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IMMUNOLOGICAL FACTORS IN BREASTFED INFANTS ALLERGIC TO COW'S MILK

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Academic dissertation

To be discussed publicly, by permission of the Medical Faculty of the University of Helsinki, in the auditorium of Skin and Allergy Hospital, Meilahdentie 2, Helsinki, on October 10th, 2003, at 12 noon.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals (I-V).

I Österlund P, Järvinen K-M, Laine S, Suomalainen H. Defective tumour necrosis factor-alpha production in infants with cow's milk allergy. Pediatr Allergy Immunol 1999: 10: 186-190.

II Österlund P, Suomalainen H. Low frequency of CD4+, but not CD8+, T cells expressing interferon-γ is related to cow's milk allergy in infancy. Pediatr Allergy Immunol 2002: 13: 262-268.

III Österlund P, von Willebrand M, Andersson LC, Suomalainen H. T-cell signal transduction in children with cow's milk allergy – increased MAP kinase activation in patients with acute symptoms of cow's milk allergy. Pediatr Allergy Immunol 2003: 14: 163-168.

IV Österlund P, Smedberg T, Schröder J, Järvinen K-M. The expression of intercellular adhesion molecules on circulating lymphocytes in relation to different manifestations of cow's milk allergy. Clin Exp Allergy 2003: in press.

V Österlund P, Smedberg T, Hakulinen A, Heikkilä H, Järvinen K-M. Eosinophil cationic protein in human milk is associated with development of cow's milk allergy and atopic eczema in breastfed infants. Pediatr Res 2003: in press.

Some previously unpublished data are also presented.

ABBREVIATIONS

AD	atopic dermatitis
ANOVA	analysis of variance
APC	antigen presenting cell
BLG	β-lactoglobulin
BSA	bovine serum albumin
CD	cluster of differentiation
CI	confidence interval
CLA	cutaneous lymphocyte antigen
CMA	cow's milk allergy
ConA	concanavalin A
CSF	colony stimulating factor
CTLA	cytotoxic T lymphocyte-associated antigen
DC	dendritic cell
ECP	eosinophil cationic protein
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GALT	gut-associated lymphoid tissue
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
MALT	mucosal associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein

MHC	major histocompatibility complex
MICA, B	MHC class I-related chain A, B
MIP	macrophage inflammatory protein
MGG	May-Grünwald-Giemsa
NK	natural killer
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA	phytohemagglutinin
PMA	phorbol 12-myristate 13-acetate
RANTES	regulated on activation, normally T-cell expressed and secreted
RAST	radioallergosorbent test
RPMI	Rosswell Park Memorial Institute
RSV	respiratory syncytial virus
RT	room temperature
sIgA	secretory immunoglobulin A
SPT	skin prick test
SSC	side scatter
TCR	T-cell receptor
TGF	transforming growth factor
Th	helper T cells
TNF	tumor necrosis factor
Tr	regulatory T cell

ABSTRACT

The allergic diseases commonly start as early as during the first months of life, when the immune system is immature. Often, cow's milk allergy (CMA) is an early manifestation of allergic disorder in infancy. The incidence of allergic diseases is increasing in westernized countries, which is suggested to be due to changes in microbial exposure. Maturation of helper T-cells (Th) with Th1 cytokine profile, required for defense against infections, has been postulated to be delayed in allergic diseases. However, the immune mechanisms behind the development of CMA are still incompletely understood.

The purpose of this study was to characterize some activation markers of immune responses in breastfed infants who were in an early stage of developing CMA, and in healthy control infants. In addition, the influence of mother's milk composition on the development of an allergic disease in the breastfed was assessed. The study comprised 131 infants who were followed-up prospectively for development of allergies, and breastfeeding mothers of 104 of them. Peripheral blood samples from the infants and breast milk samples from the mothers were collected.

The present study demonstrates a defective production of proinflammatory cytokines, IFN-γ and TNF-α from PBMCs in infants at an early stage of developing CMA. Furthermore, the IFN-γ deficiency was typical for the CD4+ T cell population. The phosphorylation of MAP kinases, reflecting the activation of leukocytes, was measured from the PBMCs of the infants. The MAP kinases were vigorously activated in the delayed manifestations of CMA and at the symptomatic phase of the disease. This activation was comparable to that for healthy infants and infants with CMA who were treated successfully with an elimination diet. Expression of co-stimulatory adhesion molecules on the circulating lymphocytes was analyzed to assess their activation state, and also in clinically different manifestations of CMA to evaluate the role of these molecules in the migration of lymphocytes to different target organs. A higher proportion of ICAM-1 expressing lymphocytes was detected in multiorgan and gastrointestinal manifestations of CMA, and a higher proportion of LFA-1 was found in cutaneous manifestations of CMA. To evaluate the influence of a cytotoxic mediator in mother's milk on the development of food allergies in the breastfed infant, the presence of eosinophil cationic protein (ECP) from mother's

milk was measured and correlated to the health status of the infant. This analysis showed that the presence of ECP in mother's milk was associated with development of food allergy and atopic dermatitis in the breastfed infant.

In conclusion, low numbers of Th cells expressing IFN-γ may play a role in the pathogenesis of CMA. Delayed-type responses are associated with a vigorous MAP kinase activity in the symptomatic phase of CMA, reflecting elevated activation of lymphocytes. The increased frequency of ICAM-1 and LFA-1 expressing lymphocytes in different manifestations of CMA may reflect the activation of lymphocytes and a possible role of these molecules in homing the lymphocytes in to their effector organ. ECP, known to be a tissue-destructive mediator of allergic inflammation, in mother's milk may contribute to the development of allergic disease in the infant.

INTRODUCTION

Cow's milk allergy (CMA) is defined as an immunologically mediated adverse reaction against cow's milk antigens (Savilahti et al. 1992). CMA is an early manifestation of allergic diseases in infancy. The symptoms of CMA frequently appear during the first months of life, usually a few days or weeks after the start of feeding with cow's milk-based formula, or even during exclusive breastfeeding (Jakobsson and Lindberg 1979, Machtinger and Moss 1986, Isolauri et al. 1999, Järvinen et al. 1999a). The prognosis of CMA, however, has been suggested to be favorable; more than 70% of the affected infants can tolerate cow's milk without symptoms at the age of 3 years (Bock 1987). However, in such infants it is common that other food allergies, hay fever and even asthma are the outcomes of "allergic march" later in childhood and adulthood.

The immune mechanisms behind development of cow's milk allergy are still incompletely understood. An inherited predisposition, together with multiple environmental contributing factors, triggers the development of allergic immune responses. There is some evidence supporting delayed maturation of helper T-lymphocytes (Th) with Th1-type cytokine profile (interferon-γ (IFN-γ)) in CMA and other allergic diseases after the physiologic Th2-type cytokine (interleukin-4 (IL-4), IL-5, IL-13) environment *in utero* (Prescott et al. 1999). The Th1-type responses required for defense against infections are thought to be induced by external factors during early postnatal life. Potential candidates for this kind of stimulation are adequate exposure to microbes and immunoregulatory effects of the mother's breast milk (Holt et al. 1997, Järvinen and Suomalainen 2001, Brandtzaeg 2002).

CMA is considered to be an example of a disturbance in the development or breakdown of oral tolerance to ingested cow's milk proteins (Mowat 1987, Suomalainen et al. 1993a). The unprocessed food antigens that pass the mucosal barrier in the newborn intestine activate the inexperienced immune system of the infant, normally resulting in a state of systemic hyporesponsiveness (oral tolerance) and the local generation of the antigen-specific immunoglobulin A (IgA) secreting B cells (Mowat 1987). Also, the commencing microbial antigens evoke systemic responsiveness and IgA production at the same time, whereas the pathogenic microbes cause activation of specific inflammatory responses (Brandtzaeg 2002). The mechanisms involved in this specific discrimination of antigens

are still largely unknown. The hypersensitivity reactions and allergic inflammation represent a failure to handle the commonplace antigens, leading to adverse immune reactions (Strobel and Mowat 1998).

CMA, like other food allergies, is a disease that is manifested in multiple effector organs, some distant from the initial sensitization site in the gut. Thus, while there being local allergic inflammation reactions, the immune reactions seen in the circulation may reflect the systemic immunologic situation of the infant. This may be a potential window for monitoring this type of multiorgan disease and investigating the pathogenesis of food allergy. The purpose of this study was to compare and define the features of the peripheral blood lymphocytes from breastfed babies with CMA relative to those without CMA.

REVIEW OF THE LITERATURE

1 MATURATION OF THE IMMUNE SYSTEM

1.1 Prenatal maturation

Numerous studies have reported that the newborn infant can display an antigen-specific T-cell reactivity to many common environmental antigens, including allergens (Kondo et al. 1992, Warner et al. 1994, van Duren-Schmidt et al. 1997, Prescott et al. 1998) and microbial antigens (Legg et al. 2002). Although immune reactions in the fetus are suppressed to prevent it from attacking the maternal tissues, antigen-specific T-cell responses at birth indicate intrauterine sensitization and priming of the fetal immune system. Both transplacental (Szepfalusi et al. 2000, Dahl et al. 1984, Holloway et al. 2000) and transamniotic (Dahl et al. 1984, Holloway et al. 2000) routes of exposure to antigens have been documented. The fetal gut as a site of antigen priming during life *in utero* has been postulated in a recent study (Jones et al. 2001). These workers documented that the human leukocyte antigen (HLA) class II-positive cells in the gastrointestinal tract of the human fetus (MacDonald et al. 1988, Jones et al. 2001) express co-stimulatory molecules needed for the initiation of antigen-specific reactivity (Jones et al. 2001).

1.2 Postnatal maturation

Helper T-cell (Th) responses are divided into different classes according to their cytokine-production patterns: Th1-type cells produce IFN-γ and IL-2, and promote cell-mediated immunity; Th2-type cells secrete IL-4, IL-5 and IL-13, and activate effector cells in the allergic inflammation (eosinophils and mast cells) and immunoglobulin (Ig)-switch to IgE; Th3-type cells produce immunosuppressive cytokine transforming growth factor-β (TGF-β) and act with Tr1-type cells, which produce IL-10, in tolerance formation (*Figure 1*) (Toms and Powrie 2001, Weiner 2001a). These two regulatory types of Th cells (Th3 and Tr1) suppress the activation of the effector Th cells (Th1 and Th2), and Th1 and Th2-type cells inhibit the activation of each other (*Figure 1*) (Weiner 2001a).

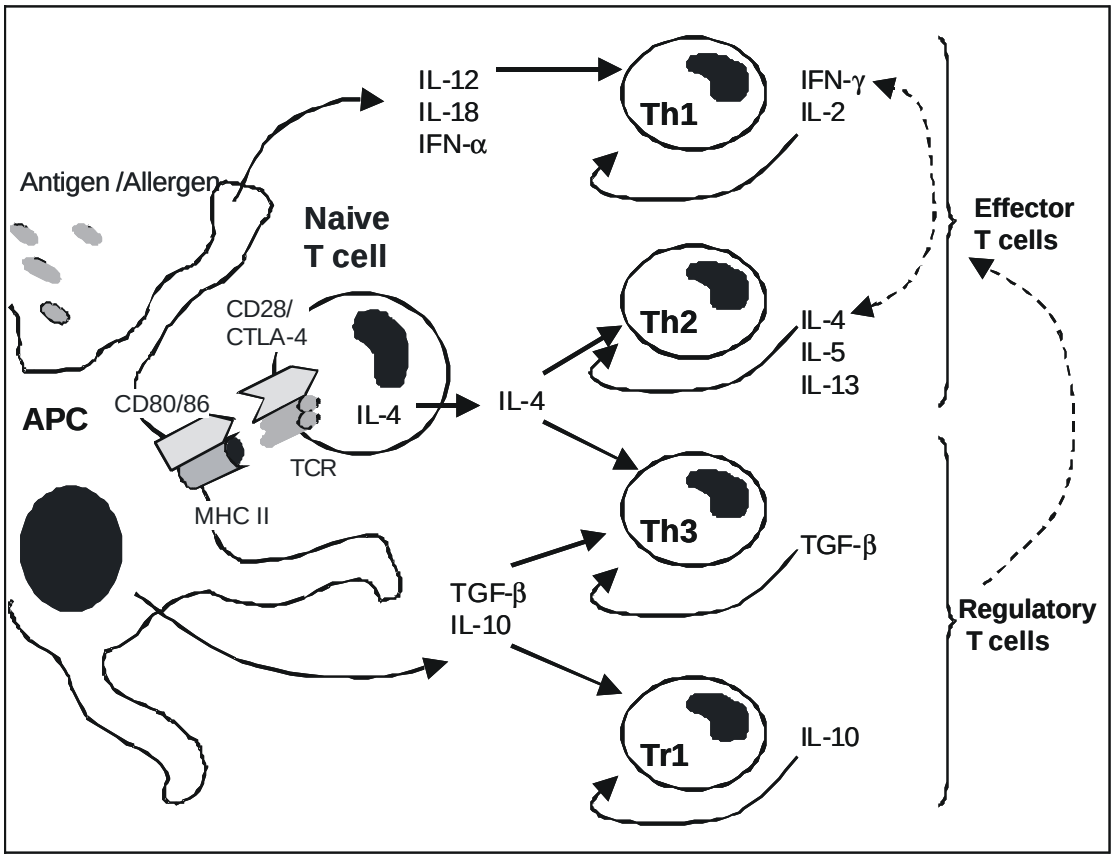

Figure 1. *Different helper T-cell subclasses and the cytokine network regulating immune responses. Continuous arrows represent the inducing response and dotted arrows represent the inhibiting response.*

Since the early 90s, it has been believed that during normal pregnancy, the fetus is surrounded by the Th2-type immune environment (Wegmann et al. 1993, Jones et al. 2001). The fetoplacental tissues especially have been shown to spontaneously secrete the Th2-type cytokines (Wegmann et al. 1993), but the maternal T cells also seem to acquire a transient state of tolerance specific for paternal alloantigens (Tafuri et al. 1995, Zhou et al. 1998) mediated with the Th2-type response (Ekerfelt et al. 1997). As a consequence, newborn infants show weak Th2-type antigen-specific responses (Holt 1995, Prescott et al. 1999), which tend towards the Th1 type of reaction, reaching a balance in early infancy (Prescott et al. 1999). In allergic disease, it is believed that this deviation towards Th1-type reactions is insufficient, resulting in a persistent Th2-type skew (Prescott et al. 1999). The selective stimulation of the immune system towards Th1-type reactions in early infancy is explained by the hygiene hypothesis (Holt et al. 1997, Strachan 2000). The prevalence of atopic diseases in children has been reported to be on the increase in countries or areas with rapid social progression, due to changes in environmental conditions early in life (von

Mutius et al. 1998, Goh et al. 1996). This has been suggested to be due to the lack of infections and other microbial pressure, which would ordinarily drive the immune system towards a Th1-type response (Holt 1995, Strachan 1996, Holt 1996). The exogenous supply of probiotics or soluble CD14 (an LPS receptor) during pregnancy and in early infancy has been reported to reduce atopic disease, although it had no effect on serum IgE levels (Kalliomäki et al 2001, Jones et al. 2002).

At birth, the immune system is inexperienced and immature, as can be seen from several phenotypic markers of immaturity (Wilson 1985). The production of both Th1- and Th2-type cytokines (IFN-γ and IL-4, respectively) appears to be deficient in neonates (Parkman 1991, Sautois et al. 1996). The production of some other cytokines regulating the inflammation responses (IL-12, tumor necrosis factor-α (TNF-α), IL-10) has also been reported to be reduced in cord blood or neonatal peripheral blood mononuclear cells (PBMCs) (Upham et al. 2002, English et al. 1988, Kotiranta-Ainamo et al. 1997, Chheda et al. 1997), although a contradictory report also exists (Karlsson et al. 2002). Conflicting evidence has also been reported concerning IL-6 production from cord blood mononuclear cells after stimulation with LPS or bacterial strains: Karlsson and co-workers (2002) reported it to be higher than that from adult mononuclear cells, whereas Sautois and colleagues (1996) had previously reported IL-6 production from cord blood and adult blood to be similar. Th1-type cytokine deficiency in neonates has also been reported at the mRNA level (Lewis et al. 1986). This impaired capacity of Th1 responses in early childhood is not regarded as being an intrinsic property of the T cells, but rather as a delay that can be overcome by appropriate stimuli, such as viral or bacterial infection (Parkman 1991, Jung et al. 1999, Upham et al. 2002).

When cytokine production from cord blood mononuclear cells was measured from newborns either with a high risk or with a low risk of allergy, decreased production of IFN-γ both by antigen or mitogen stimulation was reported to be associated with the risk of developing allergic disease later in childhood (Kondo et al. 1998, Liao et al. 1995). Moreover, due to the minimal expression of IL-4 in neonates, the authors measured IL-6, which is an important regulator of IL-4 dependent synthesis of IgE, and they found it to be increased in infants with a high risk of allergy (Liao et al. 1995). Both of the above studies reported a negative correlation between IFN-γ production and IgE levels in the cord blood

(Kondo et al. 1998, Liao et al. 1995). Another prospective study by Prescott and colleagues reported a lower level of Th2-type cytokines (IL-4, IL-6, IL-10, IL-13), in addition to IFN-γ, at birth in atopic infants than in non-atopic controls, and a rapid suppression of Th2-type responses during the first year of life in the non-atopic infants (Prescott et al. 1999). Furthermore, these workers reported a consolidation of the fetally primed allergen-specific Th2 type responses in the atopic infants (Prescott et al. 1999, Holt et al. 2000).

A concurrent increased production of IL-4 and a decreased production of IFN-γ in atopic diseases have been demonstrated after mitogen induction in many studies (Tang et al. 1993, Prescott et al. 1999, Smart et al 2002). However, Campbell and colleagues have reported only increased IL-4 production to be associated with the IgE-mediated hypersensitivity with normal IFN-γ production (Campbell et al. 1998). Tang and co-workers (1994) suggested that reduced secretion of IFN-γ was not related to abnormal regulation of transcription, but rather to a posttranscriptional defect in IFN-γ mRNA expression. Moreover, these workers demonstrated spontaneous mRNA expression of both IFN-γ and IL-4 following Th2-type skewed secretion in infants with atopic dermatitis (Tang et al 1994, Tang and Kemp 1994). In all of these studies, the use of total PBMCs did not allow clear demonstration of whether the defective IFN-γ production is due to a decrease in the number of Th1-type cells and/or IFN-γ producing cytotoxic lymphocytes (CD8+ or NK cells). This question has been approached in some studies determining IFN-γ expression at the single cell level in atopic adult patients by cytometric analysis (Jung et al. 1995, Nakazawa et al. 1997). A study by Jung and colleagues (1995) reported a decreased proportion of IFN-γ producing CD4+ cells, but a normal proportion of IFN-γ producing CD8+ cells in atopic patients. In contrast, Nakazawa and co-workers (1997) demonstrated decreased frequencies of both CD4+ and CD8+ cells expressing IFN-γ in patients with atopic dermatitis. Recently, there has been evidence that correction of an impaired Th1 response and IFN-γ production in atopic patients may be successful at the precursor T-cell level in the presence of IL-2 or IL-12 at the priming (Jung et al. 1999). Thus, the surrounding cytokine milieu at T-cell priming and during the interaction of T cell and antigen presenting cell may be crucial for the development of allergic disease (*Figure 1.*).

2 MATURATION OF THE GASTROINTESTINAL BARRIER

2.1 Gastrointestinal antigen transport

In early infancy, the gastrointestinal tract is functionally immature. At that time, the intestine absorbs more macromolecules than the mature intestine of adults (Walker 1986, Kalach et al. 2001). In infants, this leads to activation of the intestinal immune system and to the development of oral tolerance. Heyman and co-workers (1994) have observed that cow's milk protein challenge increases the production of proinflammatory cytokine TNF-α from PBMCs of infants with CMA, and that the TNF-α released increases the permeability of the intestinal mucosa in infants with CMA compared to healthy infants. Also, one recent study has reported an increased intestinal permeability in CMA infants with no negative correlation to the age, whereas in the healthy controls the intestinal permeability decreased when the child grew (Kalach et al. 2001). These results probably reflect the secondary increase in mucosal permeability due to inflammation of the intestine caused by sensitization to absorbed antigens. Moreover, the increased antigen load due to the increased permeability may evoke more adverse immune reactions and lead to further sensitization to other antigens (Holt 1990).

Antigenic macromolecules encountered via the enteric route are absorbed across the mucosa of the small intestine. There are at least four different mechanisms by which the antigens can pass the intestinal epithelial layer (Walker 1986). Large molecules may gain entry into an intestinal epithelial cell by active endocytosis, where the macromolecules are digested to smaller fragments by lysosomes, thus reducing the immunogenicity of the protein (Walker and Isselbacher 1974). The second route allows the intact macromolecules to enter via the specialized epithelium (M cells) to the Peyer's patches and thereby to stimulate the local and distant immune system (Walker and Isselbacher 1977). Thirdly, some bacterial toxins and viruses appear to enter the cells by direct penetration through the cell membrane (Goldstein et al. 1979). Lastly, leakage of large molecules through the intercellular tight junctions may also occur (Walker 1986).

2.2 Gastrointestinal maturation and factors affecting antigen transport

The maturation process of the gut mucosal barrier consists of both non-immunological and immunological components. Regarding the former, the intraluminal part of the mucosal

barrier consists of physical barrier, proteolysis and peristalsis. At the mucosal surface, the thickness of mucus contributes to the physical barrier which prevents the microvillus surface from attachment and penetration of the antigens or antigen fragments. Also, the immaturity of the microvillus membrane in newborns increases the attachment and penetration of antigens (Bresson et al 1984, Stern et al. 1984). The colonization of the intestinal microflora is also an important step in the maturation of the mucosal barrier. The normal microflora prevents overgrowth of potential pathogens in the gastrointestinal tract (Wells et al. 1988). Together with other antimicrobial agents (lactoferrin, lysozyme, glycoproteins, oligosaccharides, antimicrobial lipids), human milk provides growth-promoting factors for the favorable microorganisms in the intestinal flora (Petschow and Talbott 1991). As microbial products such as LPS are recognized as "nature's Th1 adjuvant" (Janeway 1992), the non-pathogenic normal gut flora is hypothesized to be the source of the primary signal for postnatal maturation of a balanced immune system (Björksten 1997, Björksten at al. 2001). Moreover, in animal studies it has been reported that a normal intestinal microflora is essential for the beginning of the development of normal adaptive immune functions and oral tolerance (Inagaki et al. 1996, Sudo et al. 1997).

The most important immunological part of the mucosal barrier is secretory IgA (sIgA). This immunoglobulin prevents the transport of antigens by complexing with them in the lumen or within the mucus, thereby impeding adsorption (Walker 1986). The antigens that pass the mucosal barrier of the newborn stimulate the lymphocytes within gut-associated lymphoid tissue (GALT), primarily in the Peyer's patches of the ileum. Activated lymphoblasts migrate to mesenteric lymph nodes for further maturation, and then enter the systemic circulation as plasma cells to redistribute along intestinal mucosal surfaces and produce sIgA antibodies in response to intestinally absorbed antigens (Walker and Isselbacher 1977). The secretion of IgA is decreased in the newborn (Selner et al. 1968). During this transient deficiency, mother's milk provides large amounts of specific sIgA antibodies to the breastfed infant (Machtinger and Moss 1986, Slade and Schwartz 1986). Lactating mammary glands are part of the integrated mucosal immune system, and milk antibodies reflect antigenic stimulation of mother's mucosal associated lymphoid tissue (MALT) in the gut as well as in the airways (Brandtzaeg 2002). Thus, the sIgA antibodies in mother's milk provide specifically targeted protection against the environmental antigens that the infant will be exposed to.

3 DEVELOPMENT OF ORAL TOLERANCE

Food proteins and intestinal bacteria constitute the major gut-derived antigenic challenge to the body. Exposure to these antigens has several potential outcomes, including induction of systemic immunological hyporesponsiveness (oral tolerance), systemic priming, and/or the induction of local sIgA responses (Strobel and Mowat 1998). Exposure to the high levels of different environmental antigens occurs during early postnatal life. At that point, the division of the antigens as tolerated or sensitized occurs. Induction of tolerance is seen as an initiatory Th2 response which switches to a Th3 response with TGF-β production (Strobel 2002, Weiner 2001a). The Th2-skewed immunity in the newborn is thus optimal for managing the high antigen exposure at that time.

Oral tolerance can be induced to a number of soluble antigens (Thomas and Parrott 1974, Mowat 1987), but antigens that are particulate in nature or associated with viable or replicating organisms are likely to induce active immunity (Garside and Mowat 1997, Strobel and Mowat 1998). Many different doses and regimens of single and multiple feeds induce oral tolerance successfully (Strobel and Mowat 1998). There is evidence that single administration of high doses of antigen induces T cell anergy (Garside et al. 1995a), whereas multiple low doses are more likely to generate regulatory cells (Miller et al. 1992). The immunoregulatory events after mucosal exposure of antigens have not been well characterized and remain controversial. Three major proposals for the mechanisms responsible for oral tolerance are T-cell anergy (Whitacre et al. 1991, van Houten and Blake 1996), clonal deletion (Chen et al. 1995) and active suppression (Miller et al. 1992). It is suggested that active suppression occurs with low doses of antigen and that high doses would induce T-cell anergy or deletion instead (Gregerson et al. 1993, Friedman and Weiner 1994, Chen et al. 1995, Hirahara et al. 1995). Furthermore, large antigen dose in neonate rats has been shown to induce anergy of brief duration which seems to impair the development of active suppression (Lundin et al. 1996).

3.1 Tolerogenic antigen presenting cells (APCs)

Antigen handling and presentation are the most important determinants for achieving oral tolerance (Strobel and Mowat 1998). There are several candidates for the tolerogenic APCs. Intestinal epithelial cells are speculated to be APCs due to their low expression of normal co-stimulatory molecules (CD80, intercellular adhesion molecule-1 (ICAM-1))

(Hershberg and Mayer 2000). The epithelial cells have been shown to absorb antigens and to process and sort them further (Strobel and Mowat 1998). Several groups have demonstrated that intestinal epithelial cells can present the antigen via HLA class II molecules to CD4+ T cells (Hersberg et al. 1997) and via some "non-classical" HLA class I molecules (CD1d, MICA, MICB, HLA-E) to CD8+ T cells (Hersberg et al. 1997, Braud et al. 1999, Herschberg and Mayer 2000). The professional APCs, dendritic cells (DCs), B cells and macrophages have been shown to be involved in tolerance induction, although DCs appear to play a pivotal role (Banchereau and Steinman 1998, Viney et al. 1998). Tolerance induction mainly involves two distinct DC populations, lymphoid and myeloid DCs (von Bubnoff et al. 2002). The myeloid DCs seem to exert tolerance in an immature state in the absence of inflammation and immunity after maturation, whereas lymphoid DCs appear to be involved in tolerance induction (Guéry and Adorini 1995, Grohmann et al. 1997, von Budnoff et al. 2002). Furthermore, it has been demonstrated in a mouse model that IL-12 induces the major histocompatibility complex (MHC) class II expression on DCs, whereas IL-10 reduces MHC class II expression (Koch et al. 1996).

3.2 Clonal deletion and T-cell anergy

Although clonal deletion is required for central tolerance to self-antigens, it is rarely found in peripheral tolerance to nominal antigens in normal animals. In mice transgenic for ovalbumin-specific T-cell receptor (TCR), clonal deletion of CD4+ T cells via apoptosis has been demonstrated in the spleen and gut-associated lymphoid tissue (GALT) (Chen et al. 1995). Some evidence about clonal deletion under physiological conditions has been reported through the observation that lymphocytes from orally tolerized mice display enhanced susceptibility to death by apoptosis when cultured in the absence of antigen *in vitro,* while apoptosis could not be demonstrated *in vivo* in these mice (Garside et al. 1996).

Anergy is defined as a state of T-cell unresponsiveness characterized by the absence of proliferation and IL-2 production and by diminished expression of the IL-2 receptor (Lerner et al 2000). There is substantial evidence that clonal anergy of T cells occurs in oral tolerance after exposure to a high dose of antigen (Whitacre et al. 1991, Friedman and Weiner 1994). There is evidence that the function of the tolerized lymphocytes can be rescued by the exogenous administration of IL-2 (Melamed and Friedman 1993). Clonal

anergy has been demonstrated by van Houten and Blake (1996) who showed that in mice, antigen-specific T cells persist after oral antigen exposure, but are unresponsive to restimulation with antigen *in vitro*. These studies have shown directly that T-cell anergy rather than deletion exists in oral tolerance. The induction of anergy reflects an aberrant presentation of antigen to the TCR in the absence of co-stimulatory signals from an APC (Lerner et al. 2000). The MHC class II molecules on the APC seem to be crucial for tolerance formation, because MHC class-II-deficient mice cannot develop oral tolerance (Weiner 2001). B7.1 (CD80) and B7.2 (CD86) are the most important co-stimulatory molecules. It has been shown that anti-B7.2 but not anti-B7.1 can inhibit the development of oral tolerance to a low-dose of antigen, but not to a high-dose (Weiner 2001). However, there is another co-stimulatory molecule, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) on T cells, which has been shown to be important for the induction of oral tolerance to high-doses (Bluestone 1997, Weiner 2001).

3.3 Active suppression

The antigens are presented to the lymphocytes by APCs, probably by DCs, in the context of MHC and co-stimulatory molecules (Strobel and Mowat 1998). At low doses, intestinal antigens can activate regulatory T cells (Tr). These regulatory cells can actively inhibit several aspects of the systemic immune response: IgM, IgG and IgE antibody responses, cell-mediated immune responses, T-cell proliferation, CD8+ cytotoxic T-cell responses and cytokine production (Strobel and Mowat 1998).

In the early studies, CD8+ suppressor T cells were suggested to be responsible for the active immunoregulatory mechanisms behind oral tolerance (Mowat 1987). More recent studies have shown CD4+ T cells to be more important in the induction of oral tolerance (Garside et al. 1995b, Strobel and Mowat 1998). It has been shown that oral tolerance can still be induced by depleting CD8+ T cells, and is present in CD8-knock-out mice (Garside et al. 1995b, Strobel and Mowat 1998). Also, TCRγδ T cells have been suggested to play an important role in the induction of oral tolerance. These TCRγδ T cells make up only a small percentage of all T cells, and they are proposed to have tissue-specific properties with the predisposition to home to mucosal sites (Wilson et al. 2002). The involvement of TCRγδ T cells in oral tolerance has been shown by demonstrating defects in the tolerance

formation by using antibodies against TCRγδ and in TCRγδ-knock-out mice (Strobel and Mowat 1998). The precise role of TCRγδ requires to be studied in more depth.

The intestinal mucosa expresses high basal levels of IL-4, IL-10 and TGF-β, which are upregulated further after oral administration of antigen (Weiner 2001a). These cytokines are produced by the intestinal APCs, lymphocytes and epithelial cells (Beyer et al. 2002, Weiner 2001a). This cytokine microenvironment in the gut enhances the induction of Th2 cells and regulatory Th3 and Tr1 cells (Weiner 2001b). It has been proposed that oral tolerance is induced by activation of Th2-type cells with downregulation of Th1-type responses like delayed-type hypersensitivity (Weiner 1997, Strobel and Mowat 1998). However, IL-4 induced IgE production as one of the Th2-type responses is well-suppressed in oral tolerance (Garside et al. 1995a). Thus, it seems that IL-4 and TGF-β are the initiators for activation of the Th3-type cells, which produce high amounts of regulatory cytokines such as TGF-β and provide help for IgA production (Weiner 2001b). Furthermore, TGF-β has been demonstrated to decrease the expression of CD40 and IL-12 in macrophages suppressing Th1 differentiation (Toms and Powrie 2001). TGF-β has been shown to induce the production of IL-10 (Toms and Powrie 2001), which is another important regulatory cytokine, especially in specific immunotherapy and contact dermatitis (Jutel et al. 2003, Cavani et al. 2001). On the other hand, regulation of the TGF-β response during T cell activation is modulated by IL-10 (Cottrez and Groux 2001). These regulatory cytokines (IL-10 and TGF-β) released in an antigen-specific manner are thought to induce "bystander suppression", inhibiting the surrounding immune responses to even unrelated antigens (Garside and Mowat 1997, Strobel and Mowat 1998, Weiner 2001a). This phenomenon is being studied eagerly as a therapeutic use of oral tolerance in the treatment of autoimmune diseases (Weiner 1997).

Another type of regulatory cell driven by IL-10 is the Tr1-type cell (Weiner 2001b, Toms and Powrie 2001). These cells produce the regulatory cytokine IL-10 (Weiner 2001b). Also, CD25+CD4+ regulatory T cells are considered to be one of the possible cell types actively inducing oral tolerance. These natural CD25+CD4+ T cells continuously express the membrane antigen CTLA-4, which inhibits activation of T cells by ligation of B7 (CD80/86). Administration of anti-CTLA-4 antibody completely abrogated the ability of CD25+CD4+ regulatory T cells to inhibit intestinal inflammation (Toms and Powrie

2001). A great deal more information about these cells and their relationship to the Th3 and Tr1 cells is needed.

4 IMMUNE REACTIONS IN COW'S MILK ALLERGY

4.1 Cow's milk allergy in infancy

In early childhood, CMA is frequently the first manifestation of "allergic march" (Savilahti et al. 1992). The symptoms of CMA commonly appear during the first months of life, within days or weeks after commencing feeding with a cow's milk-based formula (Goldman et al. 1963, Jakobsson and Lindberg 1979, Hill et al. 1984), or even during exclusive breastfeeding (Jakobsson and Lindberg 1979, Warner 1980, Isolauri et al. 1999, Järvinen et al. 1999a). The prognosis of CMA, however, has been suggested to be favorable; more than 70% of affected infants can tolerate cow's milk without symptoms at the age of 3 years (Bock 1987). CMA can be manifested through different organ systems, of which the skin and gastrointestinal tract are the most common ones, and infrequently the respiratory tract can be affected. Systemic anaphylaxis may even occur (Jakobsson and Lindberg 1979, Hill et al. 1984, Björksten 1987, Wilson and Hamburger 1988). The immune mechanisms behind development of CMA are still poorly understood, especially in infants with delayed type hypersensitivity (Kondo et al. 1993). CMA is considered to be an example of a disturbance in development, or a breakdown of oral tolerance to ingested milk proteins (Suomalainen et al. 1993a). Also, the imbalance between Th1- and Th2-type T cells is believed to result in adverse responses to cow's milk antigens in early infancy (Suomalainen et al. 1993b).

4.2 Hypersensitivity reactions

Coombs and Gell (1975) divided hypersensitivity reactions into four types. In CMA, as in most allergies, the best known and the most studied immune mechanism is the type I hypersensitivity reaction (Björksten 1987). It is an IgE-mediated, immediate, anaphylactic reaction, in which mast cells are activated by cross-linking IgE molecules on the cell membrane. The activated mast cell releases cytotoxic mediators, such as histamine, leukotrine and prostaglandin, from the granules. Cytokines and other chemotactic mediators are released as well, and they attract eosinophils, basophils and mast cells to the

site of inflammation. Type IV, delayed, T-cell-mediated hypersensitivity reactions have also been demonstrated in CMA (Hill et al. 1986). Type IV hypersensitivity reactions cover the cytotoxic T cell- or natural killer (NK) cell-mediated responses and macrophage activation by helper T cells. Type II (cytotoxic) hypersensitivity reactions are mediated through IgG or IgM antibodies, or through complement activation. The pathogenic antigen is recognized by antibodies which, on their own or by activating the complement system, opsonize the infected cell, and destroy it by cytolysis or phagocytosis. In the type III (immune complex-mediated, Arthus reaction) hypersensitivity reaction, the antigen opsonization by IgG or IgM creates large immune complexes that generate an inflammation reaction by complement activation and neutrophil migration, which eventually leads to tissue damage. (Reviewed from Coombs and Gell 1975) Both type II and type III hypersensitivity reactions can also be present in CMA, and there are many findings to suggest that more than one type of reaction may be implicated in a particular patient, even when there is a single clinical manifestation (Bahna 1985, Suomalainen et al. 1993a, Suomalainen et al. 1993b).

4.3 Relationship between the immune reactions and clinical outcome in CMA

Hill and co-workers (1984, 1986) have reported three immunologically and clinically different subclasses of CMA. They studied 100 young children with CMA (mean age of 16 months). In the first group, Hill and his co-workers put all the infants reacting within 45 minutes after the beginning of the challenge with cow's milk. These infants had predominantly acute cutaneous eruptions and vomiting, and a positive skin prick test with elevated specific IgE levels in serum. The second group consisted of patients with delayed (45 minutes to 20 hours) gastrointestinal symptoms, such as diarrhea and vomiting. Immunologically, these infants had less IgA antibodies in their serum than the others. The third group consisted of infants reacting after several hours or days (>20 hours) to the ingestion of cow's milk. These infants had chronic, multiorgan symptoms and heterogeneous immunological findings possibly reflecting different subclasses in this group. These delayed manifestations of CMA have been proposed to be mediated through immune complexes (Arthus reaction) or T cells (Ferguson et al. 1983, Savilahti and Kuitunen 1992). Although the clinical symptoms of CMA seem to disappear and tolerance to cow's milk antigens is achieved after age-dependent intestinal maturation, antigen-specific IgE antibodies can still be detected in serum (Sampson and McCaskill 1985, Hill

et al. 1989). Furthermore, CMA infants are usually reported to react to other food antigens as well, such as egg, soy, wheat, and the other manifestations of atopy following their recovery from CMA (Bishop et al. 1990).

4.4 Deviations in the immune response in CMA

As the cytokine observations lead us to assume, it has been demonstrated that a large number of activated CD19+ B cells and, in contrast, a low number of CD8+ T cells are present in young infants with early symptoms of developing CMA (Järvinen et al. 1998). Although the immunoglobulin secretion and class-switch together with the production of cytokines supporting the B-cell responses are deficient at birth (Watson et al. 1991, Splawski and Lipsky 1991, Durandy et al. 1995), the CD40 ligand (CD40L) is known to be present and active in neonatal T cells – to further activate the immature B cells to start producing IgM (Splawsky et al. 1996). Moreover, in CMA infants the total number of antibody-secreting B cells has been shown to rise during the challenge with cow's milk (Isolauri et al 1992, Suomalainen et al. 1992, Järvinen et al. 1999a), but the antigen-specific B-cell response is defective while the antigen unspecific response, especially in the IgM class, is strong (Isolauri et al. 1992, Suomalainen et al. 1992). This is proposed to be due to the defective regulation of B cells in CMA infants.

Eosinophilic leukocytes are effector cells in the allergic late-phase inflammation. Measurement of fecal eosinophil cationic protein (ECP) from allergic and healthy infants showed that ECP levels were higher before any elimination diet and decreased during a successful elimination diet in allergic infants (Majamaa et al 1999). Furthermore, the pre- and post-cow's milk challenge levels of fecal ECP have been reported to be higher in CMA infants reacting slowly with gastrointestinal symptoms than in infants with other manifestations of CMA (Saarinen et al. 2002). In addition, direct measurement of intestinal ECP showed that luminal challenge with cow's milk increased ECP secretion in both allergic and food intolerant patients (Bengtsson et al. 1997). All of these studies indicate that eosinophils and their mediators play an important role in the intestinal inflammation process. In CMA infants with cutaneous symptoms, serum ECP levels have been reported to be elevated during the challenge, indicating that systemic eosinophil degranulation may also be an important immunological mechanism in allergic inflammation (Suomalainen et al. 1994a).

Additionally, in CMA the antigen-specific T-cell-mediated suppression has been reported to be defective (Suomalainen et al. 1993a, Ferguson et al. 1983). In CMA infants, oral *Lactobacillus rhamnosus GG* ingestion was reported to enhance the serum IL-10 concentration, suggesting a positive immunosuppressive effect of the probiotic bacterium, but no correlation to the IL-10 concentrations in healthy infants was made (Pessi et al. 2000). The antigen-induced proliferative responses of PBMCs have been demonstrated to be elevated in infants with delayed-type gastrointestinal reactions to cow's milk (Suomalainen et al. 1994b) and egg (Fukutomi et al. 1994), but in another study also in infants with IgE-mediated egg allergy (Ng et al. 2002), in contrast to healthy controls. In addition, in the egg-sensitive allergic infants, the increased lymphocyte proliferation was associated with mixed Th1 and Th2 responses following a strong IL-10 and IFN-γ response after the development of tolerance later in childhood (Ng et al. 2002). Both lymphocyte proliferation and antigen-specific IFN-γ production from PBMCs have been shown to be abolished after oral challenge with cow's milk, with the suggestion that the antigen-specific T cells might migrate out of the circulation to the effector site of the inflammation (Suomalainen et al. 1994b, Sütas et al 1997).

Lymphocyte migration seems to have an important role in the pathogenesis of food allergy, because it typically manifests itself in several effector organs after sensitization in the gut. The cutaneous lymphocyte antigen (CLA) has been shown to be expressed more in circulating milk-specific T lymphocytes from infants with food allergy and atopic dermatitis than from healthy controls, suggesting preferential homing of these cells to the skin (Abernathy-Carver et al. 1995). This has been shown further in other allergic diseases also, such as drug allergy and contact dermatitis (Gonzales et al. 1997, Santamaria et al. 1995), but also doubt about the exclusive role of the CLA in the lymphocyte homing to the skin has been demonstrated in children with atopic dermatitis (Campbell and Kemp 1999). A few studies have reported an increased expression of the mucosal adhesion molecule α4β7 in CMA infants and food allergic adults (Eigenmann et al. 1999, Veres et al. 2001). Adhesion molecules also play a major role in the regulation of immune responses in allergic diseases (Eigenmann 2002). The pro-inflammatory cytokines have been shown to upregulate ICAM-1 and the lymphocyte function-associated antigen-1 (LFA-1) (Detmar et al. 1991, Wedi et al. 1996), and blockage of these adhesion molecules inhibits the

proliferative response of PBMCs – and even the development of the clinical and histological signs of an allergic disease (Whitcup et al. 1999, Kawamura et al. 1998).

5 IMMUNOLOGICAL COMPOSITION OF MOTHER'S MILK

Human milk is believed to impart specific immune advantages to the neonate through enhancement or induction of the still-developing neonatal immune system (Goldman et al. 1998). Prolonged breastfeeding has been recommended in order to prevent or delay development of food allergies and atopic eczema in infants (Chandra et al. 1986, Host et al. 1988, Lilja et al. 1989, Zeiger et al. 1989, Saarinen and Kajosaari 1995). Nevertheless, there have been contrary reports suggesting that severe food allergies may develop already during exclusive breastfeeding (Isolauri et al. 1999, Järvinen et al. 1999a). Furthermore, recent findings on a relation between long duration of breastfeeding and an increased risk of the breastfed infant developing atopy (Wright et al. 1999, Bergman 2002, Sears et al. 2002) suggest that the protective effect (or lack thereof) may be due to individual variations in the levels of immunological constituents in mother's milk.

5.1 Human milk as an immunomodulatory factor

Human breast milk is known to be an important immunological support system extending from the mother to her infant during the first months of life (Slade and Schwartz 1987, Pabst 1997, Goldman et al. 1998, Hanson 1998, Jones and Warner 2000). The immune system of the fetus and the newborn is relatively immature. Because of this, the evolution of mammals has created a way of transmitting protective and supportive agents through the placenta and amniotic fluid during fetal life and through breast milk after birth (Goldman et al. 1998). Some immune factors in mammalian milk are highly conserved in many mammalian species, but in contrast – due to the adaptation to dissimilar environmental microorganisms – the expression of certain antimicrobial factors differs considerably between e.g. human and cow's milk (Goldman et al. 1998). The defence system of human milk is comprised of antimicrobial agents (i.e. IgA, lactoferrin, lysozyme, oligosaccharides and antiviral lipids) (Goldman et al. 1996, May 1994), anti-inflammatory agents (i.e. IL-10, TGF-β, IgA) (Goldman et al. 1986, Strobel 2002) and immunomodulatory agents (other cytokines, leukocytes) (Eglinton et al. 1994, Goldman et al. 1996). Additionally, human milk provides factors (i.e. oligosaccharides, lactose) that promote the growth of

favorable microbial organisms in the intestinal flora (Petschow and Talbott 1991, Brandtzaeg 2002). It has been shown in several studies that several protein antigens, such as ovalbumin, β-lactoglobulin (BLG) and casein, from the diet of the lactating mother pass to human milk (Stuart et al. 1984, Chandra et al. 1986, Machtinger and Moss 1986, Sorva et al. 1994). Hence, human milk is the most important source of dietary antigens in the exclusively breastfed infant. Thus, human milk provides many factors that are important in the promotion of oral tolerance and normal development of the gastrointestinal mucosal immune system.

5.2 Dietary protein antigens in breast milk

Since food allergies can appear already during exclusive breastfeeding, the most likely source of sensitizing antigens is the mother's breast milk (Vandenplas et al. 1992). In many studies BLG, a cow's milk allergen, has been reported to pass to mother's milk in up to 75% of mothers consuming cow's milk (Stuart et al. 1984, Chandra et al. 1986, Sorva et al. 1994). The elimination diets of the lactating mothers were reported to be successful in decreasing the allergic symptoms of the suckling infant (Hattevig et al. 1989, Isolauri et al. 1999), and re-introduction of cow's milk in the maternal diet has been associated with reappearance of symptoms in a breastfed infant (Järvinen et al. 1999a). However, the persistence of symptoms of food allergy in some breastfed infants during a maternal "few foods only" diet (Järvinen and Suomalainen 2001) has evoked the suspicion that other factors in mother's milk may contribute to the allergic symptomatology of the infant (Little 2001, Hanson et al. 2002).

5.3 Milk leukocytes

Human milk consists of leukocytes that are morphologically different from those in the peripheral blood, presenting a phenotype more like tissue-specific cells (Rivas et al. 1994, Xanthou 1997, Järvinen and Suomalainen 2002). Generally, there are 10^5 to 10^7 leukocytes in 1 ml of human milk, the highest concentration of leukocytes being detected in colostrum and declining over time (Ogra and Ogra 1978, Goldman et al. 1982, Xanthou 1997, Järvinen and Suomalainen 2002). However, the average cell intake by the suckling infant remains constant since the volume of breast milk consumed increases as the infant grows older. The predominant cellular component of breast milk leukocytes is the macrophage,

comprising about 60 to 90% of the total (Smith and Goldman 1968, Ho et al. 1979, Crago et al. 1979, Rivas et al. 1994, Xanthou 1997, Järvinen and Suomalainen 2002). Neutrophils appear to be somewhat less frequent in human milk, comprising less than 30% of the total cell count. However, in colostrum the neutrophil count seems to be as high as 40 to 60% (Cargo et al. 1979, Rivas et al. 1994, Xanthou 1997, Järvinen and Suomalainen 2002). Only 3 to 10% of breast milk leukocytes are lymphocytes, the majority of which are T cells (Eglinton et al. 1994, Rivas et al. 1994, Lindstrand et al. 1997). Additionally, occasional monocytes, about 2% of eosinophils, and hardly any basophils are found (Vassella et al. 1992, Järvinen and Suomalainen 2002).

Macrophages. The human milk macrophages are known to be morphologically and phenotypically specialized tissue macrophages (Rivas et al. 1994, Rodriguez et al. 1989). Their main function is thought to be in protecting the suckling infant against infections. They have been reported to show high phagocytic activity toward bacteria (Smith and Goldman 1968, Rodriguez 1989). Although human milk macrophages are known to be only weakly activated by non-specific mitogens such as ConcanavalinA (ConA) or phytohemagglutinin A (PHA) (Xanthou 1997, Järvinen et al. 2000a), infection with RSV has been shown to enhance cytokine production by milk macrophages (Sone et al. 1997). In CMA and atopic dermatitis, the proportion of milk macrophages has been found to be lower in breastfeeding mothers than in mothers with healthy infants (Järvinen and Suomalainen 2002). Expression of the MHC class II antigen HLA-DR has been shown to be very high, being almost 100% on human milk macrophages (Rivas et al. 1994). In contrast, human milk macrophages from mothers with a CMA infant have been reported to express less HLA-DR than macrophages in the milk of mothers with a healthy infant (Järvinen et al. 1999b). Despite the expression of maternal HLA antigens on milk-derived leukocytes, the infant does not seem to respond adversely to them. This could be due to the tolerance developed to maternal HLA during pregnancy and breastfeeding (Burlingham et al. 1998, Hanson et al. 2002).

Neutrophils. The evidence about the number of neutrophils in the colostrum and mature milk of humans is conflicting: some studies have demonstrated numbers as low as 8-28% (Crago et al. 1979) and others have reported proportions of 40-60% (Xanthou 1997). The proportion of neutrophils has been shown to be significantly higher in the milk of mothers with a CMA infant than in milk from mothers with a healthy infant (Järvinen and

Suomalainen 2002). The role of neutrophils in human milk is somewhat obscure, however. The protective effect against infection of milk neutrophils seems to be less than their counterparts in blood (Buescher et al. 1993). Still, they may have a contributory role in protection against mastitis and the production of lactoferrin (Järvinen and Suomalainen 2002).

Lymphocytes. T cells account for only 3 to 11% of the total leukocyte count in milk (Rivas et al. 1994, Xanthou 1997, Lindstrand et al. 1997). They display the phenotype and functional characteristics of memory T cells (Bertotto et al. 1990). Milk T cells more often express γδ-TCR than T cells in the peripheral blood (Lindstrand et al. 1997). This gives evidence for the gut-mucosa-to-mammary-gland homing mechanism. The role of milk T cells is suggested to be the transfer of cell-mediated immunity acquired by the mother, as demonstrated by the tuberculin-reactive T cells found in the milk of tuberculin-positive mothers and after lactation also in the peripheral blood of their suckling infants (Schlesinger and Covelli 1977). Only a minority of the lymphocytes in milk are B cells (4 to 26%), which produce IgA (Bertotto et al. 1990). The proportion of lymphocytes has been reported to be reduced in the milk of mothers with an infant suffering from atopic dermatitis but with no CMA (Järvinen and Suomalainen 2002).

Eosinophils. Eosinophils are found in the human milk only in association with maternal atopy or with CMA in the breastfed infant (Vassella et al. 1992, Järvinen and Suomalainen 2002). Their precise role in the milk is unknown, but their presence has been suggested to be associated with the development of CMA in the infant (Järvinen and Suomalainen 2002).

In animal studies, leukocytes have been shown to pass through the gastrointestinal mucosa of a suckling newborn (Schnorr and Pearson 1984, Slade and Schwartz 1987, Jain et al. 1989). Thus, the breast milk leukocytes may travel through the gastrointestinal tract of the suckling infant without being destroyed. This may be due to several protective factors, neutral pH in the infant's stomach and the neutralizing effect of mother's milk in the gastrointestinal tract (Paxson and Cress 1979).

5.4 Immune mediators in human milk

Immunoglobulins. Breast milk provides multiple agents against infection to the suckling infant. These are important in the colonization of the normal microflora and in the protection against pathogenic bacteria. The immunoglobulins, mainly sIgA, but also IgM, IgG and IgD in small quantities, make the major contribution against bacteria, and IgE against parasites (Xanthou et al. 1995). sIgA constitutes about 90% of human milk immunoglobulins. It is present at high concentrations during the first days and weeks after birth, and its level decreases thereafter to a basal level of about 0.2 g/L (Machtinger and Moss 1986, Savilahti et al. 1991, Järvinen et al. 2000b). The amount of sIgA in human milk has been reported to be lower in mothers whose baby later developed CMA (Savilahti et al. 1991, Järvinen et al. 2000b), but contradictory reports exist – with no relation to the development of atopy in the infant when the mother's milk sIgA levels were low (Duchén et al. 2000). The specificity of sIgA in milk is proposed to be result of the mother's antigenic exposure at the respiratory and gastrointestinal tracts, and of the homing mechanisms between these tracts and the mammary glands, thus being dependent on the maternal immunological memory (Slade and Schwartz 1986, Pabst 1997, Hanson 1998). Besides the common microbial agents in the upper respiratory tract and in the intestinal mucosa, the specificity of sIgA is also directed against common food antigens in the mother's diet (Machtinger and Moss 1986, Slade and Schwartz 1986, Savilahti et al. 1991, Järvinen et al. 2000b). Thus, sIgA has a crucial role in the inhibition of adhesion of pathogenic microbes and absorption of undigested food antigens.

Anti-infectious agents. Other agents against infection in the human milk are less specific. Lactoferrin exists in milk in large quantities, and its anti-microbial properties are based on the binding of free iron, leading to an optimal colonization of bacteria in the intestine (Brock 1995, Pabst 1997). Anti-infectious effects have also been reported for lactose, which promotes the growth of *Lactobacillus bifidus* and thereby prevents gut colonization with pathogenic organisms (Lönnerdahl 2000, Ogundele 2001). Lysozyme and the complement system are provided from the mother's milk and they have an important role in the host defense, such as bacteriolysis and enhancement of phagocytosis (Ogundele 2001). Oligosaccharides and some glycoproteins present in human milk also have anti-microbial effects (D'Ostilio et al. 1996, Pabst 1997). In addition, the lipid fraction of milk develops anti-microbial activity in the gastrointestinal tract of the infant as a result of lipolytic activity (Isaacs 2001).

Immunomodulating agents. Human milk provides the breastfed infant with many immunomodulating agents (Pabst 1997, Hanson 1998, Goldman et al. 1998). Recently, the development and control of immune homeostasis in the neonatal intestine has been studied in the context of immune modulation by mother's milk. Soluble CD14 (an LPS receptor) was detected in human milk, and it was shown that stimulation with bacterial antigens resulted in activation of and release of proinflammatory cytokines from CD14 negative intestinal epithelial cells only in the presence of milk-derived soluble CD14 (Vidal et al. 2000, Labéta et al. 2001). The higher levels of CD14 in mother's milk are reported to be associated with lower incidence of atopy in the breastfed infant, indicating a possible Th1-type response as the mechanism (Jones et al. 2001). By administering probiotics to pregnant and lactating mothers, it has been shown that the immunoprotective effects of human milk are increased, as measured by the amount of the anti-inflammatory cytokine TGF-β (Rautava et al. 2002).

Cytokines. The immunomodulatory molecules in human milk include cytokines and growth factors. Anti-inflammatory cytokines seem to play a major role in regulating the immune responses against the considerable exposure to antigens in the neonatal gut. TGF-β appears to be the predominant cytokine in human milk, together with IL-6 (Böttcher et al. 2000a). IL-10 is also present in human milk in large quantities (Garofalo et al. 1995, Rudloff et al. 1999). The levels of TGF-β in colostral milk have been reported to positively correlate with specific IgA antibodies as well as with IgA-secreting cells in the breastfed infant, which supports the protective and oral tolerance inducing immunological nature of human milk (Saarinen et al. 1999, Kalliomäki et al. 1999). Colostral TGF-β levels have been shown to be lower in mothers with an infant with later developing IgE-mediated CMA than in mothers with an infant with later developing non-IgE-mediated CMA (Saarinen et al. 1999). Furthermore, the concentration of TGF-β in colostrum correlated negatively with the reactivity of PBMCs to cow's milk proteins in infants with CMA (Saarinen et al. 1999). These findings suggest that TGF-β in mother's milk could inhibit the IgE-mediated and cell-mediated reactions to food proteins, and instead promote IgA production in the breastfed infant (Saarinen et al. 1999, Kalliomäki et al. 1999). Human milk has also been discovered to contain soluble receptors and cytokine antagonists, such as IL-1 receptor antagonist (IL-1RA), IL-6 receptor (IL-6R), TNF-α receptors I and II

(TNF RI, TNF RII) and IL-2 receptor (IL-2R) (Srivastava et al. 1996, Buescher and Malinowska 1996, Buescher and McWilliams-Koeppen 1998). These receptors can block the bioactivity of their ligand cytokines and act as anti-inflammatory factors. The IL-2R in human milk could be important in the development of tolerance by suppressing T cell proliferation. An excess of soluble Fas in human milk has been suggested to play a role in the development of gastrointestinal tract and immune tolerance by blocking the function of Fas ligand (FasL), thereby preventing apoptosis and tissue damage (Srivastava and Srivastava 1999).

The pro-inflammatory cytokines make up another subset of cytokines in human milk. IL-1 (Söder 1987), IL-4 and IL-5 (Eglington et al. 1994, Böttcher et al. 2000a), IL-6 (Saito et al. 1991), IL-12 (Bryan et al. 1999), IL-13 (Böttcher et al. 2000a), IL-18 (Takahata et al. 2001), IFN-γ (Eglington et al. 1994) and TNF-α (Rudloff et al. 1992) have been detected in human milk. It seems obvious that these maternal cytokines could provide protection and support to the immature immune system of the newborn infant (Goldman et al. 1991, Garofalo and Goldman 1998, Garofalo and Goldman 1999, Jones and Warner 2000). The source of these cytokines in human milk has been investigated in many studies. The human milk macrophages seem to be the major source of most of these cytokines (Skansén-Saphir et al. 1993, Rudloff et al. 1992, Srivastava et al. 1996). The epithelial cells in the mammary gland have also been reported to express some of the cytokines present in milk (Takahata et al. 2001).

A variety of chemokines and different growth factors are present in milk. Of these, IL-8, growth-related peptide-α and RANTES (regulated upon activation, normal T cell expressed and secreted) have been reported to be the most potent chemotactic factors for intestinal intraepithelial lymphocytes (Garofalo and Goldman 1999). Monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), IL-16 and eotaxin have been discovered in human milk (Srivastava et al. 1996, Rudloff et al. 1999, Böttcher et al. 2000b). Regarding the colony-stimulating factors (CSFs), granulocyte, macrophage and granulocyte-macrophage CSFs have been reported (Garofalo and Goldman 1998).

Recently, a few studies have focused on cytokine expression in human milk in relation to maternal atopy or development of allergic disease in the breastfed infant. The concentration of IL-4 in milk was demonstrated to be higher in allergic mothers than in non-allergic mothers, in addition to a similar tendency in expression of the other Th2-type cytokines IL-5 and IL-13 (Böttcher et al. 2000a). A contradictory report exists, however, with no differences in cytokine expression between allergic and non-allergic mothers (Rudloff et al. 1999). The chemokines IL-8 and RANTES were found to be at higher concentrations in the milk of allergic mothers than in that of healthy mothers (Böttcher et al. 2000b). The authors suggested that in the mammary gland, these chemokines might attract leukocytes such as eosinophils and basophils from the mother's circulation and thereby transfer the susceptibility to allergic diseases to the offspring. The production of TNF-α by human milk leukocytes has been reported to be defective in mothers with an infant suffering from CMA (Järvinen et al. 2000a). These workers suggested that defective immunologic support from mother's milk may interfere with the normal development of oral tolerance to food proteins.

AIMS OF THE STUDY

The purpose of the present study was to characterize some activation markers of immune responses in infants who were in an early phase of developing CMA and in healthy controls. In addition, the influence of mother's milk composition on the development of atopy in the breastfed was assessed.

The specific aims of the present study were as follows:

1. To analyze the production of proinflammatory (TNF-α), Th1 (IFN-γ) and Th2 (IL-4) cytokines from PBMCs in infants with CMA and in healthy controls (I).
2. To evaluate the proportions of these cytokine expressing cells in different T cell subclasses (CD4+, CD8+) in CMA infants and in healthy controls (II).
3. To measure the T cell signal transduction in MAP kinase phosphorylation in infants with CMA and in healthy infants (III).
4. To further compare the MAP kinase activation in clinically different manifestations of CMA, and between symptomatic and asymptomatic CMA infants (III).
5. To analyze the expression of co-stimulatory adhesion molecules, ICAM-1, LFA-1 and Mac-1 on circulating lymphocytes from clinically different manifestations of CMA compared to healthy controls (IV).
6. To assess the correlation of mothers' milk ECP to the development of allergic disorders in breastfed infants (V).
7. To evaluate the association of different proportions of milk leukocytes with the development of allergic disease in the breastfed infant (II, IV, V).

MATERIALS AND METHODS

1 SUBJECTS

The study population comprised 131 infants and 104 of their breastfeeding mothers (*Figure 2*). Altogether, 191 blood samples from these infants and 136 breast milk samples and blood samples from the mothers were analyzed. The infants were followed prospectively from delivery: one group (n=102) because of the infant's high risk of atopy/allergy (atopic or food-allergic sibling[s] and/or parent[s]) and the other group (n=29) because of the infant's low risk of atopy/allergy (no signs of atopy in first-degree relatives). They were followed-up for at least one year to detect development of atopic dermatitis or food allergy. At the time of sample collection, the infants were aged from 2 days to 11 months. The children were born full-term, and they had no chronic diseases other than allergy nor overt infection whilst visiting the clinic.

Of the 104 mothers, 60 had an atopic constitution: hay fever, atopic dermatitis or asthma, and 44 mothers were healthy. The samples from mothers who had had mastitis during the preceding four weeks were excluded from the analyses.

2 PATIENT STUDIES

2.1 Study protocol

The infants were followed up prospectively from birth. The first blood samples and the colostral milk samples were collected at the maternity clinic. If no colostral milk sample was obtained in the hospital, the mothers collected and froze a few ml of their breast milk at home during the first week after delivery. The visits at 1, 3, 6, and 12 months included a clinical examination by a physician investigator and the blood and breast milk samples were collected. Thereafter, the health status of the infants was followed once a year. Analyses of different immunological factors were each done as an open cross-sectional study.

2.2 Ethical aspects

The Ethical Committee of the Skin and Allergy Hospital of the Helsinki University Central Hospital and the City of Helsinki approved the study protocol. Written informed consent was obtained from the parents for sample collection and longitudinal follow-up of their children. All the elimination diets and challenges with cow's milk were done based on clinical indications, and not specifically for research purposes.

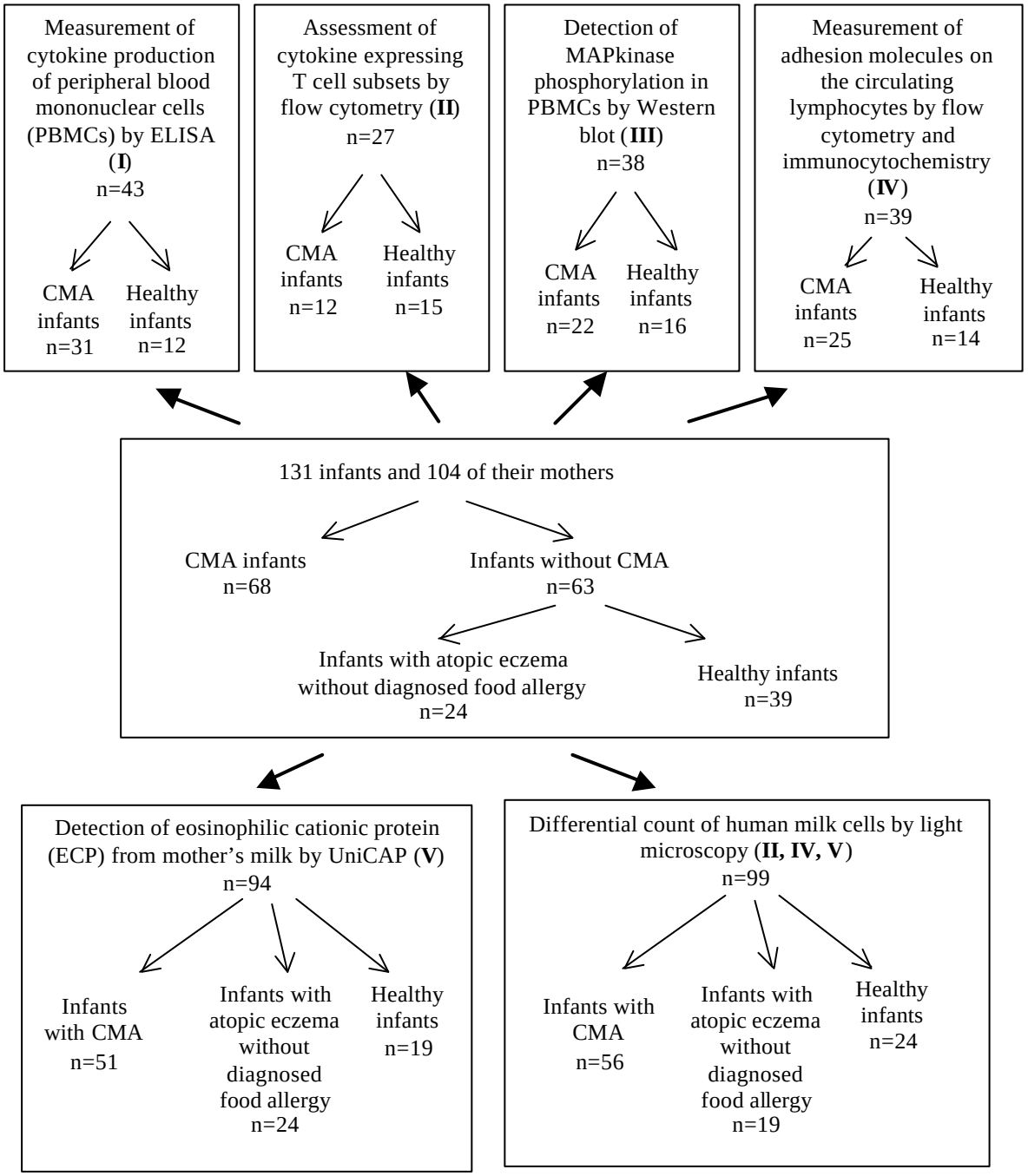

Figure 2. *Flow chart of the study infants and their mothers representing numbers of subjects included in each of the laboratory assessments.*

2.3 Cow's milk challenge protocol.

The diagnosis of CMA was confirmed by open food challenge, which has shown appropriate accuracy in infants (Isolauri and Turjanmaa 1996). During the elimination period, cow's milk was strictly eliminated from the diet of the infant and the lactating mother. If the infants needed extra formula in addition to breast milk, a tolerated formula (protein hydrolysate or amino acid-based formula) was given. After elimination of cow's milk for 2 to 4 weeks, the patients were subjected to a challenge with cow's milk. The challenge was performed through mother's milk (Järvinen et al. 1999a) or usually directly (perorally) to the infant. The challenge was started with a drop of cow's milk on the skin or lips. Thereafter, the cow's milk was given in increasing doses at half-hour intervals: 1, 10, 50 and 100 ml on day 1, and the normal milk intake appropriate to the infant's age was commenced on day 2. The challenge was immediately stopped when any adverse reaction was noticed (urticaria, flair of atopic eczema, diarrhea, profuse vomiting, abdominal pain, wheezing). Reactions occurring within an hour from the last dose on the first day were defined as immediate ones and the reactions occurring thereafter were considered to be delayed ones. The patients were followed up over the one-week period of challenge.

2.4 Skin Prick Tests (SPT)

Skin prick tests were performed on the volar side of the forearm using a commercial cow's milk extract (ALK, Denmark) according to a standard technique (Dreborg et al. 1989). Negative control solution (ALK, Denmark) and histamine dihydrochloride at 10 mg/ml (ALK, Denmark) as a positive control were used. At least a 5-mm diameter reaction was taken as an adequate histamine reaction. Reactions were read after 15 min and at least a half (3mm) of the size of histamine was considered to be a positive reaction.

2.5 Peripheral blood and human breast milk

Venous blood was drawn in heparin and in EDTA, and always at the same time in the morning. Serum samples were stored frozen at -20°C until analyzed. The breast was washed with warm water without detergents before collecting the milk with a manual breast pump in the morning. Immediately afterwards, a few 1-3 ml aliquots of the sample were made for later ultracentrifugation, and were stored frozen at -70°C.

3 LABORATORY METHODS

3.1 Cell separation

PBMCs consisting mainly of lymphocytes were isolated from blood-heparin samples by Ficoll-Hypaque (Pharmacia AB, Uppsala, Sweden) gradient centrifugation at 400 g for 30 min at 20°C. The cells were resuspended in RPMI-1640 containing 5% fetal calf serum (FCS, Sigma, Steinheim, Germany), antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml), and 2 mM L-glutamine (Sigma, Irvine, U.K.). After washing three times, the cells were counted in a Bürker chamber.

The volume of fresh milk sample was measured and it was centrifuged at 400 g at room temperature (RT) for 15 minutes to separate the fat layer. Fat and supernatant were removed, and leukocytes were collected and washed 15 minutes in RPMI-1640 containing 5% FCS, antibiotics and glutamine. After being washed 3 to 4 times, the cells were resuspended in RPMI-1640 medium and counted visually in a Bürker chamber. Human breast milk samples containing less than one million leukocytes after separation were not analyzed.

3.2 MGG staining for differentiation of leukocytes (Studies II, IV and V)

Venous blood-EDTA samples were drawn onto slides and air-dried. Thereafter, the slides were stained with May-Grünwald-Giemsa (MGG, Oy Reagena LTD, Kuopio, Finland). Cytospin preparations of breast milk cells were obtained with a sample application of 1×10^5 cells per slide (Cytospin3, Shandon, Life Sciences International (Europe) Ltd., Astmoor, Runcorn, Cheshire, U.K.). The slides were treated with Vectapond™ (Vector Laboratories Inc., Burlingame, CA, U.S.A.) before sample application to improve the fixation of breast milk cells. The air-dried slides were finally stained with Wright (Merck, Darmstadt, Germany) following MGG application. The numbers of macrophages, monocytes, lymphocytes, neutrophils, eosinophils and basophils were counted under the light microscope by two independent investigators. The results were expressed as a percentage of the cell type per 100 leukocytes. The morphology of the milk leukocytes was assessed on the slides and only the intact cells were analyzed. Breast milk samples containing erythrocytes and/or bacteria were discarded.

3.3 Determination of leukocyte surface antigens (Studies II and IV)

Flow cytometry of PBMCs (Studies II and IV). 1×10^6 PBMCs/ml were incubated at +4°C for 15 minutes with a saturating concentration of fluorescent-conjugated (FITC, PE or PerCP) monoclonal antibodies against CD4, CD8, CD3, CD19, CD23, CD16+CD56, CD45, CD14 and CD54 (ICAM-1) (Becton Dickinson (BD), San Jose, CA, U.S.A.) (*Table 1*). As a negative control for background staining, the isotype control was used. After washing, the PBMC samples were analyzed with a flow cytometer (FACScan, BD). Ten thousand cells were acquired and the data were analyzed with the CellQuest™ (BD) software. The leukocytes were gated on forward scatter (FSC)/side scatter (SSC) dot plots to study the numbers of cell subsets. The numbers of cells positive for the surface markers were expressed as a percentage of the cells within the gate.

Table 1. *Human leukocyte differentiation antigens and their specificity.*

Antigen	Distribution
CD45	Leukocytes
CD3	T cells
CD4	Helper/inflammatory T cells, monocytes, macrophages
CD8	Cytotoxic/suppressor T cells
CD19	B cells
CD23	Mature B cells, activated macrophages, dendritic cells, eosinophils, platelets
CD16	NK cells, granulocytes, macrophages
CD56	NK cells
CD14	Monocytes, macrophages, granulocytes, B cells

Flow cytometry of whole blood (Study IV). Fifty microlitres of venous blood-EDTA samples were incubated at RT for 15 minutes with a saturating concentration of fluorescent-conjugated (PE) monoclonal antibodies against CD11b (Mac-1 α^M subunit, Becton Dickinson (BD), San Jose, CA, U.S.A.). After washing, the blood-EDTA samples were incubated with FACS™ Lysing Solution (BD) for 5 min at RT, washed twice and analyzed with a flow cytometer (FACScan, BD) as described in the previous paragraph.

Immunocytochemistry (Study IV). Cytospin preparations of PBMCs were obtained with a sample application of 1×10^5 cells per slide (Cytospin3, Shandon, Life Sciences International (Europe) Ltd., Astmoor, Runcorn, Cheshire, U.K.). The cytospin slides were air-dried and stored frozen. The slides were slowly thawed and then fixed with acetone for

10 minutes at RT. After protein blocking with bovine serum albumin (BSA) solution, the slides were stained with primary antibodies. Primary antibodies to CD11a, recognizing the LFA-1 α^L subunit (Novocastra Laboratories Ltd., Newcastle, U.K.), were used at a dilution of 1:400 and incubated at RT in a humidity chamber for 30 minutes. The antigen-antibody complex was visualized using a commercial AEC kit (K3461, DAKO Corporation, Carpinteria, CA, U.S.A.) in accordance with manufacturer's instructions. In the negative control for background staining, the primary antibody was omitted. Slides were covered with Mountex (Histolab, Göteborg, Sweden). Cells were immunolabeled and stained in a single series to minimize batch-to-batch variation. Immunostained cell samples were examined microscopically and the results were expressed as percentage of positive cells.

3.4 Determination of intracellular cytokines (Study II)

Lymphocyte induction. Altogether, 1×10^6 PBMCs in one ml were incubated with 10 µg/ml Brefeldin A, 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml Ionomycin (Sigma, St. Louis, U.S.A.) at 37°C in 5% CO_2 for four hours to activate the lymphocytes for cytokine production. Brefeldin A, as well as monensin, disrupts intracellular Golgi-mediated transport and allows cytokines to accumulate, yielding an enhanced cytokine signal that can be detected by flow cytometry (Jung et al. 1993). Spontaneous cytokine production in unstimulated controls was assessed in an equivalent manner but without PMA and Ionomycin.

Intracellular staining. After activation, the cells were stained first with surface antigen-specific (CD8 or CD3) fluorescent-conjugated (FITC, PE or PerCP) monoclonal antibodies (Becton Dickinson (BD), San Jose, CA, U.S.A.) for 15 min. The cells were incubated with FACS™ Lysing Solution (BD) for 5 min at RT to lyse any possibly remaining erythrocytes, to fix the external epitopes and to assist in permeabilization. Subsequently, the cells were centrifuged for 5 min at 500 g and the Lysing Solution was changed to FACS™ Permeabilizing Solution (BD), in which the cells were incubated for 10 min at RT. After permeabilization, the cells were washed with PBS containing 0.5% BSA (Sigma, Steinheim, Germany) and thereafter the intracellular cytokines (IFN-γ, TNF-α and IL-4) were stained with fluorescent-conjugated monoclonal antibodies (BD) for 30 min. After

washing with 0.5% BSA in PBS, the cells were fixed in 1% paraformaldehyde (Sigma, Steinheim, Germany) and analyzed with a flow cytometer (FACScan, BD).

Flow cytometric analysis of intracellular cytokines. The data were collected and analyzed with a FACScan (BD) flow cytometer. Ten thousand cells were acquired and the data were analyzed with the CellQuest™ (BD) software. The lymphocytes were gated on FSC/SSC dot plots and the cytokine production was determined from that gate and displayed as single-color dot plots. The results were expressed as the percentage of cytokine expressing CD4+ or CD8+ T cells from the total CD4+ or CD8+ T-cell populations.

3.5 MAP kinase activation after mitogenic induction (Study III)

Lymphocyte induction. To induce T cells and increase mitogen-activated protein kinase (MAPK) phosphorylation, 1×10^6 lymphocytes were incubated in a humidified 5% CO_2 atmosphere at 37°C for 5 and 10 minutes together with 25 µg ConA (Pharmacia, Uppsala, Sweden) in a final volume of 1.2 ml. A control cell population was cultured with RPMI-1640 only. The cells were stored frozen at -70°C.

Western blotting and quantification. Cells (1×10^6 per sample) were lysed in a buffer containing 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40, 10 µg/ml aprotinin and leupeptin and 1 mM Na_3VO_4, and clarified by centrifugation at 13,000 g for 15 min. After the centrifugation, the supernatants were suspended in SDS sample buffer. Proteins were resolved by SDS/PAGE on 10% gels and transferred onto nitrocellulose filters. The antisera were used at 1:1000 dilution and the blots developed by the enhanced chemiluminescence technique (ECL, Amersham) according to the manufacturer's instructions. The same nitrocellulose filters were first probed with anti-phospho-MAP kinase antibodies (phospho-p44/42 MAP, New England Biolabs, MA, U.S.A.), and then stripped and reprobed with anti-MAP kinase antibodies (p44/42 MAP, New England Biolabs). The bands were scanned and their density was quantified with Fuji MacBas software (MacBAS 2.5, Fuji, Tokyo, Japan). The amount of phospho-MAP kinase in each sample was divided by the total amount of MAP kinase protein in the same sample. The results, i.e. the change in the adjusted amount of phospho-MAP kinase, were expressed as percentage values, where the level without mitogen induction was labeled 100%.

3.6 Determination of cytokine production with ELISA (Study I)

To induce lymphocytes and monocytes, 6.25×10^5 isolated cells in one ml of RPMI-1640 containing antibiotics and glutamine were cultured in a humidified 5% CO_2 atmosphere at 37°C for 48 hours, together with ConA at a final concentration of 25 µg/ml. A control cell population was cultured with RPMI-1640 only. The supernatants were collected and stored frozen at -70°C for later determination of cytokines. Subsequently, the TNF-α, IL-4, and IFN-γ produced during lymphocyte induction were determined from the thawed supernatants using a commercial ELISA kit (CLB, Amsterdam, the Netherlands) according to the manufacturer's instructions. The results of different runs were equalized, employing the comparison of standard curves, and are expressed as pg/ml. In cell cultures, the survival of cells was randomly tested with Trypan blue staining (0.4%, GibcoBRL, Life Technologies, U.K.).

3.7 Measurement of human breast milk ECP concentrations (Study V)

Fresh or frozen milk samples (1 ml) were centrifuged at 18,500 g for 60 minutes at +4°C and the translucent supernatants were collected and frozen. Human breast milk ECP concentrations were measured from ultracentrifuged milk samples in order to measure all the cellular ECP also. After thawing the frozen samples, they were diluted 1:2 in ECP Diluent (Pharmacia CAP System, Pharmacia Diagnostics, Uppsala, Sweden). ECP was measured by UniCAP™ method (Pharmacia Diagnostics) according to the manufacturer's instructions using a calibration curve with an extra concentration point of 1 µg/L. The results of different runs were equalized, employing a comparison of calibrator curves, and were expressed as µg/L. After the ultracentrifugation, satisfactory lysis of cells was randomly tested.

3.8 Radioallergosorbent test (RAST)

A commercial immunoCAP RAST-method (Pharmacia, Uppsala, Sweden) was used to measure cow's milk-specific IgE antibodies in patients' sera. These measurements were performed routinely in a clinical laboratory.

4 STATISTICAL ANALYSIS

Analysis of variance (ANOVA), Kruskal-Wallis and Mann-Whitney U tests were employed to determine the statistical significance of differences between continuous variables (I-V). Chi-Square test and Fisher exact test were applied to determine differences in proportions (III, IV, V). The correlations between data were studied with Spearman's rank correlation test (IV).

Geometric means have been presented with 95% confidence intervals (CI) and medians with ranges. Statistical significance was defined as $p \leq 0.05$. The analyses were carried out with NCSS2000 software (Kaysville, UT, U.S.A.).

RESULTS

1 CLINICAL FEATURES OF THE SUBJECTS

By the end of the follow-up, 68 of the infants (90% of whom had a positive family history of atopy) had CMA as shown by clinical cow's milk challenge, and 63 infants did not. Of the latter infants, 24 (83% with a positive family history of atopy) had atopic dermatitis. Five of these infants had suspicion of cow's milk allergy but the elimination/challenge procedure remained negative, and 7 had a positive history of reaction to egg (n=4), fish (n=1), nuts (n=1) or tomato (n=1), but no clinical challenges were performed to confirm the diagnosis. Thirty-nine infants (54% with a positive family history of atopy) were healthy. The positive family history of atopy was strongly correlated with development of atopic disease in the infant (p=0.0003, Chi-Square). Infantile colic was reported in 36% of the infants with CMA, in 17% of the infants with atopic dermatitis and in two (5%) of the healthy infants. During the follow-up, 15 (22%) of the CMA infants and 4 (6%) of the others developed asthma.

The durations of exclusive and total breastfeeding were comparable between the infants with CMA, atopic dermatitis without diagnosed food allergies and in the healthy infants (*Table 2)* (with data missing in n=18). One of the infants in this study was not breastfed, and 22 of the infants had received additional tolerated formula already from birth. Median age of onset of symptoms in infants with CMA was 1.5 months (range: 0.1-10 months). Of these CMA infants, 36 (56%) developed symptoms already during exclusive breastfeeding,

Table 2. *The duration of exclusive and total breastfeeding in different study groups.*

	CMA infants	Non-CMA infants, n=49		p-value
	n=64	Atopic dermatitis† n=24	Healthy n=25	
Duration of exclusive breastfeeding (months)	3 (0-6)	3 (0-6)	3 (0-6)	0.9
Total duration of breastfeeding (months)	7 (0-13)	7.0 (2-13)	6.5 (2-13)	0.6

Expressed as median (range).
Statistics from Kruskal-Wallis test.
†Atopic dermatitis without any diagnosed food allergies.

and in 28 infants the symptoms occurred after starting with formula or solid foods. Of the breastfed CMA infants, 18 (32%) continued to have symptoms despite the fact that the mothers were on a very restricted diet.

The median age at diagnosis of CMA was 5.1 months (range: 1.2-12 months). Of the CMA infants, 20 manifested either urticarial or eczematous skin eruptions, 6 had gastrointestinal symptoms and 39 reacted both cutaneously and gastrointestinally. Three infants had respiratory symptoms in addition to other symptoms of CMA. On clinical challenge with cow's milk, 18 patients developed urticaria immediately after introduction of a drop of cow's milk on their skin or lips. The challenge was performed through mother's milk in 7 patients (Järvinen et al. 1999a) and perorally in 43 patients. *Table 3* shows the volumes of cow's milk eliciting symptoms and the time of reaction in these perorally challenged patients. Infants with CMA manifested with gastrointestinal symptoms had reactions of significantly slower onset in the cow's milk challenge than the CMA infants with cutaneous manifestations (p=0.01, Kruskal-Wallis). Skin prick test or RAST to cow's milk was positive in 25 (38%) of the infants with CMA.

Table 3. *Time of onset of reaction and dose eliciting symptoms of cow's milk allergy in infants with immediate or delayed-type reaction after oral cow's milk challenge.*

	Cow's milk challenge	
	Immediate reaction n=14	Delayed reaction n=29
Time of onset of reaction †		
From last dose	0.5 (0.1-0.75)	4 (1-6)
From beginning	2 (0.25-4)	48 (1-168)
Dose eliciting symptoms ††		
Last dose	20 (0.3-200)	100 (10-200)
Cumulative dose	43 (0.3-360)	621 (36-3311)

† Median (range) expressed in hours. †† Median (range) expressed as ml (of cow's milk).
Immediate reaction is defined as occurring within 1 hour, and delayed reaction more than 1 hour after the last dose ingested.

Of the 105 samples collected from CMA infants or their mothers, 14 samples were obtained from the time point when the infant had as yet no symptoms suggestive of CMA [median age 0.7 months (range: 0.2-5.7 months)]. Sixty-five samples (from 52 infants and their mothers) were from the time point of a suspicion of, but as yet undiagnosed, allergy. These samples were more often from the time when the infant had acute allergic symptoms

(n=37) than from the time when infant was currently symptom-free (n=28). The remaining 26 samples were from 21 infants and 19 of their mothers, from the time point when the infant had an already diagnosed CMA and was commonly symptom-free due to a successful elimination diet.

Of the 60 atopic mothers, 34 had an infant with CMA, 15 mothers had an infant with atopic dermatitis without diagnosed food allergies, and 11 mothers had a healthy infant at the end of the follow-up. Eighty-two breast milk and blood samples were obtained from the atopic mothers. At the time of the sample collection, 10 mothers had atopic symptoms (7 with hay fever and 3 with eczema) and the rest were currently free of symptoms of atopic disease.

2 CYTOKINE PRODUCTION BY PBMCS FROM INFANTS (STUDY I)

Cytokine production by PBMCs from 43 infants was measured. Spontaneous TNF-α production in PBMCs was significantly lower in CMA infants at an early stage of developing CMA (n=31) than in the healthy infants (n=12) (p=0.017; Mann-Whitney U test). Furthermore, alteration in TNF-α production in PBMCs when induced with ConA was significantly lower in infants with CMA than in healthy controls (p=0.0003; Mann-Whitney U test) (*Figure 3)*.

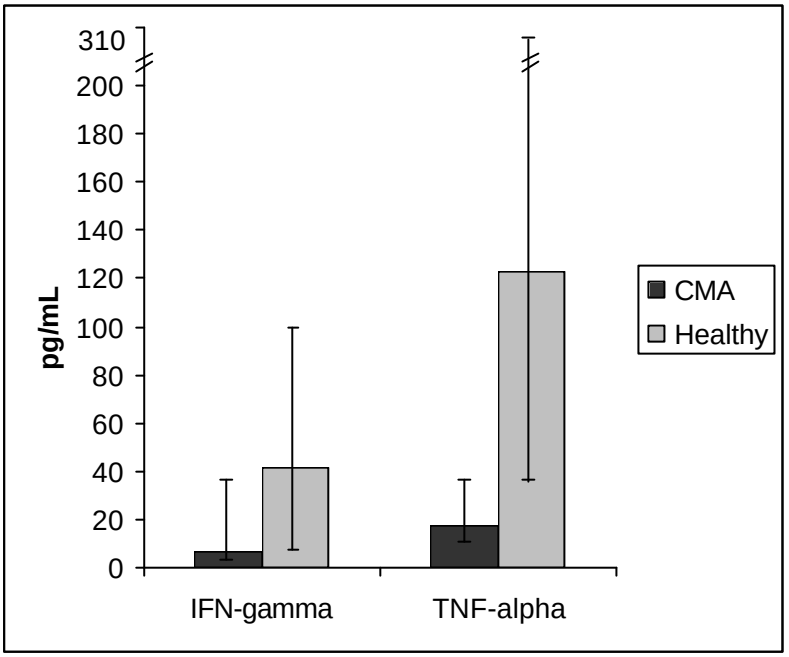

Figure 3. *Comparison of the medians of ConA-induced cytokine production in PBMCs from cow's milk allergic (CMA) infants and healthy infants. The vertical bars denote confidence intervals (95% CI).*

The mean stimulated production of TNF-α in infants with CMA did not even reach the level of spontaneous production in healthy infants: 40.9 pg/ml and 49.5 pg/ml, respectively (Study I: *Figure 1*). In infants with CMA, the change in IFN-γ production in PBMCs when induced with ConA was also strikingly lower than in healthy infants (p=0.0015; Mann-Whitney U test) (*Figure 3*). Production of these cytokines was low in all CMA infants; no association was found between cytokine production and the time of onset of the reaction during challenge or IgE/non-IgE-mediated CMA. IL-4 production was low or undetectable in both groups.

3 INTRACELLULAR CYTOKINE EXPRESSION IN T CELLS OF INFANTS (STUDY II)

Intracellular cytokines (IFN-γ, TNF-α, and IL-4) in CD4+ and CD8+ T cells of peripheral blood were detected by flow cytometry in 27 infants. There was a significant difference in the frequency of CD4+, but not CD8+, T cells expressing IFN-γ between infants who were at an early stage of developing CMA (n=12) and healthy controls (n=15). After four hours of stimulation with PMA and Ionomycin to activate cytokine expression in the presence of Brefeldin A – which disrupts the Golgi-mediated transport of proteins, thus leading to intracellular accumulation (*Figure 4*) – the frequency of CD4+ T cells expressing IFN-γ in the group with CMA (median 4.5 % [range: 2.8;8.9%]) was significantly lower than in the healthy control group (median 7.2 % [range: 2.2;20.1%]) (p=0.006; Mann-Whitney U test) (Study II: *Figure 1.a*). In the CD8+ T cells, the frequency of IFN-γ expressing cells was comparable between the infants with CMA and healthy infants (median 15.3% [range: 7.8;20.1%] and 18.4% [range: 8.7;32.5%], respectively) (p=0.15) (Study II: *Figure 1.a*). The basal cytokine expression from unstimulated samples was comparable between the study groups.

The frequencies of CD4+ and CD8+ T cells expressing TNF-α were comparable between the two study groups, p=0.22 (in CMA infants 11.5% [3.4;27.1%] and in healthy ones 15.3% [10.6;24.4%]) and p=0.43 (in CMA group 13.2% [8.5;24.2%] and in healthy group 15.9% [9.8;27.5%]), respectively (Mann-Whitney U test). The intensity of fluorescence for IL-4 was low or undetectable in both groups.

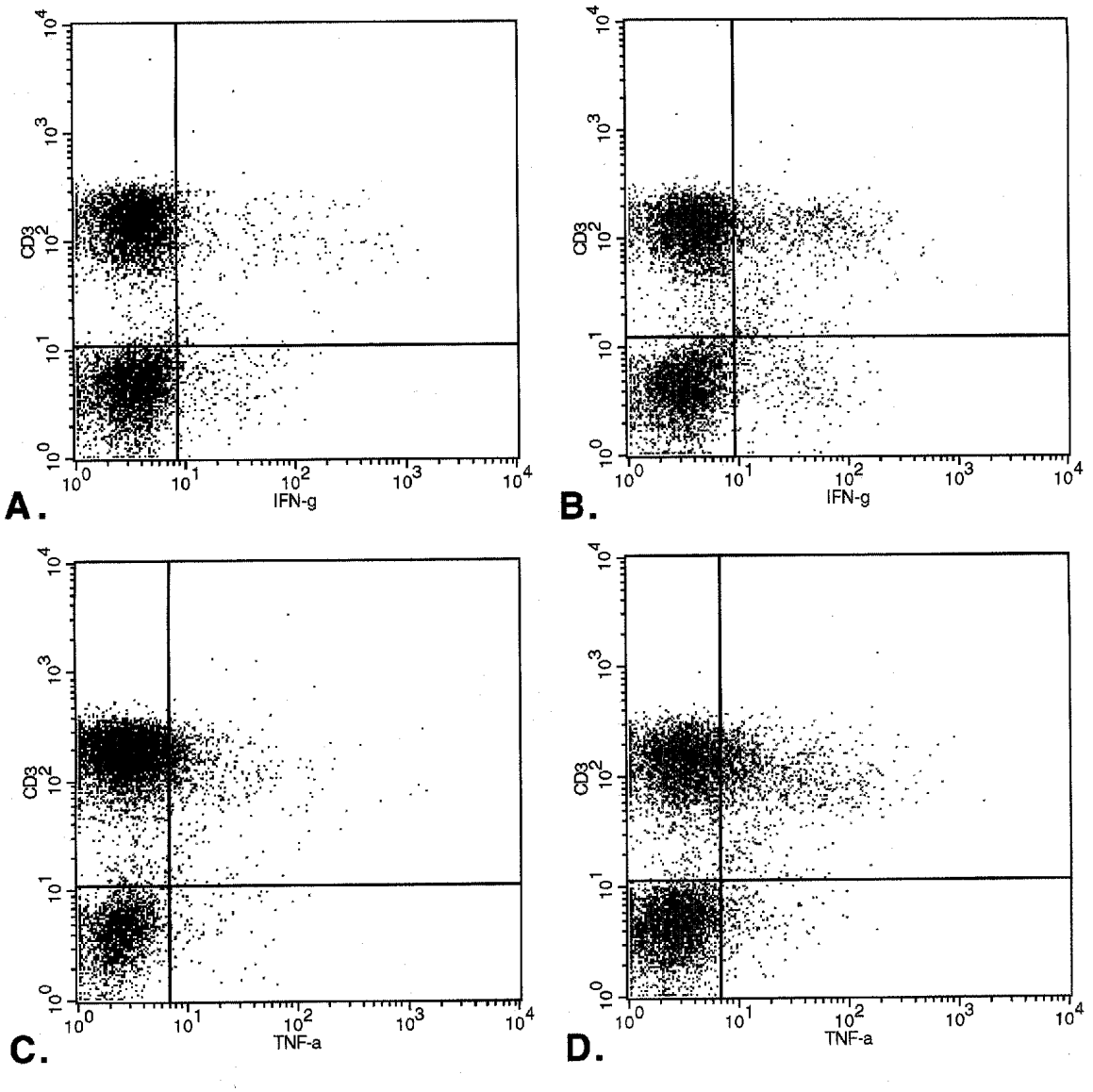

Figure 4. *Flow cytometric detection of T cells expressing the intracellular cytokines interferon (IFN)-g (A, B) and tumor necrosis factor (TNF)-a (C, D) both before (A, C) and after (B, D) mitogen induction.*

4 MAP KINASE ACTIVATION IN PBMCS OF INFANTS (STUDY III)

Activation of the MAP kinase Erk2 (p42) was investigated by stimulating PBMCs from infants with CMA (n=22) and from healthy controls (n=17) for 5 and 10 minutes, after which the phosphorylation of MAP kinase was analyzed by Western blotting (Study III: *Figure 1*). The percentage increase in MAP kinase phosphorylation after 5 minutes of incubation with ConA when compared with baseline phosphorylation (level without mitogen induction, 100%) was significantly higher in cells from patients with current

symptoms of CMA than in cells from patients free of symptoms and cells from healthy infants (p=0.02; Kruskal-Wallis test) (*Figure 5*). A time-course experiment showed that the change in MAP kinase activation in cells from infants with acute-phase CMA was still increasing after 10 minutes of mitogen induction. Cells from healthy and symptom-free infants reached their highest MAP kinase phosphorylation level as early as after 5 minutes induction (Study III: *Figure 3*). Statistically, the differences in the medians after 10 minutes of induction were still significant between these three study groups (p=0.03; Kruskal-Wallis test) (*Figure 5*).

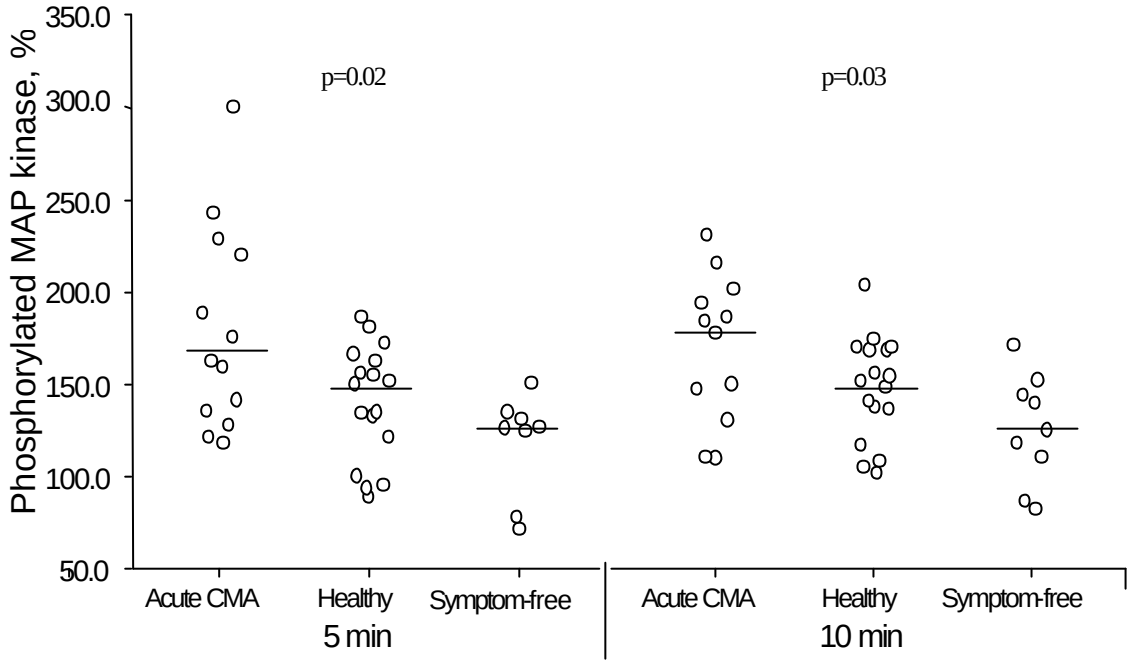

Figure 5. *The per cent change in MAP kinase phosphorylation after 5 and 10 minutes of ConcanavalinA induction (with baseline without mitogen induction as 100%). The phosphorylation level in cells from patients with acute symptoms of cow's milk allergy (CMA) was significantly higher than in cells from patients without symptoms and cells from healthy infants. The horizontal lines denote the median values: 163%, 150%, 126%, and 181%, 152%, 125%, respectively.*

Furthermore, the association between having IgE-mediated CMA, i.e. having positive Prick or RAST test to cow's milk proteins, and increased MAP kinase activation was analyzed. The increased MAP kinase activation was found to correlate positively with the non-IgE mediated CMA in the patients with acute symptoms of CMA, although the number of infants in these subgroups was quite small. Cells from infants having non-IgE-

mediated CMA showed an almost 2-fold increase in MAPK phosphorylation, while cells from infants with IgE-mediated CMA showed a 1.3-fold increase, which is in the same range as the increase in cells from healthy infants and infants with CMA but free of symptoms (Study III: *Table 1*).

5 EXPRESSION OF ADHESION MOLECULES ON PBMCS OF INFANTS (STUDY IV)

ICAM-1 expression on peripheral blood lymphocytes was measure by flow cytometry in 39 infants. It was significantly higher in the CMA group (n=25) than in the healthy control group (n=14) (p=0.03; Mann-Whitney U test). Moreover, higher ICAM-1 expression was found in the infants with CMA who presented with gastrointestinal, skin and gastrointestinal or multiorgan symptoms than in those with cutaneous symptoms only, or healthy controls (p=0.005; Kruskal-Wallis test) (*Figure 6*). The ICAM-1 expression was not associated with the positive SPT or RAST nor with the presence of current symptoms of CMA at the time of the sample collection. The ICAM-1 expression was lower in the CMA infants reacting immediately in the challenge test, who had skin symptoms more often than slowly reacting CMA infants. The expression of Mac-1 (CD11b) on mononuclear cells and total blood leukocytes, measured by flow cytometry in the 21 infants, was comparable between our two study groups (data not shown).

LFA-1 expression from peripheral blood lymphocytes of the 36 infants was analyzed by immunocytochemistry. The proportions of LFA-1 expressing lymphocytes were comparable in both CMA and control infants: median 21% (range: 14-59%) and 21.5% (range: 3-35%), respectively (p=0.53; Mann-Whitney U test). However, the expression of LFA-1 was higher in the CMA infants reacting immediately in the challenge test than in those with delayed reaction (p=0.015; Mann-Whitney U test), although the number of infants in these subgroups was small. Furthermore, LFA-1 expression was higher in the infants having positive SPT or RAST than in the infants with negative SPT and RAST (p=0.003; Mann-Whitney U test) (*Figure 6*).

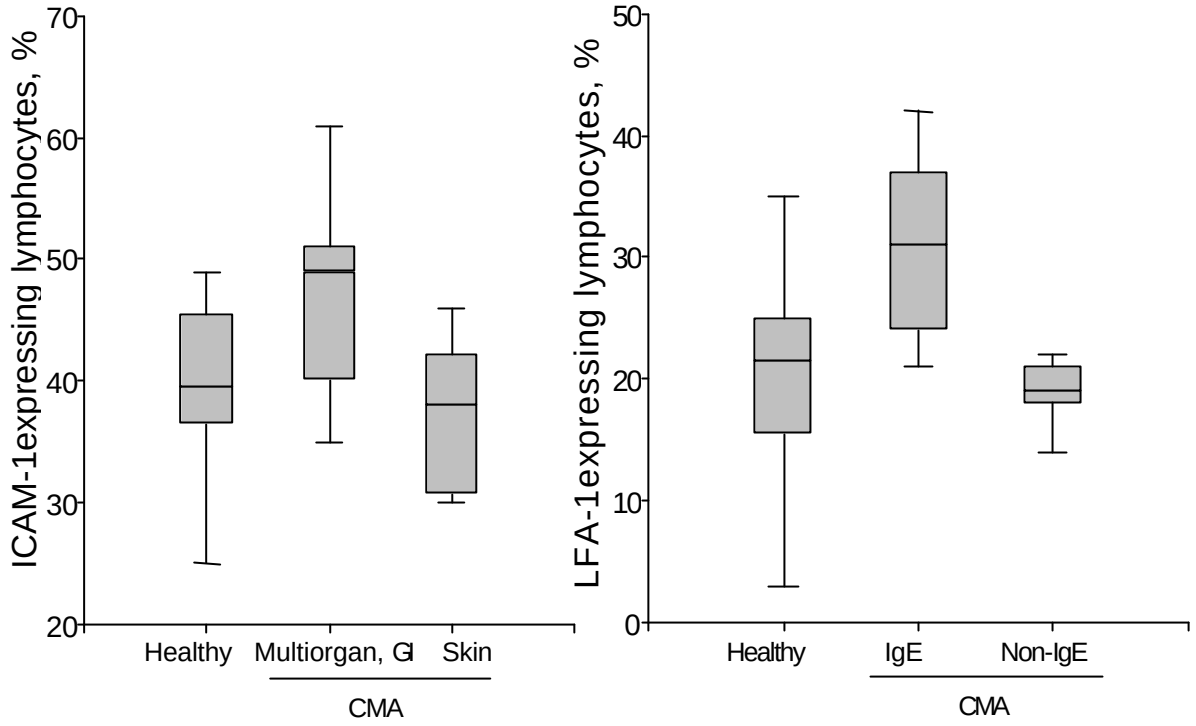

Figure 6. *Proportion of adhesion molecule (intercellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen (LFA)-1) expression by peripheral blood lymphocytes from infants with different manifestations of cow's milk allergy, and from healthy infants. The boxes represent the 95% confidence intervals with medians and the vertical lines represent the ranges.*

6 ECP CONCENTRATIONS IN MOTHER'S MILK (STUDY V)

Due to the significant difference in the composition of colostrum and mature milk, milk samples from the first week and at 3 months of lactation were analyzed separately. Colostral and early milk samples (day 1-7) were obtained from 15 mothers. The levels of ECP varied within each of the three groups: the median was 2.1 µg/L (range <2 to 92.8) in the CMA group (n=5), 5.7 µg/L (<2 to 26.3) in the AD group (atopic dermatitis without diagnosed food allergies) (n=4), and 6.9 µg/L (<2 to 55.0) in the healthy group (n=6).

Mature milk samples (taken at 3 months) from 94 mothers were examined. ECP concentration was below the detection limit (<2 µg/L in 1:2 diluted samples) in milk from all the mothers with a healthy infant, whereas detectable levels were found in milk from 27% of mothers (14/51) with a CMA infant and in samples from 42% (10/24) of those with a baby with AD (*Figure 7*). Measurable milk ECP concentrations were significantly more often detected in the mothers with infants with CMA or atopic dermatitis than in those with

a healthy infant (CMA vs. healthy: p=0.008, AD vs. healthy: p=0.001, CMA vs. AD: p=0.2; Fisher exact test). When the detection limit of the assay (2 µg/L) was used as a cut-off level, all the infants from mothers whose milk ECP was higher than this developed CMA or atopic dermatitis (specificity 1.0, sensitivity 0.32, positive predictive value (PPV) 1.0 and negative predictive value (NPV) 0.27). There was no association between mother's milk ECP levels and maternal atopy (p=0.84; Mann-Whitney U test).

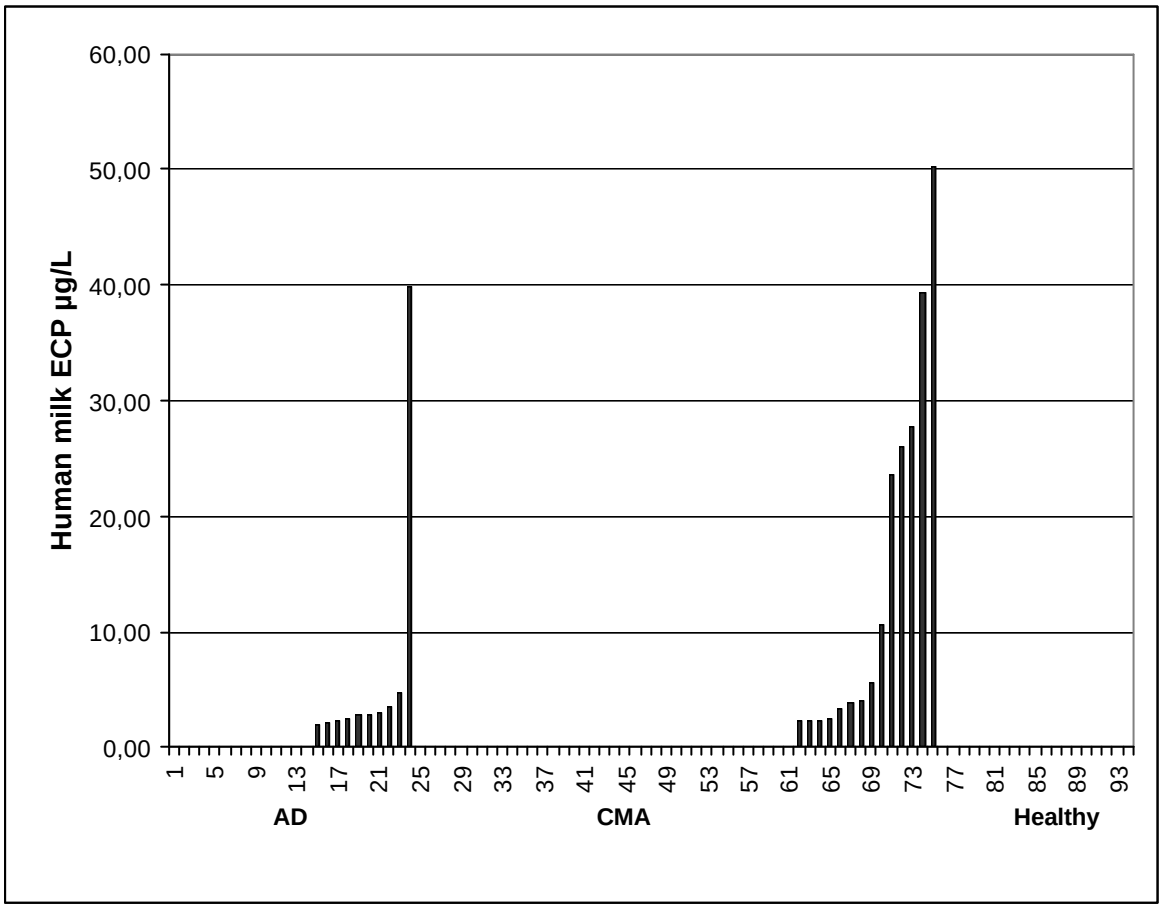

Figure 7. *The concentration of eosinophilic cationic protein (ECP) in human milk from mothers with a breastfed infant suffering from atopic dermatitis without diagnosed food allergies (AD, samples 1-24), cow's milk allergy (CMA, samples 25-75), or with an infant who was healthy (samples 76-94).*

7 LEUKOCYTE SUBSETS IN MOTHER'S MILK (STUDIES II, IV, V)

The proportion of different leukocytes in mother's milk, in relation to maternal atopy and clinical status of the breastfed infant, are presented in *Table 4.* In the milk of atopic

mothers, the percentage of lymphocytes was higher than in that of the non-atopic mothers. In this study population, the mother's milk leukocyte composition was statistically comparable in the clinically different groups of breastfed infants (CMA, atopic dermatitis or healthy).

The proportion of mother's milk neutrophils was positively correlated with the proportion of ICAM-1 expressing peripheral blood lymphocytes in breastfed infants (r=0.32, p=0.04; Spearman correlation test) (*Figure 8*). Furthermore, the mother's milk macrophages tended to show a negative correlation with the ICAM-1 expressing lymphocytes of the breastfed infant (r=-0.25, p=0.1) (*Figure 8*).

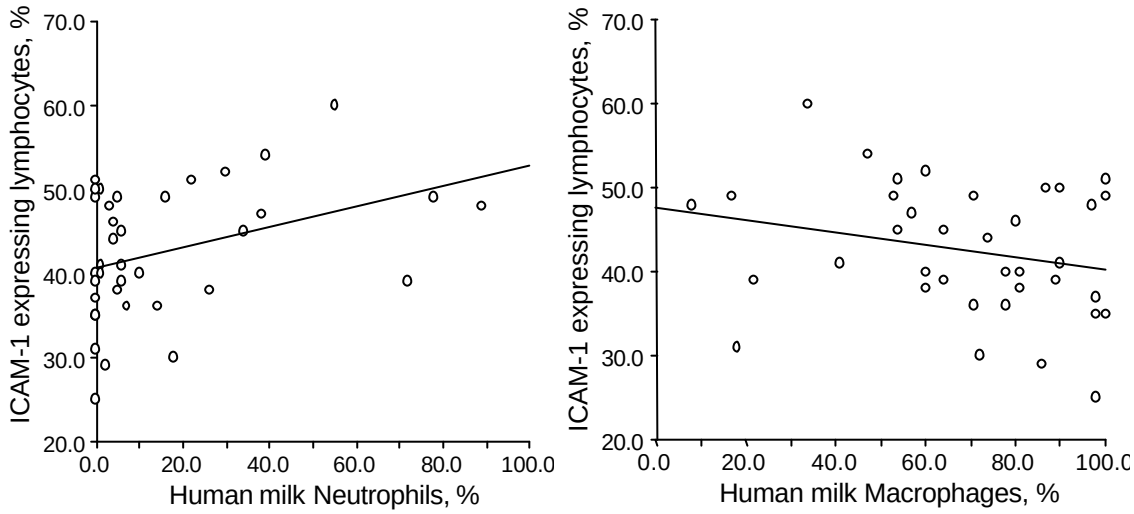

Figure 8. *Correlation of mothers' milk neutrophils and macrophages (%) with the infants' ICAM-1 expressing circulating lymphocytes (%).*

The detectable levels of ECP in human milk were associated with the higher proportion of neutrophils (p=0.02), but also the higher proportion of eosinophils had a tendency to associate to detectable ECP levels, p=0.08 (Mann-Whitney U test). In 11 of the 24 samples with detectable ECP (>2 µg/L), no eosinophils were detected in the light microscopy slides. Interestingly, three milk samples had clear eosinophilia but no ECP was detected in the milk by UniCAP method. The lower proportions of human milk macrophages were associated with detectable human milk ECP concentrations (p=0.02; Mann-Whitney U).

Table 4. *Percentages of different leukocytes in the milk of atopic (n=57) or non-atopic (n=42) mothers, and with infants having cow's milk allergy (CMA) (n=56), with infants having atopic dermatitis (AD) (n=19), or with healthy infants (n=24).*

	Milk of mothers with						
Leukocyte	atopy	no atopy	p-value	infants with CMA	infants with AD	healthy infants	p-value
Macrophages (%)	69 (13-100)	68 (5-100)	0.4	62 (5-100)	76(52-100)	71 (37-100)	0.4
Monocytes (%)	2 (0-23)	1 (0-22)	0.5	0 (0-22)	2 (0-23)	1 (0-23)	0.1
Lymphocytes (%)	10 (0-78)	5 (0-88)	0.04	8 (0-88)	5 (0-26)	6 (0-41)	0.2
Neutrophils (%)	13 (0-80)	7 (0-89)	0.3	9 (0-89)	10 (0-80)	10 (0-31)	0.8
Eosinophils (%)	0 (0-35)	0 (0-30)	0.9	0 (0-33)	0 (0-30)	0 (0-35)	0.4

Values expressed as median (range).

P-values calculated with Mann-Whitney U test or Kruskal-Wallis test.

DISCUSSION

1 IFN-γ AND TNF-α RESPONSES IN INFANTS WITH CMA

The "allergic march" starts already during the first months of life when the immune system is virgin and immature. After Th2-biased intrauterine life, the immune system is believed to become skewed towards Th1-type responses as a consequence of the external antigenic pressure (Stumbles et al. 1998). This skewing from Th2 to Th1-type responses appears to be delayed in atopic diseases (Prescott et al. 1999) and is believed to be due to changes in the microbial and antigenic environment over the past decades in Western communities (Holt et al. 1997, Strachan 2000). The studies on the higher prevalence of allergic diseases in Western lifestyle countries compared to countries with lower socio-economic conditions or developing countries have lent support to this hypothesis (Jõgi et al. 1998, Vartiainen et al. 2002). It has been suggested that a change in the immune modulating environment has led us to the increased prevalence of disorders of both Th1 (celiac disease, type 1 diabetes) and Th2 (allergies, asthma) types (Kero et al. 2001, Julge et al. 2002).

The decreased proportion of intracellular IFN-γ producing CD4+ T cells in CMA infants demonstrated in the present study (II) confirms the suggestions of defects in the number of mature and activated Th cells. However, we could not overrule the possibility that the IFN-γ deficiency might partly be a consequence of the different quantitative expression of cytokines per cell, because we did not measure the intensity of the fluorescence to assess the amounts of the expressed cytokine in one cell. Whether in the ELISA (I) or the flow cytometric analyses (II), we could not see the increased Th2-type responses reported in atopic diseases in other studies (Tang et al. 1993, Campbell et al. 1998). This could be due to the young age of our study patients, when the overall cytokine production is low, and to the nature of IL-4 in that it is expressed and responsive in very low concentrations. A more useful tool for detecting IL-4 expression would have been measuring IL-4 mRNA levels or assessing Th2-type cells by specific chemokine/cytokine receptor expression. Due to this, we can only hypothesize about the Th1/Th2 balance indirectly. However, our result clearly suggests that a reduced number of IFN-γ expressing Th cells, which are either Th1-type or Th0-type cells, is found in CMA. Because there were equal proportions of CD4+ T cells in CMA infants and in healthy infants, this decreased proportion of IFN-γ expressing CD4+

cells in infants with CMA results in a larger proportion of the IFN-γ-non-expressing cells that would presumably be Th2, Th3 or Tr1 cells.

At the single cell level, the present study has shown that there is defective IFN-γ expression in the CD4+ T cells (Th cells) of CMA infants (II), as shown also in atopic patients by another study from Jung and colleagues (1995). The flow cytometric analyses in the present study (II) showed a decreased proportion of IFN-γ producing CD4+ cells relative to all CD4+ cells, but a normal proportion of IFN-γ producing CD8+ cells (cytotoxic effector T cells). Moreover, the total proportion of CD8+ lymphocytes relative to all lymphocytes was reduced in CMA infants in the present study, which results in a decreased number of IFN-γ and TNF-α producing CD8+ cells in these infants, although the ratios of cytokine producing CD8+ cells relative to all CD8+ cells were equal in CMA and healthy infants (II). CD8+ T cells from the naive CD8+ T cell form to the effector and memory CD8+ T cell forms have been reported to be able to produce IFN-γ (Mailliard et al. 2002). Moreover, CD8+ T cells have been demonstrated to induce the Th1-promoting phenotype in DCs, i.e. in IL-12 production, whereas absence of the naive CD8+ T cells at the priming conditions leads to the IL-4 producing Th2-type T cells (Mailliard et al. 2002). These findings give evidence for the important and novel helper role of CD8+ T cells in the development of Th1-type responses. Together with the results in the present study, these findings suggest that the deficiency in the number of CD8+ T cells could further lead to the defective priming of CD4+ T cells into IFN-γ producing cells.

In the previous studies on CMA, the production of a Th1-type cytokine, IFN-γ, in PBMCs was shown to be defective (Suomalainen et al. 1993b). In the present study, this finding was confirmed and we found a similar finding for another proinflammatory cytokine, TNF-α (I). Although both of these cytokines contribute to the Th1 immune response, there were no differences in the production of these cytokines between the IgE- and non-IgE-mediated CMA. After antigen exposure, the immune system is activated to produce cytokines such as TNF-α, which has been shown to differentiate the immature DC to more mature one with enhanced expression of the T cell co-stimulatory and adhesion molecules and cytokines priming T cells (Sallusto et al. 1995, Ardavin et al. 2001). Thus, lack of TNF-α may dramatically disturb the development and maintenance of oral tolerance to dietary antigens by interfering with the activation of antigen presenting cells and decreasing the

communication between activated DCs and target T cells. This could further lead to the decreased IFN-γ production and, consequently, disturbed function of T cells, as previously demonstrated by Suomalainen and colleagues (1993b). Moreover, it has been disproved that the IFN-γ deficiency would be due to an intrinsic genetic defect, because the appropriate cytokine milieu (IL-2 or IL-12) could restore the IFN-γ production of atopic patients at the precursor T cells (Jung et al. 1999). Instead, the authors suggested that the deficient IFN-γ response is the result of an altered T-cell/APC interaction, as proposed in the present study also. If the infant's T-cell function is depleted or only weakly induced because of low TNF-α production, exposure of a child to foreign proteins might cause a local or systemic immunoinflammatory reaction.

TNF-α has synergistic effects with IFN-γ, and they are needed for adequate communication in the cellular network of the neonate (Sturgess et al. 1992). TNF-α is initially expressed as a membrane-anchored precursor, which is proteolytically processed to yield the mature cytokine (Kriegler et al. 1988). This processing is dependent on matrix metalloproteinase-like enzymes (Gearing et al. 1994). The result of the present study (II) of comparable intracellular expression of TNF-α in T cells (both in CD4+ and CD8+) between CMA infants and healthy infants could indicate that there is no defect in the expression of the TNF-α gene. This finding, compared to the results of decreased production of TNF-α from the PBMC culture supernatants from CMA infants (I), could indicate that there may be defects in the cleavage process of the TNF-α precursor. This defect could lie in the cleavage enzyme(s), or in the cytokines regulating the secretion, such as TNF-α itself. Also, the defective production of TNF-α from PBMCs but normal frequencies of TNF-α expressing T cells could reflect that monocytes are deficiently activated to TNF-α production.

2 IMMUNOLOGICAL FINDINGS IN DIFFERENT MANIFESTATIONS OF CMA

The mechanisms behind food allergy are still poorly understood. IgE-mediated food allergy has been characterized somewhat better, but the mechanisms leading to a delayed-type food allergy still remain largely unresolved. According to the results of the present study (III), in the acute phase of CMA, before the introduction of a successful elimination diet, T cells of infants with CMA become more vigorously activated, as measured by MAP kinase phosphorylation after mitogenic induction, than T cells of healthy infants or those CMA infants who are on elimination diet and free of symptoms of CMA. These results suggest high activation of the immune system in infants with acute symptoms of CMA, as compared to healthy, age-matched children. The most severely reacting infants, who had difficulties in becoming symptom-free even after being fed solely with amino acid-based formula, showed the greatest MAP kinase activation (III). This might even reflect chronic inflammation in these infants. The equivalent response in CMA infants free of symptoms and in healthy controls indicates that elimination of cow's milk proteins in the infant's diet has a normalizing effect on the function of the immune system, at least when assessed by a responding activity to mitogenic stimulation. In agreement with this particular finding, fibroblasts from psoriatic lesions, also an immunoinflammatory disease, were shown to express high MAP kinase activity, whereas no differences in MAP kinase activity were found between normal and lesion-free psoriatic skin (Dimon-Gadal et al. 1998).

Furthermore, the increased MAP kinase activation was found to be associated with non-IgE mediated CMA (III), although the numbers of subjects in these subpopulations were quite small. The strong association of non-IgE-mediated disease with the high activation of MAP kinase suggests that increased responsiveness of T cells to stimulation may mediate the prolonged and delayed-type immunoinflammatory disease. In a recent study, the inhibition of MAP kinase (Erk) phosphorylation in anti-CD3/CD28-activated naive T cells resulted in a strong induction of IL-4 production and inhibition of IFN-γ production, suggesting that the MAP kinase cascade might promote Th1 responses and inhibit Th2 responses (Smits et al. 2002). According to these previous findings and our results, the cell-mediated immune mechanisms in the delayed-type food allergy – as shown in the non-IgE mediated CMA infants in the present study (III) – would be assumed to be Th1-mediated. Moreover, in atopic dermatitis, Th1-type responses have been reported to be

associated with chronic inflammation reactions (Grewe et al. 1998) and both Th1 and Th2-type responses were even seen in parallel after continuous antigen exposure (Savolainen et al. 2001).

Interactions of ICAM-1 with LFA-1 and Mac-1 are important for their function as co-stimulatory molecules in T-cell activation (Hubbard and Rothlein 2000). Because most leukocyte functions depend on cell-to-cell contact, they are strictly controlled both at the level of specificity and strength of interaction. Thus, several molecular systems have involved, beginning with the selectin-induced weak contact between the cells, followed by the firm adhesion through integrin intercellular molecular binding, which allows the stimulating antigen presentation to occur (Gahmberg et al. 1999). In a previous study, it has been demonstrated that anti-CD54 (anti-ICAM-1) antibodies effectively suppress the proliferative responses of PBMCs, both in patients with atopic dermatitis and in healthy controls (Kawamura et al. 1998). The high proportion of ICAM-1 expressing lymphocytes found in the present study in infants with CMA (IV) might consistently reflect a high activation and proliferation of the circulating lymphocytes in these patients. Furthermore, in another study, cow's milk proteins given orally have been shown to increase the soluble ICAM-1 in infants at the ages of 3, 6, 9 and 12 months, reflecting a possible immune response against these proteins during early infancy (Paronen et al. 1996). This finding supports our suggestions about the elevated ICAM-1 expression in infants with CMA being a result of increased antigen load in these sensitized infants.

Food allergy is a disease manifested in multiple effector organs, some of which can be distant from the initial sensitization site in the gut. This suggests that migrating cells may play a major role not only in the pathogenesis of the disease, but also in the regulation of the immune response, and in determining the sites of involvement in tissue-directed allergic responses. Milk-specific lymphocytes were found to express more cutaneous lymphocyte antigen (CLA) in milk-allergic patients with atopic dermatitis, suggesting preferential homing to the skin by circulating milk-specific T cells from these patients (Abernathy-Carver et al. 1995). $\alpha 4\beta 7$ integrin is described as an integrin expressed in activated T cells with homing properties toward the gut, and has been demonstrated to be highly upregulated in β-lactoglobulin (BLG)-specific T cells from patients with CMA (Eigenmann et al. 1999). In another study, $\alpha 4\beta 7$ integrin and ICAM-1 were shown to be

elevated on the mononuclear cells in the lamina propria of the small intestinal mucosa from food allergic adults, reflecting homing properties of these molecules toward the gut (Veres et al. 2001). The high proportion of circulating ICAM-1 expressing lymphocytes in CMA infants reported in the present study (IV) was especially associated with gastrointestinal and multiorgan symptoms in CMA infants. Interactions of ICAM-1 with LFA-1 and Mac-1 are important for their transendothelial migration (Gahmberg et al. 1999). As reported in adults by Veres and colleagues (2001), the results in the present study on infants suggest that ICAM-1 could be a receptor for homing to the gut. Additionally, as most of the patients in our study with elevated levels of ICAM-1 expressing circulating cells had CMA that manifested with multiple effector organs, this might indicate that ICAM-1 reflects the systemic allergic inflammation and the migration of activated lymphocytes to the tissues.

In the present study, high expression of LFA-1 on lymphocytes was associated with IgE-mediated CMA (IV). LFA-1 has been reported to upregulate IgE synthesis in B cells (Katada et al. 1996). In mast cells, the use of anti-LFA-1 antibodies inhibited adhesion-induced mast cell degranulation (Inamura et al. 1998), suggesting a role for LFA-1 and ICAM-1 in mast cell degranulation. Moreover, eosinophils from atopic individuals have been shown to express more LFA-1 than non-atopic controls (Lantero et al. 2000). In agreement with this, infants in our study reacting immediately on cutaneous challenge and having a positive SPT had high levels of LFA-1 expressing lymphocytes (IV), which could reflect the IgE-mediated mast cell activation and eosinophil activation also.

3 HUMAN MILK AND DEVELOPMENT OF CMA

Human breast milk provides nutrients and multiple immunological factors, such as secretory IgA, to the breastfed infant (Goldman et al. 1982). It is known to be an important immunologic support system extending from the mother to her infant during the first months of life. Yet allergic diseases often develop during exclusive breastfeeding (Isolauri et al. 1999, Järvinen et al. 1999a). Recent findings on the relationship between long duration of breastfeeding and an increased risk of the breastfed infant developing atopy (Wright et al. 1999, Bergmann et al. 2002, Sears et al. 2002) have lent support to the view that the protective effect (or lack thereof) may be due to individual variations in the levels of immunological constituents in the mother's milk. In the present study, ECP from the

mother's milk was found to be related to the development of allergic disorders in breastfed infants.

Eosinophils are rarely found in human milk (Vassella et al. 1992). Vassella and co-workers (1992) have shown that the milk of allergic/atopic mothers contains a relatively high proportion (4%) of eosinophils, but no data about the health status of the breastfed infants was given in their paper. Recently, it has been shown that in the milk of mothers with a CMA infant, the proportion of macrophages is significantly smaller than in the milk of mothers with infants without CMA (Järvinen and Suomalainen 2002). Moreover, the mothers with high proportions of neutrophils (>20%) or eosinophils (>1%) in their milk more often had a CMA infant than the mothers with low proportions of neutrophils or eosinophils (Järvinen and Suomalainen 2002). In the present study, the proportions of different leukocytes bore no relation to the infant's clinical outcome as measured with non-parametrical tests of variance (*Table 4*). The study material was partially the same in these two studies: in the earlier study (Järvinen and Suomalainen 2002), the material had been collected between June 1995 and May 1998, and the sample collection was continued until September 2001 for the present study. The differences in the results are due to the use of different statistical analyses: in the earlier study the proportion of eosinophils was used as a class variant (>1% or <1%), whereas in the present study it was used as a continuous variant. Furthermore, we found a higher proportion of lymphocytes in the milk of atopic mothers (*Table 4*), which was reported in the earlier study as well (Järvinen and Suomalainen 2002).

ECP is an eosinophil-derived protein that has been demonstrated *in vivo* and *in vitro* to be tissue-destructive (Weller 1991). The present study shows that 32% of the mothers having an infant developing atopic eczema or CMA during the follow-up of two years had eosinophils and/or ECP in their milk (*Figure 7*, V). These results indicate that in these infants, mother's milk eosinophils and/or ECP may act as a contributing factor to the pathogenesis of the disease, although no causal relationship was demonstrated. They could also indicate that milk ECP levels may reflect another causal factor that predisposes an infant to food allergy, such as maternal atopy. This was not proven to be the case, however, since milk ECP was not directly associated with maternal atopy (V). In the gut of the suckling infant, eosinophils will finally break by cytolysis during cell death, even without any activation process leading to degranulation, and the mediators will be released.

Subsequently, the permeability of the gut could be enhanced, and the antigenic load could thereby increase. It has been demonstrated in previous studies that eosinophils and their mediators may have a role in the intestinal inflammation process (Chung et al. 1999, Majamaa et al. 1999, Saarinen et al. 2002). Chung and colleagues (1999) demonstrated elevated levels of eosinophil-derived major basic protein (MBP) in the intestinal mucosa of infants with cow's milk-sensitive enteropathy, but they had no evidence about the original source of the MBP. A very recent study has demonstrated a higher level of fecal ECP in CMA infants with intestinal symptoms than in those with other symptoms, or control subjects (Saarinen et al. 2002). The authors postulated that in these patients, the allergic inflammation of the intestine was caused by eosinophilic reactions. The possible contributory role of human milk eosinophils or ECP in these inflammatory processes has not been investigated previously.

4 ALLERGIC INFLAMMATION IN BREASTFED INFANTS WITH CMA

After birth, a major exposure to antigens affects the immature immune system of the newborn. The external stimulation starts to skew the innate Th2-type responsiveness towards Th1-type responses (Stumbles et al. 1998, Prescott et al. 1999). The potential candidates for the "Th1-adjuvant" have been proposed to be the adequate microbial exposure and immunologically supportive mother's breast milk (Holt et al. 1997, Järvinen and Suomalainen 2001). Recently, it has been demonstrated that the composition of mother's milk might differ from one individual to another, and this difference may be associated with the development of allergies in the breastfed infants (Järvinen and Suomalainen 2001). In the present study, we suggested that in some cases in which ECP is present in the mother's milk, breastfeeding could even be deleterious to the infant (V). Ingestion of large amounts of ECP in the mother's milk by the infant could hypothetically lead to an inflammation process in the gut mucosa and further increase the intestinal permeability to antigens. This increased antigen load might interfere with the development of oral tolerance, and result in adverse immune responses.

In the present study (I, II), the immune responses in the infants in an early phase of CMA were reported to be Th1/Th0 deficient, disturbing the balance between the T helper cells. In contrast to previous studies, we did not detect increased Th2-type responses, which may be due to use of a low sensitivity assay. Moreover, we showed that in symptomatic infants

reacting with delayed-type hypersensitivity responses, the MAP kinase activation as a response to mitogenic stimulation was more vigorous than in children reacting with type I (IgE mediated) reactions (III). The activation of the MAP kinase cascade has been reported to promote the Th1-type responses in humans (Smits et al. 2002). This suggests that the delayed-type responses in CMA infants could be Th1 polarized. A more vigorous MAP kinase activation, together with the finding of a smaller proportion of Th1-type cells in CMA infants compared to controls, might suggest that the smaller subpopulation of Th1 cells is however more reactive and active in the delayed-type responses during the symptomatic phase of CMA.

The circulating lymphocytes from CMA infants were shown in this study (IV) to express the co-stimulatory adhesion molecule ICAM-1 more often than the ones from healthy controls. This could reflect high activation of the lymphocytes in CMA infants. This finding was associated with the multiorgan and gastrointestinal tract manifestation of CMA, whereas a higher proportion of LFA-1 expressing lymphocytes was found in the CMA infants with the IgE-mediated manifestation. The expression of ICAM-1 on circulating lymphocytes might indicate their properties of homing toward the gut, as postulated for adults by Veres and colleagues (2001).

SUMMARY AND CONCLUSIONS

The "allergic march" often starts already during the first months of life when the immune system is inexperienced and immature. The intrauterine Th2-skewed responsiveness starts to change after birth towards the Th1-type responses through the external exposure to antigens. This change is delayed in infants developing an allergic disease later. However, the precise immune mechanisms behind the development of allergic inflammation processes in infants with food allergy are still incompletely understood. The purpose of the present study was to determine characteristics of the allergic inflammation reaction in CMA infants relative to those in healthy controls. In addition, the association of mother's milk ECP, a cytotoxic mediator of eosinophils in an allergic reaction, to the development of allergic disorders in the breastfed infant was assessed.

The analyses of the production of the proinflammatory cytokines, IFN-γ and TNF-α, and the anti-inflammatory cytokine IL-4 were performed from the culture supernatants of PBMCs using ELISA (I). The production of both IFN-γ and TNF-α was found to be reduced in infants with CMA (n=31) when compared to healthy controls (n=12). Furthermore, the proportion of IFN-γ expressing Th cells (CD4+) was lower in CMA infants (n=12) than in healthy infants (n=15) as measured by flow cytometric analysis (II). The decreased TNF-α production was not reflected in the proportions of TNF-α expressing cells (either in CD4+ or CD8+ cells). However, the proportion of CD8+ T cells was reduced in CMA infants as compared to healthy ones. IL-4 expression was low both in the ELISA and by flow cytometrical analysis (I, II).

To detect possible differences in the T cell signal transduction in infants with CMA (n=22) compared to healthy infants (n=17), the MAP kinase-phosphorylation levels after mitogenic induction were evaluated by Western blot (III). The proportion of the phosphorylated form of the MAP kinase was higher in the acute phase of the CMA than in the symptom-free CMA infants or healthy ones. Moreover, the MAP kinase was more vigorously phosphorylated in the CMA infants with delayed-type responses than in CMA infants with the IgE-mediated responses. To assess the possible association of the adhesion molecules with lymphocyte migration to effector organs, the expression of co-stimulatory adhesion molecules ICAM-1, LFA-1 and Mac-1 on circulating lymphocytes was analyzed

from infants with CMA (n=25) and from healthy controls (n=14) by flow cytometry (IV). The proportion of ICAM-1 expressing lymphocytes was significantly higher in patients with CMA than in healthy infants. Furthermore, the high proportion of ICAM-1 expressing cells was associated with gastrointestinal and multiorgan symptoms in the CMA infants, whereas the LFA-1 α^L expressing cells seemed to be most frequent in the IgE-mediated CMA. There was no difference in the frequency of Mac-1 α^M expressing cells between our study groups.

The mother's milk ECP concentrations were analyzed from 58 mothers with a food allergic infant, 17 mothers with an infant suffering from atopic eczema without food allergies and 19 mothers with a healthy infant (V). ECP concentration was under the detection limit (<2 µg/L) in all of the mothers with a healthy infant, whereas detectable levels were found in 28% of mothers with an infant with CMA or other food allergy and in 47% of those with a baby with atopic eczema but no food allergies. Mother's milk ECP levels showed no correlation with maternal atopy.

The main conclusions of this study are:

1. A deficiency in polyclonally induced IFN-γ expression exists in the CD4+ T cells (helper T cells) of infants with CMA. This suggests reduced Th1-type or Th0-type responses in CMA infants, leading to an imbalance in the T-helper cell-mediated immunity. The proportion of CD8+ T cells (cytotoxic T cells) was lower in CMA infants than in healthy ones. Also, there is a deficiency in the production of the IFN-γ and TNF-α in CMA infants, proinflammatory cytokines which regulate T-cell mediated immunity. These findings suggest impaired immune responses in both CD4+ and CD8+ T cell subclasses.
2. Immunological differences were seen in the clinically different manifestations of CMA. The delayed-type responses were Th1-polarized with activation of MAP kinase cascade and associated with a higher proportion of ICAM-1 expressing circulating lymphocytes. Different adhesion molecules were expressed in the multiorgan (ICAM-1) and cutaneous (LFA-1) manifestations of CMA, reflecting differences in regulation of their homing properties.
3. Clinically, more vigorous MAP kinase activation was seen at the symptomatic phase of CMA and may suggest high activation of lymphocytes during continuous allergen load, which could, in turn, result in a prolonged immunoinflammatory process.
4. The presence of ECP in mother's milk was associated with the development of allergic disorders in the breastfed infant, suggesting a contributory role for milk ECP in the development of food allergies and atopic eczema. Since ECP was only detected in one third of the milk samples from mothers with an allergic infant (food allergy or atopic dermatitis), several other factors that are contributory to the development of the allergic disease are likely to exist.

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