimulation (R. de Waal Malefyt, DNAX, personal communication). In case of IL-10 a longer stimulation time would have been optimal, since IL-10 is produced late during immune responses. For IL-4 a shorter stimulation time would have been optimal, because IL-4 peaks relatively early after activation. In case of the other cytokines analyzed, IL-5, IL-13 and IFN-γ, 16-18 hours of stimulation was optimal. Because of the limited number of T cells available, we were forced to use only this one time point with and without stimulation, at which cytokine mRNA expression and protein production were measurable.

By studying cytokine mRNA expression and protein production in freshly isolated and short-term cultured T cells the intrinsic capacity of T cells to produce cytokines could be studied precisely. Possible influences, like cytokine production by other cell populations, were prevented. Non-T cells might have a significant effect on the IFN-γ and IL-4 levels in supernatants of polyclonally activated PBMC (7). Several investigators have focussed on the

generation of T cell clones and analysis of their cytokine production. Because cloning techniques may bias the frequency of specific types of clones, we decided to study cytokine profiles in bulk populations of T cells. Analysis of cytokine mRNA expression by the purified T cell fraction rather than PBMC showed a more differential cytokine expression between the various patient groups. Interestingly, most of the significant differences in cytokine mRNA expression and protein production were found after culturing the purified T cell fraction. Polyclonal stimulation of T cells thus augmented the intrinsic differences already present in the purified T cell fraction. However, polyclonal stimulation in some of the patients did not result in stimulation of mRNA expression or protein production, in particular of IL-4. This could be due to a concomitant increase in the expression level of the HPRT (hypoxanthine phosphoribosyl-transferase) house-keeping gene. Alternatively, the differential kinetics of expression of the cytokines studied (16-18 hrs) could be the reason for a suboptimal assay time in the case of IL-4 (7). Also a high baseline expression could result in difficulties to further stimulate the cells (7).

PCR-analysis

To study cytokine and germline-ε mRNA expression in the *ex vivo* situation we optimized a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique. This semi-quantitative PCR technique has several useful characteristics: it is a sensitive technique, which needs little starting material and enables the analysis of various cytokines in one sample. The different patient samples can be compared by means of standardization by their house-keeping gene expression (HPRT) and different experiments are comparable by means of standardization with a batch of B21 cDNA, which had a positive signal for all the cytokines analyzed. Based on new insights, the calculation method of standardization for house-keeping gene expression as described in chapter 2.2., was used in a slightly modified form in chapter 3.1. and following chapters. For each individual cytokine studied the linear phase of the PCR was determined, allowing a proper and sensitive comparison of gene expression. As soon as the plateau phase was reached, differences in gene expression disappeared. Several other studies have used variations of the semi-quantitative PCR-technique that we applied (8,9). A potential reduction of the sensitivity of this method was caused by the fact that, with polyclonal stimulation, in the T cells sometimes also the HPRT mRNA expression was upregulated. In such cases, after house-keeping gene correction the cytokine mRNA expression became comparable to the unstimulated condition. This was particularly the case for IL-4. For most other cytokines the culture period of 16-18 hrs was optimal, so that the effect had less or no influence. For the analysis of IL-5, IL-10, IL-13 and germline-ε mRNA expression the PCR signals were too weak to allow proper analysis of gene-expression directly from the photograph of the gel. Therefore, Southern blotting

and subsequent radioactive hybridization were necessary. Recently, also quantitative PCR methods have been developed for analysis of cytokine gene expression, but the relatively small increase in sensitivity as compared to our semi-quantitative PCR does not, as yet, warrant its application in these studies. Besides it's labour-intensive procedure, it generally requires larger sample sizes than currently available from these young children.

Intrinsic B and T cell differences

Due to ethical reasons, we had to limit our studies to the analysis of peripheral blood B and T cells. However, many of our findings in peripheral blood T cells are in agreement with studies in adults analyzing bronchial biopsies and bronchoalveolar lavage fluids, as will be discussed later. Since IgE producing plasma cells are normally not present in the peripheral blood, our analysis of purified B cells does not fully reflect the *in vivo* situation. After stimulation, however, analysis of isotype production potential reveals possible intrinsic differences among B cells of the various patient groups. Principally, we applied our techniques for comparison between the various patient groups.

In our studies we demonstrated strongly elevated serum IgE levels in children with allergic asthma and atopic dermatitis, as compared to healthy control levels, whereas IgE levels in children with non-allergic asthma were comparable to that of the healthy children. We also showed a significant positive correlation between total serum IgE levels and the height of the radio-allergosorbent test (RAST) class in children with atopic dermatitis, whereas in children with allergic asthma the correlation between total serum IgE levels and the height of the RAST class just failed to reach significance. The predisposition to develop antigen-specific IgE antibodies upon repeated exposure to low doses of foreign proteins (pollen, animal products, house dust mite, food, etc.) is the principal feature so far that distinguishes atopic from non-atopic individuals (10). This results in higher serum IgE levels in allergic individuals than in non-allergic. Cord blood IgE levels in conjunction with a positive family history (especially in the mother), can be useful for identifying infants at risk for early development of atopic disease. High total IgE levels at two and six months after birth showed no better predictive values for allergy development than cord blood IgE levels (11,12).

Recently, evidence of a linkage of high levels of total serum IgE to loci on chromosome 5q31.1, including the cytokine gene cluster, was reported (13,14). It was also demonstrated that elevated serum IgE is coinherited with a trait for bronchial hyperresponsiveness and that a gene governing bronchial hyperresponsiveness is located near a major locus that regulates serum IgE levels on chromosome 5q (15). One specific site on chromosome 11q accounts for 20% of the 11q-associated cases of allergy. This site represents a single amino acid change in the ß chain of the high affinity IgE receptor ($F_{C\varepsilon}RI$) (16). Furthermore, genetic linkage was found between a

gene (or genes) in the T-cell receptor α region, encoded on chromosome 14, and specific IgE responses (17). Thus, there is growing insight into the genetic basis of the immunological process in allergic diseases, next to the influence of environmental factors.

Since germline-ε mRNA expression precedes switching to IgE, we investigated whether differences existed between germline-ε mRNA expression in allergic and non-allergic children. We could not demonstrate significant differences in germline-ε mRNA expression between the four groups of children that we studied. We did, however, find a tendency of increased germline-ε mRNA levels in purified B cells of children with allergic and non-allergic asthma, indicative of a switching process occurring *in vivo* selectively in asthmatic children. In children with atopic dermatitis germline-ε mRNA expression was comparable to that in healthy controls.

Although IL-4 induces germline-ε mRNA expression in B cells, we demonstrated a discrepancy in children with atopic dermatitis between the significantly increased IL-4 mRNA expression by purified T cells and normal germline-ε levels, both as compared with healthy control children. This apparent contradiction between elevated IL-4 mRNA and normal germline-ε mRNA expression may result from B cells already switched to IgE *in vivo* and becoming independent from IL-4, as has been shown in an *in vitro* model (18) and a mouse-model (19). Further support that germline-ε mRNA expression decreases with rising IgE production was produced by the inverse relationship between these parameters in the atopic dermatitis patient group.

The elevation of $CD4^+CD45RO^+$ T cells in children with atopic dermatitis, which *in vitro* are capable of giving enhanced help for switching to IgE (20,21), might contribute to the enhanced IgE levels.

We also demonstrated elevated percentages of $CD4^+CD45RA^+$ T cells in non-allergic asthmatic children as compared with healthy controls. The increase in the CD4/CD8 ratio in non-allergic asthmatics was most likely due to an elevated percentage of $CD4^+CD45RA^+$ cells. The expression of IL-4 mRNA by purified T cells in children with non-allergic asthma resulted in germline-ε mRNA expression in this patient group. The relatively high percentage of naive T cells not capable of providing efficient help for switching to IgE may account for the limited IgE production. Differences in the ratio of $CD45RA^+/CD45RO^+$ cells (22,23), together with the differential density of CD40 ligand on these cells (24), have been suggested to play a role in altered IgE synthesis in atopic individuals versus non-atopics. The isotype analysis after B cell culturing demonstrated that B cells of children with non-allergic asthma have the potency to switch to IgE under proper stimulatory conditions. The role of natural killer (NK) cells in allergic diseases has not yet been elucidated. We found a relative decrease of NK cells in children with non-allergic asthma. Because NK cells are potential producers of IFN-γ (25), this would imply that in these children relatively less IFN-γ will be present.

The contribution of NK cell derived IFN-γ in the regulation of IgE synthesis in atopics, however, is still unclear. These findings support the concept that increasing age and repeated allergen exposure will result in more $CD4^+CD45RO^+$ T cells, able to provide efficient B cell help, resulting in higher IgE levels and subsequently in allergic symptoms.
In vitro IgE production in B cell cultures of children with allergic asthma was increased after the appropriate stimulation, as compared with that of healthy control children. As discussed in chapter 3.1. our results are in line with the general belief that elevated serum IgE levels result, in a large part, from clonal expansion of B cells able to produce IgE (26,27). We did not find an increase in IgG4 production in stimulated B cell cultures of allergic children as compared to healthy controls. On the one hand, as reviewed by Aalberse *et al.* (28) changes in IgE and IgG4 serum levels are found to be closely linked (28). On the other hand, a lack of correlation between allergen-specific IgE and IgG4 has also recently been shown (29). The relative contribution of B cells undergoing direct switching from IgM to IgE (30-32) and B cells in which sequential switching takes place via IgG4 (33,34), has not yet been established.
Based on the finding that activated/memory T cells are the prime source of cytokine production, the ratio between naive and activated/memory T cells is considered to play an important role in the cytokine gene expression and switching to IgE. In atopic dermatitis more activated/memory T cells are present and B cells have already switched to IgE and have become relatively independent of IL-4, whereas in non-allergic asthma naive T cells dominate and little switching to IgE takes place. Based on these criteria children with allergic asthma display parameters both of children with non-allergic asthma and of children with atopic dermatitis, having normal levels of naive as well as activated/memory T cells (chapter 3.1).
By analyzing cytokine mRNA expression and protein production in purified T cells, intrinsic differences in T cells between patient groups could be studied. In this way the possible influence of antigen presenting cell derived factors, such as prostaglandin E_2 (PGE_2) and IL-12, on T cell-derived cytokine mRNA expression and protein production was avoided.
As discussed in chapter 3.2., we found IL-4 expression to be elevated in allergic asthmatic children (IL-4 mRNA) as well as in children with atopic dermatitis (IL-4 mRNA and protein) as compared with healthy controls. Comparable findings of increased IL-4 levels were described in asthmatic patients (35-37) and in children with atopic dermatitis (38). Recently, in adult patients with atopic dermatitis an increased frequency of IL-4 producing T cells has been demonstrated, which may be due to alterations in transcription factors (39). We could not demonstrate an increase in IL-4 mRNA expression or production in children with non-allergic asthma. This is in agreement with similar findings at the IL-4 protein level in adult patients

with non-allergic asthma (40). Since we only found increased IL-4 expression in allergic children with asthma (and atopic dermatitis) and not in children with non-allergic asthma, we suggest that the high IL-4 expression is related more to the allergic disease process than the asthmatic state of the children. We showed that the increase of IL-5 mRNA expression and protein production as observed in stimulated T cells of children with asthma was even stronger in children with atopic dermatitis. This would be consistent with a potential role of IL-5 in the inflammatory process and enhanced IgE levels in atopic dermatitis. Comparable to asthma, atopic dermatitis is often accompanied by peripheral blood eosinophilia (41). A pathogenic role for eosinophils in atopic dermatitis has emerged recently (42).

In children with allergic asthma and atopic dermatitis both IL-4 and IL-5 mRNA expression and protein production were found to be elevated as compared to healthy control children. In children with non-allergic asthma IL-4 mRNA expression and protein production were comparable to healthy control levels, whereas IL-5 protein production was elevated. We found a co-expression between IL-4 and IL-5 mRNA expression in children with allergic asthma, atopic dermatitis and healthy children. A similar co-expression of IL-4 and IL-5 was reported previously in adult allergic asthma (43,44). This co-expression may be due to the close localization of the IL-5 gene to the IL-4 in the cytokine gene cluster on chromosome 5q31 (45). On the other hand, we did not find this co-expression between IL-4 and IL-5 in children with non-allergic asthma. This may be explained by a differential expression of these cytokines or differences in the kinetics of expression. The demonstration that IL-4 and IL-5 are rarely co-expressed by human T cells at the single cell level (46), provides evidence for the independent regulation of these cytokines, as observed in non-allergic asthma. A similar discrepancy between IL-4 and IL-5 expression was observed when comparing adult patients with allergic and non-allergic asthma (40) and atopic and 'nonatopic' (intrinsic) dermatitis (47).

Between the patient groups no differences were observed either with regard to IL-13 mRNA expression and IL-13 protein production. We did, however, demonstrate a positive correlation between IL-13 protein production by unstimulated PBMC and the SCORAD index in children with atopic dermatitis, suggesting a clinical relevance of IL-13 expression, most likely via IgE. The positive correlation between IL-13 protein production by unstimulated T cells of allergic asthmatic children and the height of the RAST class gives further support for the role of IL-13 in allergic disease, as suggested before (48,49). Further research into the role of IL-13 in allergic disease remains necessary. It has been demonstrated that the majority of $CD45RO^+$ T cells produced IL-4 and IL-13 simultaneously (50). This co-expression between IL-4 and IL-13 can be explained by the close localization of the IL-13 and IL-4 gene on chromosome 5q23.31 (51) and the use of common transcription

factors (52). Although IL-4 and IL-13 share many biological activities, there are also indications for a differential regulation of IL-4 from IL-13 (53).
In addition to the increase in IL-4 mRNA expression and protein production in children with atopic dermatitis, we found support for a post-translational defect in IFN-γ production in this patient group, which was suggested previously (54-56). Children with atopic dermatitis had a decreased IFN-γ production in unstimulated T cell cultures, which was not reflected in a diminished IFN-γ mRNA expression, as compared to healthy controls. Interestingly, in children with atopic dermatitis the IFN-γ mRNA expression by stimulated T cells was found to be increased as compared to healthy controls. This might be due to an earlier and more prolonged expression of IFN-γ mRNA following polyclonal stimulation, compared with healthy controls (57). Next to a post-translational defect in IFN-γ production in children with atopic dermatitis, an increased sensitivity to stimulation might thus be intrinsically present in their T cells.
These findings do not sustain the widespread belief of selective activation of T_h2-like cells in allergic disease, but instead suggest activation of both T_h1- and T_h2-like T cells. The imbalance of IL-4 and IFN-γ secretion as described in patients with atopic dermatitis (55,56,58-60) could reflect general activation of T cells, in the presence of an intrinsic post-translational defect in IFN-γ production. During severe acute exacerbations of allergic asthma, increased serum concentrations of IFN-γ occur, which decrease as the patients improve clinically (61). IFN-γ may contribute to the local inflammation, by activating macrophages and epithelial and endothelial cells (62,63). Recently, the possible role of $CD8^+$ T cells in IgE regulation has also been suggested (64,65). Although still controversial, comparable to $CD4^+$ lymphocytes, a T_h1- and T_h2-like cytokine profile of $CD8^+$ T cells has been implied (66-68). Because of the limited number of T cells available, we were not able to discriminate between $CD4^+$ and $CD8^+$ derived cytokine expression.
In children with allergic asthma we found, comparable to children with atopic dermatitis, also a downregulation, although not significant, of the spontaneous IFN-γ production by unstimulated T cells. IFN-γ production by unstimulated and stimulated T cells of non-allergic asthmatic children was found to be decreased as compared to healthy controls, in contrast to the absence of differences in IFN-γ production in a comparable study in adults (40).
We found a striking decrease in IL-10 mRNA expression by purified T cells of children with both allergic and non-allergic asthma and with atopic dermatitis as compared with healthy controls. After stimulation of T cells IL-10 mRNA expression of the patient groups was comparable to healthy control levels, indicative of the absence of an intrinsic T cell defect.
We did show an inverse correlation between IL-10 mRNA expression by purified T cells and the SCORAD index from children with atopic dermatitis,

indicative of a clinical relevance of decreased IL-10 expression. Furthermore, it has been shown that high IL-10 producing cells may have a general inhibitory function, since they will possibly inhibit both T_h1 and T_h2 cytokine production (69), antigen-specific proliferation of T cells, as well as monocyte activation. Therefore, we hypothesize that the decreased IL-10 expression in children with asthma and atopic dermatitis results in a diminished immunosuppressive function of T cells (70-72), leading to a lack of downregulation of the inflammatory process, caused among others by increased IL-4 and IL-5 expression. Supportive for a role of decreased IL-10 expression in allergic diseases is the inhibitory effect of IL-10 on IL-4 (73) and allergen-induced (74) IgE synthesis.
Monocyte-derived IL-10 could also play a role in the suppression of inflammation as suggested by a decreased cognate interaction between monocytes and regulatory T cells (75). Recently, it was demonstrated that both recombinant IL-10 and endogenous IL-10 were able to inhibit IL-5 production by T cells costimulated by B7/CD28 signalling (76). If *in vivo* production of IL-5 would prove to depend on B7/CD28-CTLA-4 interactions, possible therapeutic applications of IL-10 might include diseases related to IL-5 hyperproduction, such as hypereosinophilic syndromes (77) or bronchial asthma.
In conclusion, we showed that T cells of children with both allergic and non-allergic asthma and atopic dermatitis had elevated IL-5 expression, whereas IL-10 expression was strongly diminished in the same groups of patients, particularly by freshly purified T cells, as compared to healthy controls. The discrepancy in IL-4 and IL-5 expression in children with non-allergic asthma, in contrast to the co-expression of these cytokines in allergic asthma, suggests that there are basic immunologic differences between allergic and non-allergic asthma. The decreased spontaneous IFN-γ secretion, together with the decreased percentages of $CD4^+CD45RA^+$ T cells and NK cells, might be indications that children with non-allergic asthma are possibly in the process of developing allergic asthma .
The demonstration of increased IL-4 expression by T cells of children with allergic asthma and atopic dermatitis, and not in non-allergic asthma, together with an intrinsic defect in IFN-γ secretion in children with atopic dermatitis, indicates a differential regulation of IL-4 and IFN-γ cytokine production in these patient groups. IL-4 and IFN-γ are cytokines which are more involved in the IgE-mediated processes taking place in allergic diseases, whereas IL-5 and IL-10 are involved in the inflammatory mechanisms found in atopic dermatitis, allergic and non-allergic asthma. The role of IL-13 in these diseases awaits further investigation.

Adhesion molecules
In allergic asthmatic patients a correlation was found between the adhesion

molecule expression (E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) on the endothelium and the eosinophil and total leucocyte infiltrate (78), suggesting that adhesion molecule expression in the vessels of the bronchi might be involved in leucocyte and particularly in eosinophil infiltration. These adhesion molecules can also be present in soluble form (79). We investigated whether differences could be detected between the levels of soluble adhesion molecules in the plasma of allergic and non-allergic children.
We did not observe any differences in the plasma levels of the soluble adhesion molecules ICAM-1 and E-selectin between the patient groups which we studied. In adult allergic asthmatic patients increased serum levels of soluble ICAM-1 (sICAM-1) and E-selectin during asthma attacks have been demonstrated (78,80). From these studies it was not clear whether the levels of soluble adhesion molecules reflected the severity of the inflammation (80) or were related to the allergic status of the patients (78). Allergen provocation of asthmatic children, in agreement with our findings, did not result in a rise in sICAM-1 expression (81).
We found a linear correlation between plasma levels of sE-selectin and the SCORAD index in patients with atopic dermatitis, indicating that sE-selectin is presumably associated with disease severity in these patients. A correlation between sE-selectin and the severity of atopic dermatitis in adult patients was described previously (82). The clinical significance of this correlation needs to be investigated in a larger group of patients.
Elevated tumor necrosis factor-α (TNF-α) levels have been described in the serum of patients with atopic dermatitis (83) and in patients with asthma during an exacerbation (84). TNF-α was, however, not detectable in the serum of the patients we studied, presumably due to its short half-life (85). Therefore, we analyzed whether differences in sTNF-receptors (sTNF-R), which are considered to be more stable (86), were present. We did not find any differences in sTNF-receptor levels in the plasma of the patient groups we studied. However, in plasma collected from patients during an asthma attack (80) or during an exacerbation of atopic dermatitis (82,87) increased levels of sTNF-R have been found. This suggests that soluble adhesion molecules and sTNF-R levels reflect disease activity, and therefore may be useful as markers for the severity of inflammation.

Comèl-Netherton syndrome
Finally, we analyzed cytokine mRNA expression and protein production in two patients with Comèl-Netherton syndrome. The data on cytokine expression in these patients should be considered as an indication of the role that T cells and cytokines may play in this disease, which is associated with high serum IgE levels.
On the basis of their clinical characteristics both patients could be identified

as Comèl-Netherton patients, but immunopathologically the two patients were different. We found in both patients with Comèl-Netherton syndrome the combination of high IL-5 expression, low IL-10 expression, high numbers of eosinophils and high IgE levels as compared with healthy controls. In case of patient 2 this was also true compared with atopic dermatitis. This may indicate that IL-5 plays a role in the eosinophilia and may contribute to the enhanced IgE levels observed in Comèl-Netherton patients (88,89). The low expression of IL-10 may result in a less potent inhibition of inflammatory responses in these patients, because IL-10, as stated above, is a strong downregulator of inflammatory responses (70-72).
Analysis of IL-4 and IFN-γ expression showed seemingly contradictory results between the two patients. High IL-4 expression, as observed in one patient, as well as low IFN-γ expression, as observed in the other, can lead to high IgE levels. Comparable to our studies in allergic children, we could not find differences in IL-13 expression between Comèl-Netherton patients and healthy control or atopic dermatitis children. The finding of low percentages of NK cells in both Comèl-Netherton patients is also interesting, since NK cells can be potential producers of IFN-γ (25). In the two Comèl-Netherton patients we studied, we did not observe a distinct association between the differences in immunopathological parameters in the two patients and their disease severity. These observations need to be confirmed in a larger patient group.

Highlights per patient group
In children with **non-allergic asthma** we found the following significant differences compared to healthy control children:

- an increased percentage of $CD4^+CD45RA^+$ naive T cells
- increased CD4/CD8 ratios
- a decreased percentage of NK cells
- a decreased IL-10 mRNA expression by purified T cells

Children with **allergic asthma** displayed, compared to healthy control children:

- increased serum IgE levels
- increased IL-4 and IL-5 mRNA expression by polyclonally stimulated T cells
- a decreased IL-10 mRNA expression by purified T cells

Children with **atopic dermatitis** had the following characteristics as compared to healthy control children:

- increased serum IgE levels
- increased percentages of $CD4^+CD45RO^+$ activated/memory T cells
- increased IL-4 mRNA expression by purified T cells

- increased IL-4 and IL-5 mRNA expression and protein production by polyclonally stimulated T cells
- a decreased IL-10 mRNA expression by purified T cells

Conclusions

The studies described in this thesis have lead to the following conclusions relating to the questions raised in the aims of the study:

- In children with atopic dermatitis relatively more activated/memory T cells are present; B cells have already switched to IgE and have become relatively independent of IL-4. In children with non-allergic asthma naive T cells dominate and little switching to IgE takes place. Children with allergic asthma have characteristics both of children with non-allergic asthma and of children with atopic dermatitis, with normal levels of naive as well as activated/memory T cells.
- Our findings do not sustain the widespread belief of selective activation of T_h2-like cells in allergic disease. Instead, these findings suggest activation of both T_h1- and T_h2-like T cells, with an extensive shift in the IL-4/IFN-γ ratio. The imbalance of IL-4 and IFN-γ secretion in patients with atopic dermatitis could reflect general activation of T cells, in combination with an intrinsic defect in IFN-γ secretion.
- The decreased IL-10 expression in children with asthma and atopic dermatitis may result in a diminished immunosuppressive function of T cells, leading to a lack of downregulation of the inflammatory process, caused among others by increased IL-4 and IL-5 expression.

Ultimately, we aim at finding parameters with a predictive value for allergy development in young children, so that preventive measures can be undertaken in time and for the appropriate risk groups. In this thesis we investigated the various parameters in a cross-sectional study. Based on the data obtained, at present a longitudinal study is in progress following high-risk children from birth onward.

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SUMMARY

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During the first years of life there is an enhanced risk of allergic sensitization that can lead to development of allergic diseases in childhood and later on in life. The very property of the immune system of young children to be in development contributes to this enhanced risk. In order to get more insight in the development of allergic diseases, we investigated the immune system of young children (0-4 years) in a cross-sectional study.

In *chapter 1.1* we discuss the influence of genetic (specific loci) and environmental factors (allergen exposure, viral infections, smoke), considered to be involved in the pathogenesis of allergic diseases. These factors may modulate the T cell function as well as several other cell types of the newborn immune system, contributing to the development of allergic disease. It is discussed that irregularities in IgE synthesis, helper T cell subsets (T_h1, T_h2, CD45RA, CD45RO), cytokines (IL-4, IFN-γ, IL-13) and possibly cell types other than T cells may play a role in the development of allergic disease in childhood. Also the potential role of cell surface markers, such as co-stimulatory molecules, activation markers and adhesion molecules is discussed. Finally, the sensitivity and specificity of current predictive factors for the development of allergic diseases, such as cord blood IgE levels, are discussed. Based on our current understanding of the immunopathology of allergic disease, new therapeutic approaches, like cytokine therapy, are considered.

Chapter 1.2 describes the patient groups we studied in this thesis. Children with asthma were selected according to the criteria of the American Thoracic Society, had a positive family history of atopy and/or asthma and did not use corticosteroids. We analyzed two subgroups of asthmatic children: an allergic asthmatic group and a non-allergic asthmatic group, based on the presence or absence of specific IgE in their serum and allergic symptoms. Children with atopic dermatitis were included in the study based on typical skin lesions and localization, a chronical recurrent course and the presence of a family history of atopy. Healthy control children were age-matched, had no specific IgE in their serum, and neither allergic symptoms nor a family history of atopy.

In *chapter 1.3* the aims of the studies presented in this thesis are described. The main question was whether intrinsic T and/or B cell differences occur between allergic and non-allergic children. Therefore, we have developed techniques to asses several functional aspects of B and T cells in the small volumes of blood that can be obtained from young children. We also apllied these techniques to characterize a related disease, Comèl-Netherton syndrome.

In *chapter 2* the techniques developed and applied to study these aims are described. In *chapter 2.1* techniques for B and T cell purification and their subsequent culturing are described. B cells were purified by positive selection, whereas T cells were negatively selected in order to avoid activation. To analyze the *ex vivo* germline-ε and cytokine mRNA expression,

aliquots of B and T cells were taken directly after purification. Intrinsic differences were analyzed by polyclonal stimulation with a T_h0 clone and IL-4 (for B cells) and Ca-ionophore and TPA (for T cells). *Chapter 2.2* describes the semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) we used to analyze germline-ε and cytokine mRNA expression. Important characteristics are standardization for inter-patient and inter-experimental variation and analysis in the linear phase of the reaction. Furthermore, specific software was developed to analyze densitometric data obtained.
Chapter 3.1 describes the analysis of possible B and T cell differences between allergic and non-allergic children, by investigating serum IgE levels, IgE synthesis after B cell stimulation and *ex vivo* germline-ε and IL-4 mRNA expression. Based on the expression of these parameters in children with atopic dermatitis, we postulate that B cells from children with atopic dermatitis have already switched to IgE *in vivo* and have become relatively independent from IL-4. The elevated percentage of activated/memory T cells observed in children with atopic dermatitis may contribute to the enhanced IgE levels. Although *in vitro* B cells from children with non-allergic asthma had the potency to switch to IgE, *in vivo* limited switching to IgE takes place in these children. This is possibly due to the high percentage of naive T cells, not able to deliver adequate help for switching to IgE. We hypothesize that with increasing age and repeated allergen exposure, relatively more naive T cells will develop into activated/memory T cells. This may result in higher IgE levels and subsequently in allergic symptoms.
In *chapter 3.2* we provide evidence that the high IL-4 expression observed in children with allergic asthma is related more to the allergic disease process than to the asthmatic state of the children. In children with atopic dermatitis we demonstrated a discrepancy between the increased IFN-γ mRNA expression and the reduced IFN-γ protein production in unstimulated T cells, suggesting a post-translational defect in IFN-γ production. No differences were found in IL-13 mRNA expression or protein production between the patient groups. We did, however, show significant correlations between the extent of IL-13 expression and clinical parameters in children with allergic asthma and atopic dermatitis.
As described in *chapter 3.3* we found that the IL-5 mRNA expression and protein production in different T cell fractions of children with allergic and non-allergic asthma as well as in atopic dermatitis were increased as compared to healthy controls. This increased IL-5 expression is probably related to the eosinophilia and inflammation in both types of asthma and atopic dermatitis. We demonstrated that both the IL-4 and IL-5 expression are increased in children with allergic asthma and atopic dermatitis. In children with non-allergic asthma only the IL-5 expression was found to be increased. Furthermore, a strong decrease in IL-10 mRNA expression by purified T cells of children with allergic and non-allergic asthma and children with atopic

dermatitis was found as compared with healthy control children. The decreased IL-10 expression in these diseases may result in a diminished immunosuppressive function of T cells. This may account for the reduced downregulation of the inflammatory process in these diseases.

Chapter 4 describes the analysis of plasma levels of soluble (s)ICAM-1, sE-selectin, TNF-α and sTNF-receptor subunit 55 and 75 in children with allergic and non-allergic asthma, atopic dermatitis and healthy controls. This analysis was of special interest because soluble adhesion molecules and soluble TNF-receptor levels may reflect disease severity. Although plasma levels of these molecules, except for TNF-α, were detectable in all children studied, no significant differences in the levels of these molecules were found between the studied patient groups and the healthy controls. We did, however, find an association between the levels of sE-selectin and disease severity in children with atopic dermatitis.

In *chapter 5* we applied the developed techniques for studying cytokine mRNA expression and protein production, to characterize cytokine profiles in two young children with Comèl-Netherton syndrome. Comèl-Netherton syndrome is a disease characterized by atopic dermatitis-like skin eruptions, hair abnormalities, elevated IgE levels and food allergy. Both the high IL-4 expression detected in one child with Comèl-Netherton syndrome and the low IFN-γ expression in the other child, can explain the high IgE levels in the children. The relatively high IL-5 expression detected in both patients indicates that IL-5 may play a role in the eosinophilia and the high IgE levels. As we found a low IL-10 expression, this may account for a decreased inhibition of the inflammatory reactions in these patients.

In *chapter 6* the composition of the patient groups, the analysis methods used and the results presented in this thesis are discussed against the background of literature. The following conclusions relating to the study aims are presented:

- Based on the finding that activated/memory T cells are the prime source of cytokine production, the ratio between naive and activated/memory T cells is considered to play an important role in the cytokine gene expression and switching to IgE. In children with atopic dermatitis relatively more activated/memory T cells are present; B cells have already switched to IgE and have become relatively independent of IL-4. In children with non-allergic asthma naive T cells dominate and little switching to IgE takes place. Children with allergic asthma have characteristics both of children with non-allergic asthma and of children with atopic dermatitis, with normal levels of naive as well as activated/memory T cells.
- Our findings do not sustain the widespread belief of selective activation of T_h2-like cells in allergic disease. Instead, our findings suggest

activation of both T_h1- and T_h2-like T cells, with an extensive shift in the IL-4/IFN-γ ratio. The imbalance of IL-4 and IFN-γ secretion in patients with atopic dermatitis could reflect general activation of T cells, in combination with an intrinsic defect in IFN-γ secretion.
The decreased IL-10 expression in children with asthma and atopic dermatitis may result in a diminished immunosuppressive function of the T cells, leading to a lack of downregulation of the inflammatory process, caused among others by the increased IL-4 and IL-5 expression.

SAMENVATTING

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Gedurende de eerste levensjaren bestaat er een verhoogd risico op allergische sensibilisatie, hetgeen kan leiden tot ontwikkeling van allergieën op de kinderleeftijd en in het latere leven. Het feit dat het immuunsysteem van jonge kinderen nog sterk in ontwikkeling is, draagt bij tot dit verhoogde risico. Om meer inzicht te krijgen in de ontwikkelingen van allergieën, onderzochten wij het immuunsysteem van jonge kinderen (0-4 jaar) door middel van een transversale studie.

In *hoofdstuk 1.1* wordt de invloed besproken van genetische (specifieke loci) en omgevingsfactoren (allergeen expositie, virale infecties, rook), waarvan gedacht wordt dat ze betrokken zijn bij de pathogenese van allergische aandoeningen. Deze factoren kunnen de functie van T cellen, maar ook van verscheidene andere celtypen van het neonatale immuunsysteem moduleren en daardoor bijdragen tot de ontwikkeling van allergieën. Er wordt besproken hoe afwijkingen in de IgE synthese, T helper cel subsets (T_h1, T_h2, CD45RA, CD45RO), cytokinen (IL-4, IFN-γ, IL-13) en mogelijk andere celtypen, een rol kunnen spelen in de ontwikkeling van allergie op de kinderleeftijd. Ook de potentiële rol van celmembraanmarkers, zoals co-stimulatoire moleculen, activatiemarkers en adhesiemoleculen, wordt bediscussiëerd. Tenslotte worden de sensitiviteit en specificiteit van de huidige predictieve factoren voor allergieontwikkeling, zoals de bepaling van IgE spiegels in navelstrengbloed, besproken. Gebaseerd op onze huidige kennis van de immunopathologie van allergische aandoeningen worden nieuwe therapieën, zoals cytokine therapie, overwogen.

Hoofdstuk 1.2 beschrijft de patiëntengroepen die wij bestudeerd hebben in dit proefschrift. Kinderen met astma werden geselecteerd volgens de criteria van de "American Thoracic Society". Zij hadden een positieve familiegeschiedenis met betrekking tot atopie en/of astma en gebruikten geen corticosteroïden. Twee subgroepen van kinderen met astma werden geanalyseerd: een groep met allergisch astma en een groep met niet-allergisch astma, gebaseerd op de aan- of afwezigheid van specifiek serum IgE en allergische symptomen. Kinderen met atopische dermatitis werden geïncludeerd in de studie op basis van typische huidafwijkingen en de lokalisatie daarvan, een chronisch terugkerend verloop van de ziekte en de aanwezigheid van een positieve familiegeschiedenis voor atopie. De gezonde controle kinderen hadden dezelfde leeftijdsverdeling, hadden geen serum specifiek IgE, geen allergische symptomen en ook geen positieve familiegeschiedenis voor atopie.

In *hoofdstuk 1.3* worden de doelstellingen beschreven van de gepresenteerde studies. De belangrijkste vraag van dit proefschrift was of er intrinsieke verschillen in T en/of B cellen bestaan tussen allergische en niet-allergische kinderen. Daartoe hebben wij methoden ontwikkeld om een aantal functionele aspecten van B en T cellen te onderzoeken in kleine volumina bloed die van jonge kinderen kunnen worden afgenomen. Wij hebben deze

technieken ook toegepast om een gerelateerde ziekte, het Comèl-Netherton syndroom, te karakteriseren.
In *hoofdstuk 2* worden de technieken beschreven die ontwikkeld en toegepast werden in ons onderzoek. *Hoofdstuk 2.1* beschrijft de technieken voor de zuivering van B en T cellen en de daarop volgende kweekcondities. B cellen werden gezuiverd middels positieve selectie, terwijl T cellen negatief geselecteerd werden, ter voorkoming van activatie. Om de *ex vivo* germline-ε en cytokine mRNA expressie te analyseren, werden onmiddellijk na de celzuivering monsters van B en T cellen afgenomen. De intrinsieke verschillen werd geanalyseerd door middel van polyclonale stimulatie met een T_h0 kloon en IL-4 (voor B cellen) en met behulp van Ca-ionofoor en TPA (voor T cellen). *Hoofdstuk 2.2* beschrijft de semi-kwantitatieve polymerase ketting reactie, waarmee wij de germline-ε en cytokine mRNA expressie hebben geanalyseerd. Belangrijke karakteristieken van deze methode zijn de standaardisatie voor variatie tussen patiënten en tussen experimenten onderling en de analyse in het lineaire gebied van de reactie. Voorts werd er speciale software ontwikkeld om de verkregen densitometrische data te analyseren.
Hoofdstuk 3.1 beschrijft de analyse van mogelijke B en T cel verschillen tussen allergische en niet-allergische kinderen, door het onderzoeken van serum IgE spiegels, IgE synthese na B cel stimulatie en *ex vivo* germline-ε en IL-4 mRNA expressie. Gebaseerd op de expressie van deze parameters in kinderen met atopische dermatitis, postuleren wij dat B cellen van kinderen met atopische dermatitis *in vivo* al geswitcht zijn naar IgE en grotendeels onafhankelijk van IL-4 zijn geworden. Het verhoogde percentage geactiveerde/geheugen T cellen dat werd waargenomen in kinderen met atopische dermatitis draagt mogelijk bij aan de verhoogde IgE spiegels. Hoewel B cellen van kinderen met niet-allergisch astma *in vitro* de potentie hadden om te switchen naar IgE, vond er *in vivo* beperkte switching naar IgE plaats in deze kinderen. Dit werd mogelijk veroorzaakt door het hoge percentage naïeve T cellen, die niet in staat zijn voldoende hulp te leveren voor switching naar IgE. Wij formuleerden daarom de hypothese dat met toenemende leeftijd en herhaalde blootstelling aan allergenen, relatief meer naïeve T cellen zich zullen ontwikkelen tot geactiveerde/geheugen T cellen. Dit kan resulteren in hogere IgE spiegels en vervolgens in allergische symptomen.
In *hoofdstuk 3.2* leveren wij bewijs voor het feit dat de hoge IL-4 expressie, die werd waargenomen in kinderen met allergisch astma, meer gerelateerd was aan het allergische ziekteproces, dan aan de astmatische staat van de kinderen. Wij laten zien dat er in kinderen met atopische dermatitis een discrepantie bestaat tussen verhoogde IFN-γ mRNA expressie en verlaagde IFN-γ eiwit productie door ongestimuleerde T cellen, hetgeen duidt op een post-translationeel defect in IFN-γ productie. Er werden geen verschillen gevonden in IL-13 mRNA expressie of eiwitproductie tussen de patiëntengroepen. Wij toonden echter wel significante correlaties aan tussen

de mate van IL-13 expressie en klinische parameters bij kinderen met allergisch astma en atopische dermatitis.
Zoals beschreven in *hoofdstuk 3.3*, werd gevonden dat de IL-5 mRNA expressie en eiwitproductie in verschillende T cel fracties van kinderen met allergisch en niet-allergisch astma en kinderen met atopische dermatitis verhoogd waren in vergelijking met gezonde controles. Deze verhoogde IL-5 expressie is waarschijnlijk gerelateerd aan de eosinofilie en het ontstekingsproces in beide soorten astma en in atopische dermatitis. Ook bleek er een relatie te bestaan tussen de toegenomen IL-4 en IL-5 expressie in kinderen met allergisch astma en atopische dermatitis. Bij kinderen met niet-allergisch astma was alleen de IL-5 expressie toegenomen. Voorts werd een sterk verlaagde IL-10 mRNA expressie gevonden in gezuiverde T cellen van kinderen met allergisch en niet-allergisch astma en kinderen met atopische dermatitis, vergeleken met gezonde controle kinderen. De verlaagde IL-10 expressie in deze ziekten kan resulteren in een verminderde immunosuppressieve functie van T cellen, en dus in minder remming van het ontstekingsproces in deze ziekten.
Hoofdstuk 4 beschrijft de analyse van oplosbaar ICAM-1, oplosbaar E-selectine, TNF-α en oplosbare TNF-receptor subunit 55 en 75 plasma spiegels in kinderen met allergisch en niet-allergisch astma en atopische dermatitis, vergeleken met gezonde controles. Deze analyse was vooral interessant omdat de spiegels van oplosbare adhesiemoleculen en oplosbare TNF-receptoren mogelijk een maat zijn voor de ernst van de ziekte. Hoewel de plasmaspiegels van deze moleculen, met uitzondering van TNF-α, detecteerbaar waren in alle onderzochte kinderen, werden geen significante verschillen gevonden in de spiegels van deze moleculen tussen de patiëntengroepen en de gezonde controles. We vonden echter wel een associatie tussen de hoeveelheid oplosbaar E-selectine en de ernst van de ziekte in kinderen met atopische dermatitis.
In *hoofdstuk 5* pasten we de ontwikkelde technieken om de cytokine mRNA expressie en eiwitproductie te bestuderen toe op de karakterisering van het cytokineprofiel van twee jonge kinderen met het Comèl-Netherton syndroom. Dit profiel werd vergeleken met dat van kinderen met atopische dermatitis en met gezonde controles. Comèl-Netherton syndroom is een ziekte die gekenmerkt wordt door atopische dermatitis-achtige huidafwijkingen, haarabnormaliteiten, verhoogde IgE spiegels en voedselallergie. Zowel de hoge IL-4 expressie die werd gedetecteerd in één kind met Comèl-Netherton syndroom als de lage IFN-γ expressie in het andere kind kunnen de oorzaak zijn van de waargenomen hoge IgE spiegels. De relatief hoge IL-5 expressie, die gedetecteerd werd in beide patiënten met Comèl-Netherton syndroom, wijst erop dat IL-5 mogelijk een rol speelt in de eosinofilie en de hoge IgE spiegels die worden waargenomen in patiënten met Comèl-Netherton. De lage IL-10 expressie die werd gevonden, is mogelijk verantwoordelijk voor een afgeno-

men remming van ontstekingsreactie in deze patiënten.
In *hoofdstuk 6* worden de samenstelling van de patiëntengroepen, de gebruikte analysemethoden en de gepresenteerde resultaten bediscussiëerd tegen de achtergrond van de literatuur. De volgende conclusies worden met betrekking tot de doelstellingen van de studie gepresenteerd:

- De ratio tussen naïeve en geactiveerde/geheugen T cellen speelt een belangrijke rol in de cytokine genexpressie en switching naar IgE. Bij kinderen met atopische dermatitis zijn relatief meer geactiveerde/geheugen T cellen aanwezig; B cellen in deze kinderen zijn al geswitcht naar IgE en relatief onafhankelijk geworden van IL-4. Bij kinderen met niet-allergisch astma domineren naïeve T cellen en vindt er relatief weinig switching naar IgE plaats. Kinderen met allergisch astma blijken kenmerken te hebben van kinderen met niet-allergisch astma en van kinderen met atopische dermatitis. Zij hebben zowel normale percentages naïeve als geactiveerde/geheugen T cellen.
- Onze bevindingen vormen geen ondersteuning voor de wijdverbreide gedachte dat alleen T_h2-achtige cellen selectief worden geactiveerd in allergische ziekten. In plaats daarvan wijzen onze bevindingen op een activatie van zowel T_h1- als T_h2-achtige cellen, waarbij een grote verschuiving in de IL-4/IFN-γ ratio optreedt. De dysbalans tussen IL-4 en IFN-γ secretie in patiënten met atopische dermatitis reflecteert mogelijk een algemene T celactivatie, in combinatie met een intrinsiek defect in IFN-γ secretie.
- De verlaagde IL-10 expressie bij kinderen met astma en atopische dermatitis kan resulteren in een verminderde immunosuppressieve functie van de T cellen, hetgeen ertoe leidt dat het, onder andere door verhoogde IL-4 en IL-5 expressie veroorzaakte, ontstekingsproces minder geremd wordt.

ABBREVIATIONS

AA	allergic asthma
ABTS	2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)
AD	atopic dermatitis
APC	antigen presenting cell
BAL	bronchoalveolar lavage
bp	basepair
BSA	bovine serum albumin
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	copy deoxyribonucleic acid
CLA	cutaneous lymphocyte-associated antigen
CNS	Comèl-Netherton syndrome
dNTP	deoxynucleotide triphosphate
EASIA	enzyme-amplified sensitivity immuno assay
e.g.	exempli gratia (for example)
ELAM	endothelial-leucocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
FACScan	fluorescence activated cell scanner
Fc	crystallizable fragment
$Fc_{\varepsilon}RI$	high affinity receptor for IgE
$Fc_{\varepsilon}RII$	low affinity receptor for IgE
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAH	goat anti human
GAM	goat anti mouse
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
HC	healthy controls
HPRT	hypoxanthine phosphoribosyl-transferase
ICAM	intercellular adhesion molecule
i.e.	id est (that is)
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LFA	lymphocyte function associated antigen
LPS	lipopolysaccharide
mAb	monoclonal antibodies
MACS	magnetic activated cell sorter
MAH	mouse anti human
MHC	major histocompatibility complex
MNC	mononuclear cells
mRNA	messenger ribonucleic acid

n	number in study or group
NAA	non-allergic asthma
NK	natural killer
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PGE	prostaglandin
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
r	recombinant (e.g. rIFN-γ)
R	receptor (e.g. in IL-4R)
RAST	radio-allergo-sorbent test
r_s	Spearman's rank correlation coefficient
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase-polymerase chain reaction
s	soluble (e.g. in sE-selectin)
SAFT	skin application food test
SCORAD	severity scoring of atopic dermatitis
TCR	T cell receptor
TGF	transforming growth factor
T_h	T helper
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
VCAM	vascular cell adhesion molecule
VLA	very late antigen
YM	Yssel's medium

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Nederhorst den Berg, augustus 1996

CURRICULUM VITAE

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1988 - 1989	:	Postdoctorale 1-jarige universitaire lerarenopleiding Rijks Universiteit Utrecht
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- Gebruik van radionucliden bij wetenschappelijk onderzoek, Utrecht
- Proefdierkunde (als bedoeld in artikel 2, tweede lid, van het Dierproevenbesluit), Utrecht
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- Opleiding tot immunoloog (Stichting voor opleiding tot Medisch-Biologisch Wetenschappelijk Onderzoeker), Rotterdam

When I am driven to the ground
And I feel that I'm going down
It's good to know that You're around
To help me hold on

Oh, when my light is going pale
And I feel that I'm bound to fail
Your love will still prevail
To help me hold on

When I can't face another day
When I am hurt by what people say
When my strength is slippin' away
You help me hold on

Paul Field

DaNiElle, Details, 1994