

Department of Dermatology, Skin and Allergy Hospital
University of Helsinki
Helsinki, Finland

**HUMAN MILK IMMUNOLOGY
IN RELATION TO THE DEVELOPMENT OF
COW'S MILK ALLERGY IN THE BREAST-FED**

Kirsi-Marjut Järvinen

ACADEMIC DISSERTATION

To be publicly discussed by permission of the Medical Faculty of the University of Helsinki, in the auditorium of the Hospital for Skin and Allergic Diseases, Meilahdentie 2, Helsinki, on September 1st, 2000, at 12 noon.

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Supervised by:

Hanna Raitio (*nee* Suomalainen), M.D., Ph.D.
Skin and Allergy Hospital, Department of Dermatology
Helsinki University Central Hospital
Helsinki, Finland

Kaisu Juntunen-Backman, M.D., Docent
Skin and Allergy Hospital, Department of Allergology
Helsinki University Central Hospital
Helsinki, Finland

Reviewed by:

Erkki Savilahti, M.D., Professor
Hospital for Children and Adolescents
University of Helsinki
Helsinki, Finland

Markku Viander, M.D., Docent
Department of Medical Microbiology
University of Turku
Turku, Finland

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1. SUMMARY

In the light of epidemiologic studies up until now, the effect of breastfeeding on the development of allergic diseases and cow's milk allergy (CMA) in the breast-fed had remained controversial. This may in part be due to individual variations in the levels of immunological constituents in mother's milk. To investigate the impact of distinct immunological factors in human milk on the breast-fed infant's risk for developing CMA, the presence of several immunologic components in the colostrum and milk of the mothers of newborns and infants was studied prospectively. Levels of various immunological factors were correlated with the offspring's clinical response to cow's milk challenge. Further, clinical manifestations of CMA in addition to the immune responses to cow's milk evoked were measured during a cow's milk challenge performed through mother's milk.

The proportions of distinct leucocyte subsets in human milk were studied in mothers of infants with CMA and in those of healthy infants. In the milk of the latter, the predominant leucocyte was the macrophage, whereas in the former, the proportion of macrophages was significantly smaller, and large proportions of eosinophils and neutrophils were found more often. These abnormalities in mothers' milk were in relation to an imbalance in the CD4+ and CD8+ T-cell ratio and with disturbed B-cell function that were associated with development of CMA in the breast-fed infant. When HLA-DR expression on milk macrophages was investigated in a subgroup of mothers, it was less frequent in those with infants with CMA. These results imply that sufficient numbers of functional milk macrophages may be critical in development of oral tolerance to food antigens in the offspring. Since HLA-DR is necessary for the presentation of antigens to T cells, the central role of the milk macrophages may be the presentation of foreign food antigens to the breast-fed infant's relatively naive and immature immune system. Moreover, these findings suggest that the presence of high numbers of eosinophils and neutrophils in mother's milk may be harmful to the breast-fed, since their cytolysis may potentially lead to the release of high amounts of highly cytotoxic mediators including ECP in the infant's gut. This may lead to an immunoinflammatory process similar to that seen in asthma and to increased gut permeability, and may thus contribute to development of CMA.

Further study of the function of human milk leucocytes required examination of the production of certain cytokines *in vitro*. Consistent with our findings of a low number and less frequent expression of the HLA-DR of milk macrophages, the level

of TNF- α in milk was lower in mothers having babies with CMA. TNF- α , a cytokine involved in the maturation process of dendritic cells necessary for presentation of processed antigen to T cells, may be an important factor in regulating development of oral tolerance in the gut mucosa of breast-fed infants. These results indicate that any lack of TNF- α in human milk may downregulate the differentiation of dendritic cells in the breast-fed, thereby playing a role in development of CMA.

The levels of total IgA in colostrum and milk were significantly lower in those mothers whose babies later developed CMA, whereas levels of IgA antibodies to cow's milk were comparable in the two groups of mothers. Since secretory IgA is considered the most important protective factor at mucosal level, its lack in milk may lead to an increased exposure of the intestinal mucosa of the breast-fed infant to potential allergens and thus enhance the risk for development of food allergies.

Clinical symptoms and immune responses in the peripheral blood of the breast-fed were evaluated during a cow's milk challenge performed through mother's milk. After a 2- to 4-week cow's milk elimination diet of both mother and infant, increasing doses of cow's milk or other dairy product were given at 1-hour intervals to the breastfeeding mother. Transfer of β -lactoglobulin (BLG) in mothers' milk was assessed. Most of the infants with CMA reacted to this untraditional cow's milk challenge procedure by showing typical symptoms of CMA, and the level of BLG was simultaneously increased in the milk of a subgroup of mothers. At the same time, there was a significant rise in total number of immunoglobulin-secreting cells of the IgA and IgG classes in the peripheral blood of infants. These findings give reason to believe that allergic reactions to food antigens transferred in the mothers' milk to the breast-fed, may be more common in infants with CMA than previously thought. They further imply that a strict elimination diet for the breast-feeding mother may be required in most cases of an infant with food allergies.

The present study may be the first to report that several aberrations in the cellular, cytokine, and immunoglobulin composition of human milk are related to development of CMA in the breast-fed. It therefore provides fresh insight into the etiopathogenesis of CMA. Measuring such components of mother's milk may become a useful tool for assessing those newborns at an increased risk for developing CMA. Although human milk is the best source of nutrition for healthy infants, the present study questions the general recommendation of prolonged breastfeeding in the prevention of development of food allergies in allergic families.

2. LIST OF ORIGINAL PUBLICATIONS

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

- I** Järvinen K-M, Suomalainen H. Leucocytes in human milk and immune responses in cow's milk-allergic infants. Submitted.
- II** Järvinen K-M, Juntunen-Backman K, Suomalainen H. Relation between weak HLA-DR expression on human milk macrophages and cow milk allergy (CMA) in suckling infants. *Pediatr Res* 1999;45:76-81.
- III** Järvinen K-M, Laine S, Suomalainen H. Defective tumour necrosis factor-alpha production in mother's milk is related to cow's milk allergy in suckling infants. *Clin Exp Allergy* 2000;30:637-643.
- IV** Järvinen K-M, Mäkinen-Kiljunen S, Suomalainen H. Cow's milk challenge via human milk evokes immune responses in suckling infants with cow's milk allergy. *J Pediatr* 1999;135:506-12.
- V** Järvinen K-M, Laine S, Järvenpää A-L, Suomalainen H. Does low IgA in human milk predispose the infant to the development of cow's milk allergy? *Pediatr Res* 2000, in press.

Some previously unpublished data are also presented.

3. ABBREVIATIONS

ANOVA	analysis of variance
APC	antigen presenting cell
BALT	bronchus-associated lymphoid tissue
BLG	β -lactoglobulin
CD	cluster of differentiation
CI	confidence interval
CMA	cow's milk allergy
Con A	Concanavalin A
DC	dendritic cell
ECP	eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
ELISPOT	solid phase enzyme-linked immunospot assay
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
GALT	gut-associated lymphoid tissue
HLA	human leucocyte antigen
IFN	interferon
Ig	immunoglobulin
IL-1-13	interleukins 1-13
ISC	immunoglobulin-secreting cell
MHC	major histocompatibility complex
NK cells	natural killer cells
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
RAST	radioallergosorbent test
sASC	specific antibody-secreting cell
sCD23	soluble CD23 antigen
sIgA	secretory immunoglobulin A
SPT	skin prick test
TGF	transforming growth factor
Th1, Th2	subtypes 1 and 2 of helper T cells
TNF	tumour necrosis factor

4. INTRODUCTION

Breastfeeding and human milk afford to the infant a number of benefits. Prolonged breastfeeding has been recommended to prevent or delay the development of atopic disease (Businco *et al.* 1983, Chandra *et al.* 1985, Businco *et al.* 1987, Høst *et al.* 1988, Zeiger *et al.* 1989, Saarinen and Kajosaari 1995, Oddy *et al.* 1999). However, other studies do not confirm the preventive effect of breastfeeding, even in combination with a maternal elimination diet, against the development of atopic disease (Chandra *et al.* 1986, Lilja *et al.* 1989, Lilja and Oman 1991). Some studies have even reported an increased risk for development of atopic eczema and food allergies in breast-fed infants (Kaplan and Solli 1979, Kramer and Moroz 1981, Cogswell and Alexander 1982, Taylor *et al.* 1983). Although breastfeeding has been widely recommended, especially for infants at high risk for allergy, investigations into the composition of human milk in atopic mothers are few. Hardly any data are available on the effect of distinct immunological factors in human milk on the breast-fed infant's health status (Machtiger and Moss 1986, Savilahti *et al.* 1991).

Cow's milk allergy (CMA) is defined as an immunologically mediated adverse reaction against cow's milk antigens (Savilahti *et al.* 1992). Symptoms of CMA commonly appear during the first months of life, within days or weeks of commencing feeding with a cow's milk-based formula, or even during exclusive breastfeeding (Jakobsson and Lindberg 1978, Gerrard 1979, Machtiger and Moss 1986, Sorva and Mäkinen-Kiljunen 1994, Isolauri *et al.* 1999). The most likely cause of symptoms in these infants has been suggested to be the small amounts of cow's milk proteins ingested by the lactating mother and transferred into breast milk (Stuart *et al.* 1984, Kilshaw and Cant 1984, Machtiger and Moss 1986, Vandenplas *et al.* 1992, Sorva and Mäkinen-Kiljunen 1994). Clinical relief of symptoms of CMA in an infant has been reported upon maternal withdrawal of cow's milk (Jakobsson and Lindberg 1978, Gerrard 1979, Sorva and Mäkinen-Kiljunen 1994). However, strict elimination of cow's milk and cereal antigens from the mother's diet does not always lead to disappearance of allergic symptoms in an infant reacting to these proteins. This suggests that the dietary antigens may not be the only factors in human milk causing symptoms of food allergy in breast-fed infants.

Colostrum and milk contain numerous potentially immunologically active components such as leucocytes, cytokines, and immunoglobulins (Slade and

Schwartz 1987, Xanthou *et al.* 1995, Wagner *et al.* 1996, Goldman *et al.* 1997, Bernt and Walker 1999, Hanson 2000). A breast-fed infant ingests an average of 10^8 leucocytes per day with breastfeeding, often continuing for several weeks. Maternal cells and cytokines may reside biologically intact in the gut of the breast-fed infant due to special characteristics of human milk cells and of the newborns gastrointestinal tract. Human milk is believed to impart specific immune advantages to the neonate through enhancement or induction of the still-developing neonatal immune system (Slade and Schwartz 1987, Xanthou *et al.* 1995, Wagner *et al.* 1996, Cummins and Thompson 1997, Bernt and Walker 1999, Goldman 2000, Hanson 2000). Evidence is, however, lacking as to the direct influence of human milk leucocytes and cytokines on the recipient. In this study, we sought to determine the influence of these immunologic components in human milk on the infant's risk for developing CMA.

5. REVIEW OF THE LITERATURE

5.1. General effects of breastfeeding on infant's health

Multiple advantages of breastfeeding over formula feeding have been advanced from a variety of disciplines. The benefits for infants are believed to lie in four general areas: optimal growth and nutrition, defense against infections, the enhancement of maternal-infant bonding, and avoidance of allergic diseases (Cunningham 1979). Human milk provides the newborn with nutrients, growth factors, and anti-infectious substances important for host defense against infections (Goldman *et al.* 1982). During past decades, breastfeeding has been suggested to account for the decrease in number and severity of infants' intestinal and respiratory infections (Cunningham 1979), as well as of septicemia and meningitis (Winberg and Wessner 1971). The protective activity of breastfeeding against infections is explained by antimicrobial factors such as the immunoglobulins, leucocytes, lysozyme, lactoferrin, and bifidus factor present in human milk and a lessened risk of contamination with pathogenic microorganisms, particularly when hygiene is poor. Although knowledge is still lacking, breastfeeding may impart specific immune advantages to the neonate through enhancement or induction of the still-developing neonatal immune system (Slade and Schwartz 1987).

5.2. Breastfeeding in prevention of allergy

5.2.1 *Epidemiological studies*

The effect of breastfeeding - combined with avoidance of cow's milk and solid foods - on reduction in incidence of atopic disease and CMA in the first years of life remains an unsettled issue. Several studies suggest that (prolonged) breastfeeding prevents or delays the development of atopic disease in infants (Businco *et al.* 1983, Chandra *et al.* 1985, Businco *et al.* 1987, Høst *et al.* 1988, Zeiger *et al.* 1989, Saarinen and Kajosaari 1995, Oddy *et al.* 1999). Others do not confirm the putative advantages of breastfeeding (Kaplan and Solli 1979, Kramer and Moroz 1981, Cogswell and Alexander 1982, Taylor *et al.* 1983, Lilja *et al.* 1989, Lilja and Oman 1991). This inconsistency may result from problems in the design of these studies (Atherton 1983, Björkstén 1983). The published reports include prospective as well as retrospective studies, but critical evaluation should be confined only to the

prospective ones. Some studies relied on parental diagnosis of atopic diseases alone. Duration of breastfeeding may be influenced by a family history of allergy, which should be taken into account. Further, some studies have evaluated the effect of maternal avoidance of potent sensitising foods, such as eggs, fish, and cow's milk, while others merely compared duration of breastfeeding and commencement of bottle feeding or solid foods on development of allergic diseases. Duration of breastfeeding and follow-up of the patients in the studies varies widely. Most of the prospective studies indicate that in infants with a clear family history of allergy, breastfeeding delays the onset of allergy for several years (Atherton 1983, Björkstén 1983). The ultimate long-term effect of breastfeeding is, however, another issue.

5.2.2 Possible mechanisms of allergy prevention by breastfeeding

The principal advantage of breastfeeding in the reduction of sensitisation in infants and of symptoms of allergy in an already sensitised baby has been widely assumed to be the relative scarcity of food antigens in human milk. At the same time, in animal studies, small antigen amounts administered parenterally have been reported preferentially to induce immunoglobulin (Ig)E antibody responses (Jarrett 1984). These findings offer a reason to consider the low antigen content in breast milk a mixed blessing, since it could result in low-dose sensitisation in breast-fed babies (Björkstén 1983).

Other mechanisms may contribute to the protective effect of human milk. Human milk contains IgA antibodies against a number of common foods (see Chapter 3.3). Such antibodies may reduce the entry of such antigens through mucosal surfaces and lower the risk for sensitisation. Human milk also directly affects the neonate's immune system, because it contains a factor that stimulates IgA synthesis in the infant (Roberts and Freed 1977, Pittard and Bill 1979, Allardyce and Wilson 1983, Juto 1985). Further, cell-mediated immunity is transferred from mother to infant through human milk (Schlesinger and Covelli 1977). Such support from human milk for infant immunity may be crucial, since allergy appears to be associated with primary functional abnormality of the immune system, possibly defective suppressor function (Suomalainen *et al.* 1993a, Järvinen *et al.* 1998). Infants with an increased risk for such a defect in the function of immune system may especially benefit from breastfeeding, assuming that the milk contains sufficient immune regulatory factors.

Three additional possible mechanisms of protection have been proposed. Human milk shows antimicrobial activity, which probably accounts for the lower incidence of infections in breast-fed babies than in formula-fed ones (Winberg and Wessner 1971).

A lower incidence of infections may in turn reduce risk for sensitisation, since respiratory tract infections, for example, have been associated with increased risk for sensitisation and development of atopic allergy (Frick *et al.* 1979). Secondly, breastfeeding has been proposed to affect the gastrointestinal flora in a direction that may be favourable in terms of prevention of sensitisation (Björkstén 1983), since introduction of cow's milk promotes the growth of gram-negative endotoxin-producing bacteria with adjuvant properties that enhance sensitisation (Matthew *et al.* 1977). This hypothesis, however, conflicts with the recent finding that indoor endotoxin exposure early in life may protect against allergen sensitisation by enhancing type 1 immunity (Gereda *et al.* 2000). Thirdly, certain hormones, identified in human milk, such as cortisol, plus growth factors such as epidermal growth factor (Carpenter 1980), insulin-like growth factor (Corps *et al.* 1988), milk growth factor (Kanda *et al.* 1994), and transforming growth factor (TGF) (Saito *et al.* 1993), may support the anatomic integrity of the mucosal barrier in the infant.

5.3. Human milk components

Human milk originates in lactating mammary tissue. The basic structural unit is the alveolus, which consists of lactating cells that secrete milk into an adjoining lumen (Patton and Keenan 1975). The lumen connects to a duct system that drains the collected milk to outlets at the skin's surface. Individual arteriovenous capillary systems provide each alveolus with the individual nutrients needed for producing milk. Milk lipid, lactose, and the majority of milk proteins are produced in the lactating cells (Patton and Keenan 1975, McPherson and Kitchen 1983). Human milk contains cells, soluble mediators, immunoglobulins, lactoferrin, oligosaccharides, enzymes, peroxidases, lysozyme, secretory component, bifidus factor, growth factors, hormones, and foreign food antigens. Occasional bacteria and several viruses (rubella, CMV, hepatitis B, vaccinia) have been observed in milk, either passing from the maternal circulation or entering the milk by reflux from the infant during suckling (Ogra and Ogra 1979). Maternal histocompatibility antigens are also present in the cells of milk (Beer *et al.* 1974). The following will focus purely on immunologic components.

5.3.1 Leucocytes

Total number and origin of milk leucocytes. Human colostrum contains 2 to 4 x 10⁶ cells/ml, the number of which decays rapidly in four days post partum and decreases more gradually thereafter (Ogra and Ogra 1978, Goldman *et al.* 1982). It is

estimated that on average 2 billion each of polymorphonuclear leucocytes and mononuclear cells are ingested by the breast-fed baby during its first four days (Murphey and Buescher 1993). The mean total cell count in preterm colostrum has been found to be significantly higher than in full-term colostrum (Jain *et al.* 1991). This contrasts with the finding of Rodriguez *et al.* (1989) who detected a slightly larger number of leucocytes in milk of mothers delivering preterm, but the difference was not statistically significant (0.55×10^6 vs. 0.42×10^6 cells/ml). Alcohol consumption has been associated with an increase in number of leucocytes in human milk (Na *et al.* 1997). It is likely that human milk leucocytes originate from blood (Goldman and Goldblum 1996). No leucocytes, other than a few macrophages, appear in the mammary gland until late pregnancy and throughout lactation. The vast majority of B cells that home to the mammary gland transform into plasma cells that remain sessile in the mammary gland (Goldman and Goldblum 1996). In contrast, other leucocytes attracted to the site from the maternal circulation, probably due to the presence of chemoattractant factors (Michie *et al.* 1998, Böttcher *et al.* 2000b), traverse the mammary epithelium and become part of the milk secretions.

Mononuclear phagocytic cells. In the milk of healthy women delivered full-term, the predominant cellular component (60 to 90% of milk cells) is the macrophage (Smith and Goldman 1968, Ho *et al.* 1979, Eglinton *et al.* 1994), with a morphology resembling that of tissue macrophages (Pitt 1979). Despite expressing the monocyte markers Leu-M₃ and Leu-M₅, they also appear phenotypically more similar to tissue macrophages (Xanthou 1997). Additionally, occasional monocytes are found (Smith and Goldman 1968, Ho *et al.* 1979). The structural and functional characteristics of breast milk macrophages are not completely defined. They display unusual morphology, including many lipid-filled vacuoles, milk fat globules, and casein micelles (Smith and Goldman 1968, Smith *et al.* 1971, Crago *et al.* 1979, Ho *et al.* 1979, France *et al.* 1980, Baldus *et al.* 1995). Studies of mothers that have delivered preterm and at full term have shown that milk macrophages are a fully mature tissue macrophage population (Rodriguez *et al.* 1989). They adhere to glass, although less than do their counterparts in peripheral blood (Miler *et al.* 1990). They are activated, as indicated by their high motility (Özgaragoz *et al.* 1988), but their migratory activity and chemotaxis have also been shown to be significantly less than those of less mature blood monocytes (Clemente *et al.* 1986, Thorpe *et al.* 1986, Rodriguez *et al.* 1989). They have been demonstrated to mount a respiratory burst after *in vitro* stimulation (Tsuda *et al.* 1984, Cummings *et al.* 1985, Speer *et al.* 1985; 1986). Activation, as indicated by induction of the oxidative burst and prostaglandin production, has been suggested to occur through the IgA receptors they contain (Robinson *et al.* 1991). Moreover, they show high phagocytic activity (Smith and Goldman 1968, Goldman and Smith 1973, Rodriguez *et al.* 1989), but the number of

particles engulfed per cell has been reported to be markedly lower than for blood leucocytes (Miler *et al.* 1990). They have also been demonstrated to kill ingested *Candida Albicans* (Cummings *et al.* 1985). That they exhibit strongly carbohydrate antigens in addition to peptide ones may be the result of cytokine-mediated stimulation or increased phagocytic activity (Baldus *et al.* 1995). They also possess the capability of producing toxic oxygen radicals for intracellular killing of microorganisms (Tsuda *et al.* 1984). Some authors suggest that, as elsewhere in the body, human milk macrophages may provide the first line of defense against pathogens (Waksman 1979).

The HLA-DR is a subgroup of the major histocompatibility complex class II, responsible for antigen presentation to compatible T cells (Leyva-Cobian and Clemente 1984). Expression of HLA-DR on human milk macrophages has previously been demonstrated to be very high, almost 100% (Rivas *et al.* 1994). Milk macrophages have been shown to have the capacity to present antigens and to induce T-cell proliferation (Oksenberg *et al.* 1985, Vandenplas *et al.* 1992). Mori and Hayward (1992) demonstrated that about 20% of milk mononuclear cells have antigen-presenting characteristics *in vitro*. Immunologically, in their *in vitro* responses to specific and nonspecific stimuli, mononuclear cells in human milk resemble gut-associated lymphoid tissue (GALT) or bronchus-associated lymphoid tissue (BALT) (Parmely *et al.* 1977), but they may also be responsible for longer-term immune changes by virtue of their endocrine functions (Pabst 1997). They produce cytokines (Skansén-Saphir *et al.* 1993), lysozyme, C3 and C4 complement, lactoferrin, and a large combination of immunoglobulins (Goldman and Smith 1973, Weaver *et al.* 1984, Goldman and Goldblum 1989). Milk macrophages are also able to secrete prostaglandin E₂ and plasminogen activator (Blau *et al.* 1983, Le Deist *et al.* 1986).

Neutrophils. According to the literature, neutrophils are rare in human milk (8-28%) in breast-feeding mothers (Smith and Goldman 1968, Eglinton *et al.* 1994). However, some authors report as high as 40-60% of neutrophils (Ho *et al.* 1979, Crago *et al.* 1979). Human milk polymorphonuclear cells are functionally exudate cells with less locomotive, adherence, microbicidal, and stimulated respiratory burst capabilities than those of their counterparts in blood (Ho and Lawton 1978, Kohl *et al.* 1980, Weaver *et al.* 1984, Thorpe *et al.* 1986, Buescher and McIlheran 1993, Grazioso and Buescher 1996). Although the interpretation was initially that such lower adherence, polarity, and motility are due to inhibitors in human milk (Thorpe *et al.* 1986), further investigations suggest that they are typical for activated neutrophils, as evidenced by their high expression of the activation marker CD11b and decreased expression of L-Selectin (Keeney *et al.* 1993). In an older study, the phagocytic ability of human

neutrophils was demonstrated, however, to be comparable to that of peripheral blood (Ho *et al.* 1979). Inhibition of neutrophil function *in vitro* in colostrum and mature milk has been associated with antioxidant activities (Grazioso and Buescher 1996).

The explanation for why these cells are present in colostrum is obscure. Smith and Goldman (1968) suggested that their presence may represent a response to the engorgement of the breast. Alcohol consumption has been detected to increase their number in milk (Na *et al.* 1997). The ability to sequester pathogens, thereby preventing their attachment to the gut wall and subsequent colonisation of the gut, has been proposed as their function (Ho and Lawton 1978). Yet more recently, Buescher and McIlheran (1993) have concluded that, owing to their lower phagocytic and microbicidal characteristics, they do not seem to provide significant anti-infective protection to the breast-fed infant. However, they release lactoferrin, which has important anti-inflammatory functions (Kiljstra 1991).

T cells. Only 3 to 9% of human milk leucocytes are lymphocytes (Smith and Goldman 1968, Crago *et al.* 1979), with T cells accounting for 74 to 83% of them (Bertotto *et al.* 1990, Jain *et al.* 1991, Wirt *et al.* 1992). They display predominantly the phenotype and functional characteristics of memory T cells (Bertotto *et al.* 1990). The great majority of T cells express antigens involved in intercellular adhesion (LFA-1, ICAM-1) and T-cell activation (CDw29, HLA-DR) (Bertotto *et al.* 1990, Gibson *et al.* 1991, Wirt *et al.* 1992). Milk T cells also exhibit good responsiveness to a variety of bacterial and viral antigens (Smith and Goldman 1968, Parmely *et al.* 1976, Ogra and Ogra 1978), and produce significant amounts of interferon (IFN)- γ (Bertotto *et al.* 1990). These characteristics suggest that milk T cells may be antigen-pulsed T cells capable of mounting a secondary immune response.

In the studies using flow cytometry, the mean CD4+ and CD8+ ratio of T cells in human milk has been reported to be 1, meaning that the proportion of CD8+ cells is higher than in peripheral blood (Wirt *et al.* 1992, Eglinton *et al.* 1994). However, an older study using indirect immunoperoxidase staining and monoclonal antibodies claimed the ratio was 1.6 (Jain *et al.* 1991). Further, milk T cells have 2- to 3-fold higher percentages of activated CD8+ (HML-1+ or VLA-1+) cells than does blood (Gibson *et al.* 1991, Eglinton *et al.* 1994).

The phenotypic pattern of T cells may result from T cell-activating substances in milk and/or selective homing of T cells to the breast (Wirt *et al.* 1992). The same authors have suggested that the paucity of memory T cells in the newborn period and early infancy is compensated, at least partly, by the transfer of maternal memory T lymphocytes in human milk. A variety of labelling and receptor studies have

established that milk T cells come from T cells stimulated in the GALT or BALT, which then follow the same route as IgA-bearing B blasts to the mucosae (Parmely *et al.* 1976, Parrot 1979, Waksman 1979, Richie *et al.* 1982, Keller *et al.* 1986, Bertotto *et al.* 1990, Bertotto *et al.* 1991). Hence, colostrum T cells react to antigens which may reasonably be presumed to have acted on T cells in GALT or BALT and their draining lymph nodes (Parrott 1979). At least certain subpopulations of unprimed T cells are also found in colostrum and early milk, although small in number (Wirt *et al.* 1992).

B cells. B cells comprise 4 to 26% of total milk lymphocytes (Bertotto *et al.* 1990, Jain *et al.* 1991, Wirt *et al.* 1992). They have been found to produce IgA, as first demonstrated by Murillo and Goldman (1970). A high proportion of colostrum B lymphocytes show production of the antibodies directed against *Escheria coli* antigen following oral immunisation (Ahlstedt *et al.* 1975), representing evidence that B cells migrate from GALT to the mammary gland. Evidence from labelling and receptor studies further supports this hypothesis (Roux *et al.* 1977, Bush and Beer 1979). Primed B cells stimulated to blast-transformation and bearing specific membrane IgA migrate in large numbers from the Peyer's patches to draining mesenteric lymph nodes. Further, they travel to the body by way of efferent lymph and the bloodstream to the lamina propria of mucous membranes throughout the body, including the mammary glands, where they evolve into IgA-secreting plasma cells (Roux *et al.* 1977). The migration to the mammary gland becomes a major pathway only during late pregnancy and lactation (Roux *et al.* 1977). Lymphocyte migration appears to be directed by cell-surface molecules termed "homing-receptors", which are leucocyte-endothelial adhesion molecules that interact selectively at areas of specialized endothelium on postcapillary venules to capture the lymphocytes in particular lymphoid organs (Slade and Schwatz 1987). These high endothelial venules express specific surface proteins that have been designated as vascular addressins (Carlos and Harlan 1994).

Natural killer (NK) cells. NK cells represent a small proportion of colostrum cells and display low cytotoxic activity (Moro *et al.* 1985, Wirt *et al.* 1992). In contrast to peripheral blood, the majority of colostrum NK cells exhibited a degenerated appearance with many vacuoles and no electron-dense granules (Moro *et al.* 1985).

Eosinophils. Eosinophils account for about 2% of milk cells (Vassella *et al.* 1992, Eglinton *et al.* 1994). Vassella *et al.* (1992) has reported that the number of eosinophils in human milk is positively correlated with their number in peripheral blood, suggesting migration of eosinophils from peripheral blood to mammary gland as described for lymphocytes. In that study, eosinophil count was significantly

higher in the milk of atopic women (4%) than in that of nonatopic mothers. Their function in human milk is unknown.

Basophils. Basophils are seldom found in the milk (0.1% of cells) of atopic mothers (Vassella *et al.* 1992). Their number is positively correlated with the number of eosinophils in peripheral blood, with a tendency towards higher basophil counts in the milk of atopic women. The authors suggest that the capacity of basophils to release histamine in the gut of the infant might increase the permeability of the gut mucosa and probably the risk for sensitisation to food allergens.

Other cells. Occasional epithelial cells appear in human milk (Crago *et al.* 1979, Ho *et al.* 1979).

5.3.2 Soluble mediators

Cytokines are glycoproteins, secreted predominantly by activated T cells, monocytes, and macrophages that have effects on a variety of cells of the immune system and also on numerous other cells and systems throughout the body. Human milk contains cytokines such as interleukin (IL)-1 (Söder 1987), IL-4 (Eglinton *et al.* 1994), IL-5 (Böttcher *et al.* 2000a), IL-6 (Saito *et al.* 1991), IL-8 (Basolo *et al.* 1993), IL-10 (Garofalo *et al.* 1995), IL-12 (Bryan *et al.* 1999), IL-13 (Böttcher *et al.* 2000a), IL-16 (Böttcher *et al.* 2000b), IFN- γ (Basolo *et al.* 1993), tumour necrosis factor (TNF)- α (Rudloff *et al.* 1992), and TGF- β (Noda *et al.* 1984). In addition, human milk mononuclear cells have also been demonstrated to show a potential, upon stimulation, for production of lymphokines such as IL-2 and IL-3 (Skansén-Saphir *et al.* 1993). Furthermore, maternal cells in human milk have been found to contain mRNA for various cytokines, revealing a unique cytokine profile for human milk (Srivastava *et al.* 1996).

Chemokines are a novel class of small chemotactic cytokines. In milk, chemokines such as IL-8 (see above) and growth-related peptide- α (Srivastava *et al.* 1996) that are mainly chemotactic to neutrophils, have been demonstrated. Milk also includes chemotactic factors for monocytes, basophils, and eosinophils such as monocyte chemotactic protein and RANTES (Srivastava *et al.* 1996, Michie *et al.* 1998, Böttcher *et al.* 2000b) and eotaxin (Böttcher *et al.* 2000b). Colony-stimulating factors such as granulocyte colony-stimulating factor, macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have also been discovered in human milk (Garofalo and Goldman 1998).

Clear evidence exists that the production by neonatal cells of several cytokines found in human milk or their cognate mRNAs is either slightly (TNF- α), moderately (GM-CSF) or markedly reduced (IL-6, IL-8, IL-10, IFN- γ) as compared with production by adult T cells and other cellular sources (Garofalo and Goldman 1998).

IL-1. IL-1 is a proinflammatory cytokine responsible for the induction of cytokines. Söder (1987) was the first to report the presence of IL-1 in human milk in 1987. The preliminary studies suggest that it originates from milk macrophages. This finding was confirmed by Munoz *et al.* (1990), who reported IL-1 at high concentrations in all colostrum samples. However, further studies were unable to detect any IL-1 in human milk (Basolo *et al.* 1993, Srivastava *et al.* 1996) until Hawkes *et al.* (1999), who reported a decrease in its concentration during the first 12 weeks of lactation. The biological function of IL-1 in milk remains to be defined. Since IL-1 has been found to stimulate the activation of B cells, Söder (1987) has suggested that IL-1 may participate in the regulation of humoral immune responses in the mammary gland. It is also claimed that IL-1 contributes to the well-known pyrogenic activity of milk (Dinarello 1984).

IL-4. IL-4 is a characteristic Th2-type cytokine inducing IgE production of B cells and has been found in less than 25% of milk samples (Eglinton *et al.* 1994, Rudloff *et al.* 1999, Böttcher *et al.* 2000a). Böttcher *et al.* (2000a) report that concentrations of IL-4 are higher in colostrum from allergic mothers than from nonallergic mothers, in contrast to the earlier finding of Rudloff *et al.* (1999). Some investigators, including Srivastava *et al.* (1996), have found no IL-4 in human milk at all. Human milk mononuclear cells have been proposed to be their origin because these have been demonstrated to have a potential for the production of IL-4 upon stimulation (Skansen-Saphir *et al.* 1993). According to Böttcher *et al.* (2000a), the concentration of IL-4 in milk correlates with that of IL-13.

IL-5. IL-5 is involved in B cell growth and chemotaxis and differentiation of eosinophils. It has been detected in less than 10% of milk samples in small concentrations (up to 11 pg/mL) (Böttcher *et al.* 2000a).

IL-6. IL-6 is involved in the stimulation of B and T cells, although has recently been suggested also to have anti-inflammatory and immunosuppressive properties (Tilg *et al.* 1997). It also stimulates IgA synthesis (Fujihashi *et al.* 1991), and has been associated with the local production of IgA in the breast (Saito *et al.* 1991). In 1991, Japanese investigators reported that substantial amounts of IL-6 are present in human whey and show a positive correlation with number of milk leucocytes (Saito *et al.* 1991). Leucocytes can thus be held responsible for IL-6 production.

Furthermore, Rudloff *et al.* (1999) documented considerable concentrations of IL-6 in human milk. Basolo *et al.* (1993) and later Palkowetz and colleagues (1994) demonstrated that IL-6 and IL-8 production in milk originates from epithelial cells. The further findings of considerable amounts of IL-6 in human milk (Eglington *et al.* 1994, Sone *et al.* 1997, Hawkes *et al.* 1999) are in contrast with those of Srivastava *et al.* (1996), who found this cytokine only in low concentrations. The concentration of IL-6 decreases during 12 weeks of breastfeeding, and its concentration is associated with no other cytokines (IL-1, TNF- α and TGF- β) (Hawkes *et al.* 1999). Böttcher *et al.* (2000a), however, have recently reported the presence of IL-6 in most of their milk samples, and its concentration was positively correlated with that of IL-10, TGF- β and total IgA in colostrum. Recently, Saarinen *et al.* (1999a) reported that IL-6 was measured in less than 50% of milk samples, and the percentages of samples with low IL-6 content were similar in the mothers of infants with CMA and in those of healthy babies.

IL-8. IL-8 is known to attract leucocytes to the site of inflammation (Matsushima *et al.* 1992), but any similar function in milk remains to be seen. IL-8 is reported to be present in milk in high concentrations of 3684 pg/ml (Palkowetz *et al.* 1994), and has been shown to originate from mammary gland epithelial cells (Basolo *et al.* 1993, Palkowetz *et al.* 1994, Michie *et al.* 1998). More recently, maternal alcohol consumption was found to increase significantly the concentration of IL-8 in the milk (Na *et al.* 1997). In another recent study, the level of IL-8 was higher in breast milk from allergic than from nonallergic mothers (Böttcher *et al.* 2000b).

IL-10. In 1995, Garofalo and colleagues found IL-10 in colostrum at a concentration of 3304 pg/ml, which is higher than for other cytokines, except for IL-8. Some IL-10 mRNA, but no protein product was detected in cultured mammary epithelial cells. The authors report that some IL-10 was associated with preparations of human milk leucocytes; the data did not, however, indicate that the cells were producing the cytokine, although this possibility could not be ruled out. Rudloff *et al.* (1999) have reported a more frequent presence of IL-10 in the milk of allergic mothers than of nonatopic ones. Since IL-10 inhibits the production of proinflammatory cytokines (Fiorentino *et al.* 1991, de Waal Malefijt *et al.* 1991), attracts CD8+ T cells (Jinquan *et al.* 1993), and also limits the participation of Th1 cells in delayed hypersensitivity (Fiorentino *et al.* 1989), it is suggested also to aid in regulating mucosal defenses (Garofalo *et al.* 1995). Recently, levels of IL-10 in milk were found to correlate with those of IL-6, TGF- β , and total IgA (Böttcher *et al.* 2000a).

IL-12. IL-12, a Th1-type cytokine, has been shown to enhance lymphocyte-mediated cytotoxicity (Robertson *et al.* 1992) and T cell proliferation (Bertagnolli *et al.* 1992).

It has been detectable in 62% of human milk samples (Bryan *et al.* 1999), although it was not detected in earlier reports (Srivastava *et al.* 1996, Sone *et al.* 1997). A supply of exogenous IL-12 from the milk of a breast-feeding mother has been suggested to assist in diverting the predisposed Th2 responses of the *in-utero* environment towards Th1-type responses (Bryan *et al.* 1999).

IL-13. IL-13 is another Th2-type cytokine. In a recent paper, IL-13 was detected in human milk of one-third of the samples in concentrations of up to 89 pg/mL (Böttcher *et al.* 2000a). Levels of IL-13 tended to be higher in allergic mothers than in nonallergic ones (Böttcher *et al.* 2000a).

IL-16. IL-16 was very recently found in 48% of human milk samples (Böttcher *et al.* 2000b). In addition to its chemoattractant properties, it is also a competent growth factor for CD4+ T cells (Cruikshank *et al.* 1991), which the authors claimed could explain their observation of enhanced proliferation of T cells under stimulation with human milk.

IFN- γ . IFN- γ is a typical Th1-type cytokine that antagonizes several effects of IL-4, such as IgE synthesis. This cytokine has been detected in human milk samples in concentrations of 100 to 760 pg/ml (Basolo *et al.* 1993). The authors tentatively suggest that its production is due to passenger leucocytes in milk, since it was not found in cultures of mammary epithelial cells (Basolo *et al.* 1993). Rudloff *et al.* (1999) or Skansen-Saphir *et al.* (1993) found no spontaneous IFN- γ production, but Skansen-Saphir *et al.* (1993) reported that its production was stimulated in 2 to 11% of milk leucocytes. They suggest that its production might contribute to the antimicrobial defense in the recipients by influencing B cell and macrophage activation. In a recent paper, Saarinen *et al.* (1999a) demonstrated only 21 to 29% of the milk samples to contain detectable levels of IFN- γ , levels comparable in mothers with an infant with CMA and in those with a healthy baby.

TNF- α . TNF- α is a proinflammatory and regulatory lymphokine produced by activated macrophages and T lymphocytes (Akira *et al.* 1990). Mammary epithelial cells produce but do not secrete TNF- α (Basolo *et al.* 1993). Milk macrophages likewise have been demonstrated to produce TNF- α (Sone *et al.* 1997). It appears in breast milk in sufficient quantities (approximately 620 pg/ml) potentially to affect the recipient infant (Rudloff *et al.* 1992). Such high levels (up to 2900 pg/ml) have been confirmed by other authors (Buescher and Malinowska 1996, Na *et al.* 1997, Hawkes *et al.* 1999), although Srivastava *et al.* (1996) report extremely low levels. This factor has numerous effects on the function of the immune system, although its function in human milk remains obscure.

TGF- β . Anti-inflammatory cytokine TGF- β regulates normal cell growth, development, and tissue remodelling following injury. It has also been suggested to induce IgA responses and IgA isotype switching in B cells (van Vlasselaer *et al.* 1992), as well as oral tolerance (Ishizaka *et al.* 1994) and maintenance of intestinal barrier function (Planchon *et al.* 1994). Noda *et al.* (1984) reported its presence in human milk, and since then other investigators have confirmed this finding (Saito *et al.* 1993, Srivastava *et al.* 1996, Hawkes *et al.* 1999, Kalliomäki *et al.* 1999, Saarinen *et al.* 1999a, Böttcher *et al.* 2000a). Its concentration has been reported to be much lower in colostrum from women delivering preterm than in those delivering at term (Srivastava *et al.* 1996). TGF- β was the predominant cytokine in human milk, with its concentration significantly higher in colostrum than in mature milk (Böttcher *et al.* 2000a), although Hawkes *et al.* (1999) reported no significant decrease in its concentrations during the first 12 weeks of lactation. Recent findings suggest that TGF- β is significantly lower in the milk of mothers with an infant with IgE-mediated CMA or atopic eczema (Kalliomäki *et al.* 1999, Saarinen *et al.* 1999a). It has also been shown to correlate with IgA levels in human milk (Böttcher *et al.* 2000a), with the total number of IgA-producing cells in the peripheral blood of the infants (Kalliomäki *et al.* 1999), and with IL-6 and IL-10, of which both are involved in IgA synthesis (Böttcher *et al.* 2000a). Saarinen *et al.* (1999a) demonstrated that the concentration of TGF- β 1 in colostrum is positively correlated with IgA antibodies to BLG and with IgG antibodies to α -casein and whole formula, but negatively with the diameter of a skin prick test response to cow's milk and with lymphocyte indices to α -casein and BLG. Based on these findings, they suggest that in an infant prone to CMA, the TGF- β 1 content of the mother's colostrum may promote IgG-IgA antibody production and inhibit IgE- and cell-mediated reactions to cow's milk.

5.3.3 Immunoglobulins, lactoferrin, and oligosaccharides

Immunoglobulins. The immunoglobulins are glycoproteins with oligosaccharide sequences attached to their heavy and infrequently to their light chains. Human milk contains appreciable amounts of the IgA, IgG, and IgM (Xanthou *et al.* 1995). Small amounts of IgD and IgE classes have also been detected in milk (Xanthou *et al.* 1995, Duchén and Björkstén 1996). IgG, IgM, and IgD in milk seem to mediate antibody-dependent cytotoxicity and opsonic activity against bacteria (Xanthou *et al.* 1995). IgE combines with antigens in the gut lumen and releases chemical mediators that cause increased vascular permeability (Xanthou *et al.* 1995)

IgA makes up about 90% of human milk immunoglobulins. In common with other secretions such as nasal fluid and saliva, the chief immunoglobulin of human milk is of the secretory type of IgA (sIgA). It is present at its highest concentrations in the first few days postpartum (in the colostrum) (Hanson *et al.* 1975), and then falls away progressively to a basal level of 0.2 to 0.3 g/L (Savilahti *et al.* 1991, Machtinger and Moss 1986). A number of sIgA antibodies to the common bacteria, viruses, and fungi to which the mother has been exposed have been described in human milk (Ogra *et al.* 1983). Studies have also established the presence of IgA antibodies in human milk to food proteins such as black beans and soybean (Cruz *et al.* 1981), cow's milk proteins (McClelland and McDonald 1976, Hanson *et al.* 1977, Machtinger and Moss 1986, Savilahti *et al.* 1991), and gliadin (Mascart-Lemone *et al.* 1991). Breast-milk IgA to casein or whole milk has been detected in 84% of milk donors (Machtinger and Moss 1986).

Human milk sIgA is produced locally in the lactating mammary gland, being elaborated by B cells situated proximal to the ductal epithelium. These local plasmacytes are derived from the gut-associated lymphoid tissue where they have been exposed to specific enteric antigens such as microbes and foods, and thus migrate to various mucosal sites and exocrine glands like the breast (Hanson *et al.* 1979, Slade and Schwatz 1987). The antibodies bind to the polymeric Ig-receptor, or secretory component, on the basal portion of the glandular epithelial cells, are transported through them, and appear on the mucosal membrane (Hanson 1998). There is evidence that also those lymphocytes shed into the colostrum are capable of producing immunoglobulin (Slade and Schwartz 1987, Murillo and Goldman 1970). Levels of the milk IgA antibodies to food antigens have not been shown to be influenced by the antigenic load in the mother's diet (Mascart-Lemone *et al.* 1991).

IgA antibodies in colostrum and human milk appear to be particularly important during the first few days of life, when the infant's mucosal IgA production is deficient (Hanson *et al.* 1977, Perkkiö and Savilahti 1980). After ingestion, maternal milk IgA antibodies have been suggested passively to protect the infant by reducing or preventing antigen entry across the immature gastrointestinal epithelium (Walker 1979). In an experimental model, Walker *et al.* (1975) have shown that intestinal antibodies can prevent resorption of native proteins and instead increase the uptake of degraded material, probably by binding the antigens and exposing them to intestinal enzymes. IgA antibodies may also play a role in excluding potential food allergens from human milk by forming immune complexes that can be phagocytosed by human milk macrophages (Walker 1979).

Studies have reported that infants with symptom scores highly suggestive of allergic disease had received human milk containing smaller quantities of antibodies to whole cow's milk and casein than did infants without clinical manifestations (Machtinger and Moss 1986). This was supported by the demonstration of Savilahti *et al.* (1991) that seven mothers whose infants developed CMA manifested by cutaneous symptoms produced milk containing less IgA throughout lactation than did the milk of those nonatopic mothers with a healthy infant. More recently, Calbi and Giacchetti (1998) have claimed that breast milk contains less IgA at birth in mothers of children who develop atopic eczema. However, contrary results have also been reported recently with regard to total and anti-cow's milk IgA levels in milk (Saarinen 2000, Duchén *et al.* 1999).

Lactoferrin. Lactoferrin is a 80-kDa single-chain glycoprotein produced by epithelial cells, neutrophils, and mononuclear phagocytes. It is the principal whey protein in human milk, and more than 80% of the protein is in apo form. Its concentration in human milk ranges from about 7 g/l in colostrum to about 1 g/l in mature milk (Masson and Heremans 1971). Because apolactoferrin binds ferric ions (Masson and Heremans 1966), it is able to compete with the iron-binding properties of bacteria. As a result, microorganisms cease to multiply (Stephens *et al.* 1980), contributing to the anti-infective properties of human milk.

Oligosaccharides. Human milk, compared with milk from other species, is unique, because of its high concentration of complex oligosaccharides (Kunz and Rudloff 1993). Their antiadhesive qualities very effectively reduce bacterial and viral adherence in the upper respiratory and gastrointestinal tracts (Zopf and Roth 1996). In addition, by facilitating receptor glycosylation, they may facilitate attachment to intestinal epithelium or entry into the circulation of bioactive factors such as TGF- β (Pabst 1997).

5.3.4 Dietary protein antigens

It is widely accepted that potentially allergenic macromolecules are absorbed by the normal adult gut and transmitted in human milk (Kilshaw and Cant 1984, Stuart *et al.* 1984, Chandra *et al.* 1986, Machtinger and Moss 1986, Sorva and Mäkinen-Kiljunen 1994). Ovalbumin has been found in human milk at maximal levels 4 or 6 h after ingestion, and is of normal molecular size and indistinguishable from native ovalbumin by the radioimmunoassay (Kilshaw and Cant 1984). The same study also detected egg-derived ovomucoid in milk.

β -lactoglobulin (BLG) has been regarded as one of the most important proteins causing symptoms of CMA, and has been shown by enzyme-linked immunosorbent assay (ELISA) to be present in human milk in concentrations up to 16 $\mu\text{g/l}$ (Kilshaw and Cant 1984, Stuart *et al.* 1984, Chandra *et al.* 1986, Sorva and Mäkinen-Kiljunen 1994). With a highly sensitive method having a detection limit of 0.002 $\mu\text{g/l}$, BLG was found in the milk of 75% of mothers consuming cow's milk (Sorva and Mäkinen-Kiljunen 1994). In earlier studies the maximal BLG level was detected after 8 to 12 hours of milk intake and demonstrated to vary inter- and intraindividually (Axelsson *et al.* 1986, Høst *et al.* 1990). In work by Sorva and Mäkinen-Kiljunen (1994) BLG was found in 1 or 2 hours or at both times after an oral cow's milk load in half of the samples. Casein has also been detected in human milk from half of the mothers tested on a cow's milk-containing diet by a sensitive ELISA (Stuart *et al.* 1984, Chandra *et al.* 1986). It has been demonstrated for BLG that human milk may contain intact protein, not only immunologically active peptides (Kilshaw and Cant 1984).

Several groups report the disappearance of symptoms in infants already sensitised upon abstinence of the mother from certain foods and the subsequent reappearance of symptoms in the infants upon their reintroduction to the mother's diet (Gerrard 1979, Sorva and Mäkinen-Kiljunen 1994). Several studies suggested that cow's milk protein in human milk can cause infantile colic (Jakobsson and Lindberg 1978, Iacono *et al.* 1991, Lucassen *et al.* 1998). These reports indicate such small amounts of food proteins or their split products can bring out the symptoms of food allergy in the suckling infant. Gerrard (1979) reported that as little as 5 ml of cow's milk ingested by the mother caused appearance of symptoms of CMA in the breast-fed.

5.4. *In vivo* fate of milk-derived immunoreactive factors

Human milk leucocytes are able to resist trypsinization (Ceriani *et al.* 1979, Keeney *et al.* 1993). Further, it has been shown with milk macrophages that they can tolerate large variations in environmental pH, temperature, and osmolality (Cress and Paxson 1977), characteristics that would allow them to survive in the gastrointestinal tract. The relatively neutral pH of the stomach of a small infant, together with the buffering capacity of breast milk, protect the milk cells, which then may reside biologically intact in the gut of the suckling infant (Mason 1962, Paxson and Cress 1979). Macrophages fed to newborn mice survived for at least 4 hours in the gastrointestinal tract, in some cases localized in the mucosal tissue, and in one case were found in the spleen (Hughes *et al.* 1988). Moreover, breast milk leucocytes have been shown to enter the blood stream of neonatal lambs (Schnorr and Pearson

1984), the spleen of newborn baboons and newborn mice (Jain *et al.* 1989), and the GALT in rats, newborn calves, and lambs (Seelig and Billingham 1981, Puente *et al.* 1984, Seelig and Head 1987). Further, substantial numbers of lymphocytes of the maternal HLA type (up to 10%) occur in the circulation of human newborns; foster nursing permits one to establish that these are transferred post-partum (Beer *et al.* 1975).

In early life, there is no evidence of intragastric protein digestion: low concentrations are found of trypsin, chymotrypsin and H⁺ (Koldowsky 1985). High concentrations of the antiproteases α 1-antichymotrypsin and α 1-antitrypsin, which impede proteolysis, are present in human milk (Linberg *et al.* 1982). Furthermore, the soluble factors in human milk, such as IL-1 and lactoferrin, have been reported to be relatively acid- and protease-resistant (Samson *et al.* 1980, Brines and Brock 1983, Dinarello 1984). Moreover, some of the cytokines, such as IL-10, in human milk may be protected from gastrointestinal digestion by compartmentalisation into bile salts (Garofalo *et al.* 1995). Previous human studies have shown that urinary excretion of lactoferrin, IgA, and secretory component, all of which are present in high amounts in human milk, is higher in infants fed breast milk than in those fed formula, suggesting that immune factors in human milk may be absorbed in the gut of the nursing infant and secreted in the urine, or that their production may be stimulated by uptake of immune factors present in human milk (Goldblum *et al.* 1989). Evidence thus also exists that milk-derived soluble proteins can persist in the recipient long enough to be biologically active.

sIgA is much more resistant to acid conditions and to the proteolytic enzymes of the gut (trypsin and pepsin) than serum is IgA (Kenny *et al.* 1967), and is found intact in considerable amounts in the feces (Kenny *et al.* 1967, Ogra *et al.* 1977). This is probably due to the buffering action of the milk and the dilution of acid that occurs during feeding (Kenny *et al.* 1967). However, using poliovirus antibody activity as a marker, milk immunoglobulins are absorbed for a short period of 18 to 24 hours after birth (Ogra *et al.* 1977), though the importance of this to the infant is unknown. That study showed that about 10 to 15% of ingested IgA appeared to be absorbed, and approximately 60% of IgA excreted in the feces. Almost ten years later, Klemola *et al.* (1986), however, reported that less than 0.1% of mumps virus-specific IgA antibodies was absorbed in active form in the intestinal tract of preterm or full-term neonates.

It has been discovered that an infant's active immune response to specific antigens given during the first year of life develops differently in breast-fed and formula-fed infants. Transfer of antigen-specific lymphocyte responses, such as tuberculin

immunity or specific killer T-cell-mediated resistance to tumors, has been observed in breast-fed newborns (Beer *et al.* 1975, Schlesinger and Covelli 1977, Head and Beer 1979). It remains unknown, however, whether such reactivity in these newborns represents uptake of antigen-sensitized intact cells or a simple absorption of mediators released by the T lymphocytes during their passage through the intestinal tract (Ogra *et al.* 1977). In a study of infants vaccinated as neonates towards BCG (*Bacillus Calmette-Guérin*), the breast-fed babies had a significantly higher lymphocyte blast transformation response to purified protein derivative after BCG vaccination than did those who were never breast-fed (Pabst *et al.* 1992). Antibody responses after *Haemophilus influenzae b* conjugate vaccination were also higher in breast-fed than in formula-fed infants, but nonspecific cell-mediated immunity was not significantly affected (Pabst and Spady 1990). Similarly, breast-fed children at 21 to 40 months of age had higher titres to polio and diphtheria toxoid and higher concentrations of salivary secretory IgA antibodies to polio and to tetanus and diphtheria than did formula-fed children (Hahn-Zoric *et al.* 1990).

5.5. Normal immune response to dietary protein antigens

5.5.1 Cells and mediators involved in infant immune responses

Antigen-presenting cells (APCs). APCs are a heterogeneous group of bone marrow-derived cells, commonly leucocytes, shown to have an immunostimulatory capacity (Steinman *et al.* 1986). They reside mainly in the skin and lymphatic organs (Steinman *et al.* 1986), but their precise role in the induction of oral tolerance is yet to be determined. It has been claimed that mucosal dendritic cells (DC) and gastrointestinal epithelial cells both are likely to play important roles in that process, at least based on rodent models (Strobel and Mowat 1998). As immature cells, they are scattered throughout the body in nonlymphoid organs. They pick up and process antigen, but paradoxically are unable to effectively present the processed antigen to T cells (Steinman 1991). In the second phase they migrate to regional lymph nodes and home to T-cell zones. During this process of maturation, they lose their antigen-capturing capacity and become mature immunostimulatory dendritic cells that trigger naive T cells recirculating through these areas (Austyn 1992). In addition to DCs, endothelial cells can be induced by cytokines to express class II MHC molecules and thereby function as APCs (Hershberg and Mayer 2000). B lymphocytes also express class II MHC molecules and have been shown to take part in antigen presentation (Chesnut and Grey 1981).

T lymphocytes. T cells are defined by a specific antigen-recognizing receptor comprising an antigen-recognizing molecule (T idiotype) and a complex of invariant polypeptides (CD3). Immature thymocytes can possess both CD4 and CD8 molecules on their surface during their development in the thymus, but the cells finally leaving the thymus are single-positive, either CD4+ or CD8+. Mosmann *et al.* (1986) have shown two different subsets of T cells of the helper-phenotype. Th1 cells execute cell-mediated immune responses, whereas Th2 cells assist in antibody production for humoral immunity (Mosmann *et al.* 1986). Recently, the differentiation of naive Th cells has been reported to be dependent on the type of DC, with DC1 inducing Th1, and DC2 inducing Th2 differentiation (Rissoan *et al.* 1999). Patients with severe atopic disorders have been reported to show a decreased ratio of Th1 and Th2 cells (Romagnani 1990). In addition, allergen-specific T-cell clones derived from atopic patients usually exhibit a Th2-like profile of cytokine secretion (Del Prete 1992). Evidence from animal and human experiments has given rise to the notion that an appropriate balance between suppressor and helper T cells is important for the induction of oral tolerance (Suomalainen *et al.* 1993a, Weiner *et al.* 1994, Järvinen *et al.* 1998, Strobel and Mowat 1998).

B cells. The majority of human peripheral blood B lymphocytes express IgM and IgD molecules, whereas IgG, IgA, and IgE are expressed in only a few of these cells. By contrast, in specific locations in the body, B cells bearing IgG, IgA, and IgE are present in larger numbers; for example IgA-bearing cells in the intestinal mucosa (Cooper 1987). The majority of B lymphocytes also carry MHC class II-antigens on their surface, needed for communication with T lymphocytes (Benacerraf 1986). The leucocyte antigen CD23, a low-affinity receptor for IgE, is expressed on B lymphocytes following activation by a number of stimuli (Gordon *et al.* 1989). Its soluble form, sCD23, is released upon activation (Yanagihara *et al.* 1990) and is involved in the regulation of IgE synthesis (Bonney *et al.* 1988, Yanagihara *et al.* 1990). CD23 may also be involved in presentation of antigens to T cells and in cell adhesion (Gordon *et al.* 1989). Increased levels of CD23 or sCD23 appear in atopic diseases (Colver *et al.* 1989, Yanagihara *et al.* 1990), asthma (Hoeger *et al.* 1994), and CMA (Järvinen *et al.* 1998).

The CD5 leucocyte antigen is expressed both on T and on B cells; B cells co-expressing CD5 can generate polyreactive, low-affinity antibodies (Burastero *et al.* 1988). In early life, B cells bearing the surface CD5 marker predominate, but in adults they represent only 10-25% of the peripheral blood B lymphocytes (UytdeHaag *et al.* 1991), thus reflecting the immaturity of the immune system in childhood (Erkeller-Yuksel *et al.* 1992). The precise role of these cells is unclear, but they are thought to

function as the first line of defence against environmental agents (Casali and Notkins 1989).

Neutrophils. Over 90% of the peripheral blood granulocytes are neutrophils. They contain two main types of granules; primary (azurophilic) granules containing lysosomal enzymes such as myeloperoxidase, and secondary (specific) granules containing lactoferrin in addition to lysozyme and human neutrophil lipocalin. When chemoattractants are released, for example from other leucocytes or bacteria at intravascular sites, neutrophils are capable of adhering to endothelial cells and moving through the capillary wall into tissues. Neutrophil infiltration is an early event in IgE-mediated reactions (Kay 1988). In general, the neutrophilic granulocyte is a cytotoxic and potentially tissue-injuring cell participating in the destructive processes and symptoms seen in a variety of inflammatory diseases including asthma. The precise role of neutrophils in food allergy remains unresolved.

Eosinophils. In healthy, non-allergic subjects, eosinophils constitute only 5% of peripheral blood leucocytes, circulating only briefly and thereafter being distributed into tissues (Altman and Gleich 1990). The blood/tissue ratio of eosinophils has been estimated to be 1:100 (Altman and Gleich 1990). Eosinophils have multiple functions; they can induce inflammatory injury to infectious agents, regulate or modulate inflammatory processes, and harm host tissue (Nutman 1987, Weller 1991). Therefore eosinophils are now more often considered likely to cause inflammatory damage (Kay 1988), for example in patients with eosinophilic gastroenteritis and atopic eczema (Katz *et al.* 1984, Leiferman *et al.* 1985). Challacombe and his colleagues (1986) found in patients with CMA an elevated number of eosinophils in lamina propria to be associated with elevated serum IgE levels and positive cow's milk-specific RAST. Others have reported a higher number of eosinophils in the duodenal mucosa of children with untreated CMA, and a lower number of these cells after the withdrawal of cow's milk from the diet (Kosnai *et al.* 1984). Suomalainen and co-workers (1994) reported, in patients with cutaneous symptoms, an increase in the level of eosinophil cationic protein (ECP) as a result of eosinophil degranulation in serum during positive cow's milk challenge.

Basophils. Only 0.5 to 1% of peripheral blood leucocytes are basophils, but in allergic patients the basophil count may reach 2% during the pollen season (Kay 1988). Basophils are characterised by cytoplasmic granules containing histamine and eosinophil chemotactic factor of anaphylaxis. In allergic patients, appropriate stimuli, usually allergens, can induce degranulation of basophils and thereby cause adverse symptoms. In a study by Prahl *et al.* (1988), a correlation emerged between clinical reactivity to cow's milk and *in vitro* basophil histamine release in patients with

cutaneous symptoms of CMA manifested with urticaria. Räsänen and her colleagues (1992) have found that elevated basophil histamine-release activity is associated with an immediate reaction to a clinical cow's milk challenge.

Platelets. Platelets are myeloid cells derived from megakaryocytes in the bone marrow. They not only act in blood clotting, they also express low-affinity receptors for IgE, which may suggest that they can be sensitised in allergic patients similarly to mast cells, basophils, and macrophages (Kay 1988).

IL-4 and IFN- γ . In human beings, the T cell-derived IFN- γ , together with IL-4, are the two main signals that regulate IgE synthesis (Pene *et al.* 1988). IL-4 enhances directly the generation of IgE antibodies, but this effect can be abrogated by IFN- γ (Pene *et al.* 1988). The ability of stimulated leucocytes to produce IFN- γ is defective in healthy neonates (Wilson *et al.* 1986), but the exact age when T-cell IFN- γ production achieves the adult level is unclear. IFN- γ plays an important role in the modulation of antigen-specific and non-antigen-specific immune responses (Lewis and Wilson 1990). Defective IFN- γ production of peripheral blood mononuclear cells (PBMCs) has been reported in children with CMA (Suomalainen *et al.* 1993b).

TNF- α . TNF- α is a regulatory lymphokine which, like some other factors, has been found to act synergistically with IFN- γ to upregulate HLA class II expression (Pujol-Borrell *et al.* 1987). By activating macrophages, TNF- α augments the number of receptors for itself on cell surfaces, making the cells more responsive to it and thus further increasing their activation and secretion of TNF- α (Janeway and Travers 1996). TNF- α production also induces adhesion-molecule expression (Gamble *et al.* 1985), chemotaxis of monocytes (Mushtaha *et al.* 1989), and intraepithelial lymphocyte proliferation and migration (Ebert 1998). In an experimental model, TNF- α may induce the maturation of monocytic APCs (Cumberbatch and Kimber 1992, Sallusto and Lanzavecchio 1994).

5.5.2 Development of infant's immune responses to dietary proteins

The systemic immune system of an infant is relatively naive at birth; during the first days and weeks of life the child encounters numerous environmental antigens. During the past, investigators have focused on the development of humoral immune responses to oral antigens. According to Tainio *et al.* (1988), IgG antibodies are already present in plasma at birth. The later increase in specific IgG depends on the type of feeding (Vaarala *et al.* 1995). Oral introduction of cow's milk proteins in early infancy elicited clear-cut IgG antibody production against these antigens in all

infants, demonstrating intestinal exposure to cow's milk antigens (Tainio *et al.* 1988, Vaarala *et al.* 1995), humoral response being strongest with neonatal introduction of milk proteins (Tainio *et al.* 1988). This reactivity decreased with age (Tainio *et al.* 1988, Vaarala *et al.* 1995, Jenmalm and Björkstén 1998), and at the age of one year no difference existed between those infants who received cow's milk in early infancy and those who began receiving it later (Tainio *et al.* 1988, Vaarala *et al.* 1995). However, high levels were found up to 8 years in children with early exposure to cow's milk (Jenmalm and Björkstén 1998). In that study, high levels of IgG4 antibodies to BLG were detected at 8 years in children with atopic symptoms and sensitivity to allergens. Further, Oldaeus *et al.* (1999) reported that the levels of IgG antibodies to BLG were significantly higher in the atopic infants. In contrast to these findings, Duchén *et al.* (1997) reported lower levels of IgG1 and IgG4 antibodies to BLG in atopic individuals at 4 years. Jenmalm and Björkstén (2000) examined the relationship between cord blood IgG antibodies to food and inhalant allergens and the development of atopic symptoms and sensitisation in children through the first 8 years of life. High levels of IgG antibodies to inhalant, but not food, allergens were associated with less development of atopy in these children.

Specific IgA and IgM antibodies increase more slowly, the levels of CM-specific IgA being higher among formula-fed than breast-fed infants (Kaila *et al.* 1994). However, production of plasma IgA and IgM antibodies to cow's milk is stimulated even during exclusive breastfeeding (Tainio *et al.* 1988).

IgE antibodies to cow's milk and BLG, too, were more common in infants that received regular cow's milk-based formula than in those who received partially or extensively hydrolysed formula (Oldaeus *et al.* (1999). Transient low-level IgE responses to food antigens may be seen in young infants without clinical disease (Hattevig *et al.* 1984), but and their levels were significantly higher in the atopic and allergic infants (Hattevig *et al.* 1984, Oldaeus *et al.* (1999).

The development of cellular immunity to food antigens in humans is less well understood. Feeding infants cow's milk-based formula induces systemic cellular responses to cow's milk proteins, but with a later decline in T-cell response. Furthermore, exposure to cow's milk proteins after the age of 9 months resulted in depressed cellular responsiveness to these proteins (Vaarala *et al.* 1995). An enhanced proliferative response of PBMCs to cow's milk proteins has been detected in patients with CMA (Räsänen *et al.* 1992, Tainio and Savilahti 1990), although contrary findings also exist (Eigenmann *et al.* 1995).

5.5.3 Development of oral tolerance

Because environmental antigens from food and microbial flora are in constant contact with mucosal surfaces, they provide a continuous stimulus to the entire immune system. Although a frequent result of such stimulation is the induction of mucosal and systemic immunity, an alternative outcome is a state of unresponsiveness or tolerance. This phenomenon, called “oral tolerance”, is a state of systemic immunologic unresponsiveness induced by prior oral administration of dietary antigens (Mowat 1987). Oral tolerance of both humoral and cellular immunity has been evidenced in experimental animals (Challacombe and Tomasi 1980, Hanson 1981, Strobel and Ferguson 1984, Strobel and Mowat 1998) exists also in humans (Strobel 1992, Husby *et al.* 1994).

The prevention of excessive antigen absorption from the lumen involves a dual process: activation of a local antigen-specific IgA response and depletion of systemic immune responses to specific antigens. After contact with antigens and their activation in Peyer’s patches, the B lymphocytes travel to the mesenteric lymph nodes. Subsequently, they migrate back to the intestinal mucosa via the systemic circulation, where, following contact with the antigen, they secrete IgA that binds the antigen and prevents its further absorption (Challacombe and Tomasi 1980, Kagnoff 1982). In the situation where intraluminal antigens are not adequately excluded locally by secretory antibodies or the non-immune defence mechanisms in the gut, several suppressive systems are activated. T suppressor cells activated in Peyer’s patches or in mesenteric lymph nodes are able to travel to extraintestinal lymphoid tissues where they suppress systemic immune responses (Challacombe and Tomasi 1980).

In adults, continuous oral antigenic stimulus leads to induction of T-cell, but not B-cell, tolerance (Husby *et al.* 1994). Similarly in infants, feeding with cow’s milk-based formula induced systemic humoral and cellular responses to cow’s milk proteins, but T-cell response later declined, supporting the concept of oral tolerisation (Vaarala *et al.* 1995). The B-cell system has also been demonstrated to become tolerant through antigen feeding, but generally this requires larger amounts of antigen (Challacombe and Tomasi 1980). Sensitivity to tolerance induction also varies among T-cell subsets, so that Th1 cells seem to be more easily tolerized than the Th2 cells (Burstein *et al.* 1992). In experimental animals, induction of tolerance is greatly affected by dose, frequency, and type of antigens (Kagnoff 1982). Exposure to cow’s milk proteins in human beings after the age of 9 months resulted in depressed cellular and humoral responsiveness to these proteins compared to an earlier exposure, suggesting that induction of oral tolerance is also an age-dependent phenomenon (Vaarala *et al.*

1995). Tolerance may occur by a number of mechanisms, including clonal anergy, clonal depletion, or suppression. With regard to orally induced tolerance, strong evidence exists that T-cell-mediated suppression plays an important role in oral tolerance to protein antigens and in downregulation of systemic immune responses (Weiner *et al.* 1994, Strobel and Mowat 1998). However, even after suppressor T cells can no longer be identified, the animals remain unresponsive to the antigen they are fed (Richman *et al.* 1978), suggesting the presence of an additional mechanism such as clonal anergy.

5.6. Cow's milk allergy

5.6.1 Sensitisation to cow's milk proteins

Postnatal sensitisation. Clinical symptoms of CMA often appear during the first months of life, usually within days or weeks after the commencement of nutrition with a cow's milk-based formula (Jakobsson and Lindberg 1978, Savilahti *et al.* 1992), indicating that sensitisation to cow's milk occurs frequently when cow's milk is introduced to the infant's diet in large amounts. However, symptoms may appear even earlier, whilst the infant is only breast-fed (Machtiger and Moss 1986, Jakobsson and Lindberg 1978, Gerrard 1979, Sorva and Mäkinen-Kiljunen 1994, Isolauri *et al.* 1999), or at the first intake of cow's milk (Gerrard and Shenassa 1983, Høst *et al.* 1988). In these cases sensitisation to cow's milk must have occurred *in utero* or via human milk. It has been suggested that infants might also become sensitised by inhalation or by direct antigen contact, since dietary antigens are found in the home environment (Dreborg 1995). Further, evidence indicates that IgE antibodies recognize ubiquitous antigens and structurally related human proteins (Cantisani *et al.* 1997), suggesting a possible cross-reactivity between human milk and dietary antigens, and thereby another possible means of sensitisation.

Postnatal sensitisation against cow's milk has been suggested to occur through human milk of presumably exclusively breast-fed infants, especially those at risk (Gerrard 1979, Chandra *et al.* 1986, Zeiger *et al.* 1986), but this is not conclusively reported. The retrospective nature of these studies fails to rule out the possibility that some or all of these infants may have inadvertently been given cow's milk directly (Høst *et al.* 1988), and the possibility naturally cannot be excluded that initial sensitisation had already occurred *in utero*. Doubts remain whether very small amounts of food antigens transmitted via the milk could sensitise the infant, although they can produce allergic symptoms in infants already sensitised.

Prenatal sensitisation. Evidence in favour of sensitisation *in utero* is accumulating. Feiterna-Sperling *et al.* (1997) report a case of manifestation of CMA directly after birth, confirming the finding of Kuroume *et al.* (1976). Enhanced cord blood T-cell reactivity and specific IgE antibodies to cow's milk proteins have been attributed to high-risk babies, suggesting allergy development (Michel *et al.* 1980, Kondo *et al.* 1992, Piastra *et al.* 1994). A more recent study provides further evidence that *in utero* exposure can result in priming to common allergens from 22 weeks of gestation on (Jones *et al.* 1996).

5.6.2 Hypersensitivity to cow's milk

CMA is defined as an immunologically mediated adverse reaction to cow's milk antigens (Savilahti *et al.* 1992). It is a condition involving a disturbance or breakdown in the development of oral tolerance. At birth, the infant immune system is relatively naive and immature, evidenced by the immaturity of the immunology and the gastrointestinal barrier of the infant (Strobel 1988). In genetically predisposed infants, ingested antigens may lead to the production of antigen-specific IgE antibodies or other abnormal immune responses in association with clinical symptoms of CMA.

The development of food allergy involves both genetic predisposition and exposure to environmental antigens (Strobel 1988). The risk for development of food allergies is higher in infants than in adults, which is considered to reflect a relationship between food allergy and immaturity of the gut and the immune system (Strobel 1988). In infancy, the immune system is relatively naive and incapable of effective antigen exclusion (Andersson *et al.* 1981). Immaturity of the gut involves defective antigen processing and digestion, and increased permeability of the gut mucosa (Walker 1987). Although T-cell-mediated immune responses and cytokine production are comparatively mature at birth, the capacity to generate soluble mediators such as IL-4 and IFN- γ is low, at least during the first weeks of life, reflecting the functional immaturity of the immune system (Wilson 1985, Lewis and Wilson 1990). The precise immune mechanisms responsible for CMA are unknown.

Several reports on immune defects in children with CMA are available (Isolauri *et al.* 1992, Suomalainen *et al.* 1992, Hill *et al.* 1993, Suomalainen *et al.* 1993a, Suomalainen *et al.* 1993b, Suomalainen *et al.* 1994, Österlund *et al.* 1999). The total number of antibody-secreting B cells, measured by the ELISPOT, increases during positive oral cow's milk challenge (Isolauri *et al.* 1992, Suomalainen *et al.* 1992). The antigen-specific response is defective, and in comparison, the antigen-non-specific

response, especially in the IgM class, is strong. Furthermore, the total percentage of B cells and of activated B cells is significantly higher in infants with very early symptoms of CMA than in healthy controls, indicating a defect in the regulation of B-cell function (Järvinen *et al.* 1998). IFN- γ and TNF- α production of PBMCs is significantly lower in patients with CMA than in control children (Suomalainen *et al.* 1993b, Österlund *et al.* 1999). Further, Hill *et al.* (1993) demonstrated that CMA patients with immediate reactions generated less IFN- γ than the patients with late reactions or healthy controls. In addition, T-cell-mediated suppression is defective in the patients with active CMA (Suomalainen *et al.* 1993a). However, as these defects are not confined to patients with food allergy, they are therefore not specific for CMA.

CMA may involve any of the hypersensitivity types described by Coombs and Gell (1975). Type I hypersensitivity reactions are responsible for immediate-type reactions, mediated by IgE antibodies, which bind to high-affinity receptors on mast cells and basophils (Sampson 1988, Wershil and Walker 1988). This leads to degranulation, and the released mediators, i.e., histamine, prostaglandins, and serotonin, are mainly responsible for the rapidly manifested symptoms characteristic of anaphylaxis (Coombs and Gell 1975, Sampson 1988) or for local reactions in the skin seen as erythema and urticaria (Savilahti 1981, Hill *et al.* 1984, Räsänen *et al.* 1992). The proportion of IgE-mediated CMA has ranged from 30% to 100%, depending on patient selection and criteria for the diagnosis of CMA (Hill *et al.* 1984, Räsänen *et al.* 1992).

According to Wershil and Walker (1988), no evidence exists for type II hypersensitivity in CMA, although thrombocytopenia and villous cell damage in the intestine of patients with CMA is thought to be linked with type II hypersensitivity reaction (Fontaine and Navarro 1975). Type III hypersensitivity is based on immune complex formation (Coombs and Gell 1975) and has been suggested to be connected with gastrointestinally manifested CMA (Matthews and Soothill 1979) and enteropathy in delayed allergic reactions (Wershil and Walker 1988), although this is not confirmed by Tainio and Savilahti (1990).

Type IV hypersensitivity is based on the activation of antigen-specific T cells, which recruit other cell types by producing cytokines and histamine-releasing factor (Kelso 1989). These cell-mediated reactions may be responsible for delayed-type symptoms (de Weck 1988), the mechanisms of which are poorly known (Kondo *et al.* 1993, Werfel *et al.* 1997). It has also been suggested that delayed reactions to foods would be due to a late-phase IgE-reaction, even when no specific IgE to foods is detectable (Maurer *et al.* 1994).

6. AIMS OF THE STUDY

The purpose of the present study was to determine the immunologically active components of human milk and to evaluate their possible association with the development of CMA in the infant.

The specific aims were:

1. To identify the proportions of different leucocyte subsets in human milk and leucocyte subsets in the peripheral blood of infants with CMA and of healthy infants (**I**).
2. To evaluate the function of the predominant leucocyte, the macrophage, in human milk of mothers with an infant with CMA and of those with a healthy infant by measuring HLA-DR expression on milk macrophages (**II**).
3. To determine some cytokines (IL-4, IFN- γ and TNF- α) of human milk in mothers with an infant allergic to cow's milk in comparison with those with a healthy infant (**III**).
4. To investigate the clinical manifestations of CMA and immune response to cow's milk evoked during a cow's milk challenge performed via mother's milk in infants with active CMA (**IV**).
5. To measure the passage of maternal dietary BLG to breast milk during cow's milk challenge via human milk (**IV**).
6. To study the concentration of total IgA and IgA antibodies to cow's milk in human milk during the breast-feeding period in relation to subsequent development of CMA in the breast-fed (**V**).

7. SUBJECTS AND METHODS

7.1. Subjects

The subjects were enrolled in the study between April 1995 and May 1998. The study population comprised 100 breast-feeding mothers and their infants (*Figure 1*). Of the infants, 61 were followed prospectively from birth; 30 of these were at high genetic risk for allergy (at least one sibling with severe food allergy diagnosed by open challenge), 7 were at moderate risk for allergy (atopic parent, n=7) and 24 were at low risk for allergy (no allergic individuals among first-degree relatives). They were followed up during the first year of life to detect any symptoms suggestive of food allergy. An additional 39 babies were included in the study as young infants either because of symptoms suggestive of CMA and referral to the clinic (n=33) or to serve as healthy controls (n=6). At the time of the visits, all the infants were aged from 2 days to 12 months. They were born at full term, and had no chronic diseases apart from allergy nor any infections whilst visiting the clinic.

All the atopic mothers were symptom-free (no hay fever, eczema, urticaria, asthma) during the breast-feeding period. The mothers took no medication during the study, and those with mastitis during the preceding 4 weeks were excluded.

7.2. Diets

Foods suspected to cause the symptoms in the infant - e.g., cow's milk, cereals (rye, wheat, oats, and barley), egg, and fish - were eliminated from the mother's and the infant's diets. The healthy infants had diets appropriate for their ages.

7.3. Study protocol

The infants that were followed up from birth were usually seen for the first time in the maternity clinic. At this point, the first peripheral blood and milk samples were taken from the mothers and infants. If this was impossible, they attended the hospital soon after their discharge from the maternity clinic. The next visits and collection of

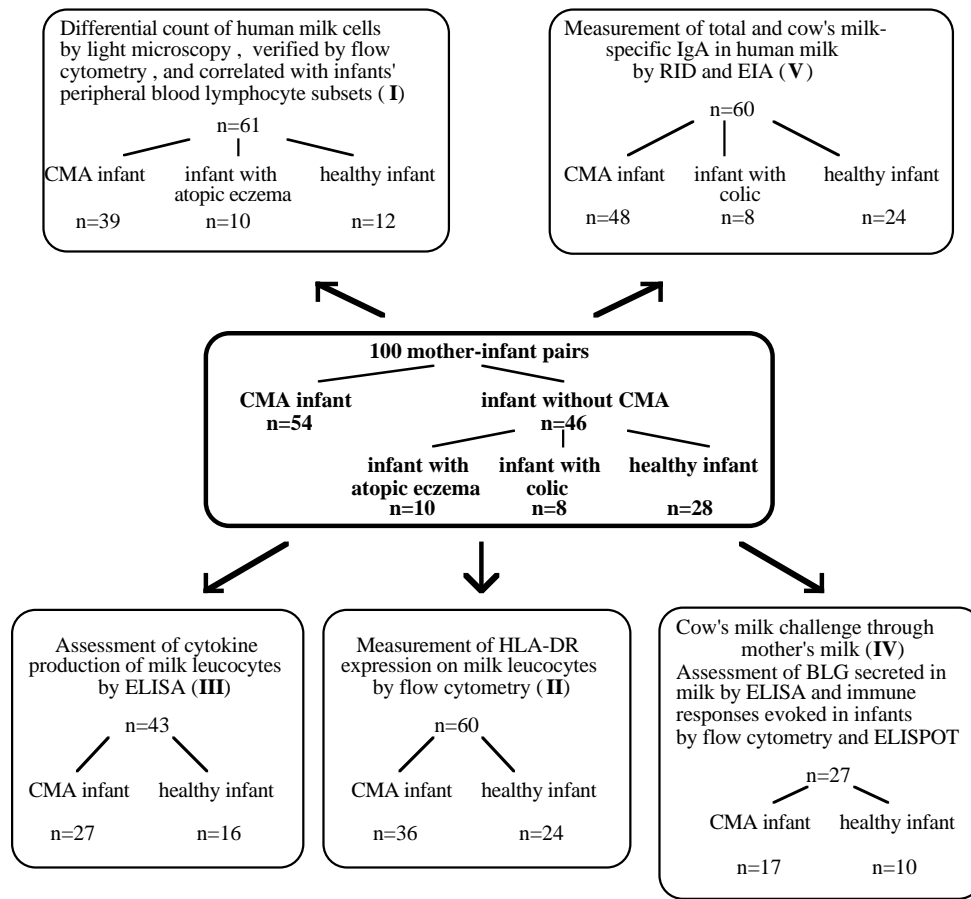


Figure 1. Flowchart of the 100 mothers and their infants, representing numbers of mother-infant pairs included in individual laboratory assessments.

samples were at 1, 3, 6, and 12 months of age. Additional visits were included in the case of arousal of suspicion of food allergy for further examination, initiation of elimination diets if needed, and subsequent clinical challenges. The infants recruited into the study because of suspicion of food allergy were selected among the patients referred to the hospital. They were examined, put on an elimination diet, and challenged thereafter. Detailed advice to the mother on performing a cow's milk and/or cereal elimination diet was always given by a nutritionist. The mothers were free to decide for themselves as to duration of exclusive breastfeeding. Infants older than 3 months started on solid foods according to the recommendations of Finnish Child Health Centre: at the age of 3 to 4 months, potato and fruit/berry sauces, at 5 months, wheat and oats, and at 6 months, meat are recommended for the child's diet.

In food-allergic children, solid foods are basically started at the same ages, but egg, fish, tomato, citrus fruits, and nuts are avoided, and wheat and oats are replaced with rice at 5 months.

7.4. Skin prick test (SPT)

The SPT was performed with a commercial cow's milk extract (Soluprick®, Allergologisk Laboratorium A/S, Hørsholm, Denmark) on the volar side of the forearm, with a 1 mm, one-peak lancet with a shoulder. Histamine dihydrochloride, 10 mg/mL (Soluprick®), served as a positive control. Reactions were read at 15 min and regarded as positive if the mean diameter of the wheal was at least 3 mm, the negative control being 0 mm and the positive control at least 5 mm at the same time.

7.5. Oral cow's milk challenge

Oral cow's milk challenge was performed after a 2- to 4-week cow's milk elimination diet, by an open, clinical cow's milk challenge. Those infants challenged first through mother's milk were subjected to oral cow's milk challenge thereafter. The challenge was started with a drop of cow's milk on the skin or lips. Thereafter, increasing doses of adapted formula (Tutteli®, Valio Ltd., Helsinki, Finland) containing 1.4 g/l of BLG (Mäkinen-Kiljunen S, personal communication) in infants less than 1 year of age, or cow's milk (>1 year of age), were given at 1-hour intervals: on day 1: 1, 10, 50, 100 ml; on day 2, the normal milk intake appropriate for that age was commenced. After any adverse reaction, the challenge was discontinued, and the patient was examined by a pediatrician or a dermatologist.

7.6. Cow's milk challenge performed through mother's milk

Cow's milk challenge through mother's milk was performed after a 2- to 4-week cow's milk elimination diet of both mother and infant. The challenge was started with a drop of adapted cow's milk-based formula (Tutteli®) on the skin or lips of the infant. Thereafter, increasing doses of cow's milk or other dairy product were given at 1-hour intervals to the mother: on day 1: 100, 200, and 400 ml; from day 2 on, free consumption of cow's milk was allowed (at least 500 ml per day recommended). The infant was breast-fed normally at 1, 2, 3, and 4 hours after the beginning of the challenge, and thereafter according to the infant's needs. The infants showing indistinct reactions during the cow's milk challenge via human milk were perorally

challenged with adapted formula (Tutteli®) to confirm the diagnosis, as described in the last paragraph. After any adverse reaction, the challenge was discontinued, and the patient was examined (K-M J).

7.7. Samples

7.7.1. Human milk

After the breast was washed with warm water, but without detergents, breast milk samples were collected in the morning by light manual expression or with a breast pump and processed immediately. The volume of each milk sample was measured, and it was centrifuged (400 x g, 20°C, 15 min). Subsequently, fat was removed, and supernatant was collected, frozen, and stored at -70°C until further determination of human milk IgA. The cells were resuspended in RPMI 1640 medium, containing penicillin (100 IU/ml) and streptomycin (100 µl/ml), glutamine (600 µl/ml), and 5% fetal calf serum (FCS). The cells were washed four times with RPMI 1640 before further analysis.

Cytospin preparations of milk cells were obtained with a sample application of 1×10^5 cells (Cytospin3®, Shandon, Life Sciences International (Europe) Ltd., Astmoor, Runcorn, Cheshire, United Kingdom). To improve the attachment of breast milk cells to the slides, the slides were treated with Vectapond™ (Vector Laboratories Inc, Burlingame, CA, USA) before sample application, and then stained with May-Grünwald-Giemsa for differentiation of leucocytes.

7.7.2. Peripheral blood

Venous blood was drawn and heparinized from the infants just before the commencement of the challenge and a week later. PBMCs, mainly lymphocytes, were obtained by Ficoll-Hypaque (Pharmacia AB, Uppsala, Sweden) centrifugation of the blood diluted 1:4 with RPMI 1640 medium. The cells were resuspended in RPMI 1640 medium containing penicillin and streptomycin, glutamine, and 5% FCS, and washed three times. After isolation, leucocytes were visually counted in a Bürker chamber for cell counting.

Venous blood-EDTA samples from the mothers and infants were drawn onto slides and stained with May-Grünwald-Giemsa for differentiation of peripheral blood lymphocytes.

7.8. Laboratory methods

7.8.1 Light microscopy

The numbers of macrophages, monocytes, lymphocytes, neutrophils, and eosinophils of venous-blood EDTA preparations and human milk cytopsin preparations were counted under light microscope, with results are expressed as percentage of the cell type per 100 leucocytes.

7.8.2 Flow cytometry

Flow cytometric analysis allows to enumeration of leucocyte subsets. Flow cytometric analyses of milk leucocytes and PBMCs of the mothers and the infants were performed at every visit, and on day 1 and day 8 of the cow's milk challenge via mother's milk from the infants' peripheral blood. For this purpose, human milk and peripheral blood cell suspensions at a cell concentration of $1 \times 10^6/\text{ml}$ in RPMI 1640, with antibiotics, glutamine, and 5% FCS, were incubated at 4°C for 15 min with a saturating concentration of phycoerythrin-labelled monoclonal antibodies against CD14, CD8, CD23, and CD16, and fluorescein isothiocyanate-labelled antibodies against CD45, CD3, CD4, CD19, and HLA-DR (Beckton Dickinson Immunocytometry Systems, Mountain View, CA, USA.) according to manufacturer's instructions (Macey 1994) (*Table 1*). Flow cytometric analysis was then performed, with the same instrument settings for every analysis. The leucocytes were finally gated to study the numbers of cell subsets. The numbers of cells positive for the surface markers are expressed as percentages of the cells within the gate.

Table 1. Human leucocyte differentiation antigens and their specificity.

Antigen	Distribution
CD45	Leucocytes
CD3	T cells
CD4	Helper/inducer T cells, monocyte subset
CD8	Cytotoxic/suppressor T cells, NK-cell subset
CD19	Precursor B cells and B cells
CD23	Activated B cells, monocytes, dendritic cells, eosinophils, platelets
CD5	Mature T cells, B-cell subset
CD14	Monocytes, macrophages, dendritic cells, granulocytes, B cells
CD16	NK cells, granulocytes, macrophages, monocytes

7.8.3. Determination of TNF- α , IFN- γ , and IL-4

To induce breast milk leucocytes and PBMCs, 6.25×10^5 isolated cells in 1 ml of RPMI 1640 containing antibiotics, glutamine, and 10% FCS were cultured in a humidified 5% CO₂ atmosphere at 37°C for 48 h with Concanavalin A (Con A, Pharmacia, Uppsala, Sweden) at a final concentration of 25 $\mu\text{g/ml}$; a control cell population was generated with RPMI 1640 only. The supernatants were collected and stored at -70°C. Subsequently, the TNF- α , IL-4, and IFN- γ produced during lymphocyte induction were determined from the thawed supernatants with commercial ELISA kits (CLB, Amsterdam, Netherlands) according to manufacturer's instructions. The results of the different runs were equalized, employing the comparison of standard curves, and are expressed as pg/ml.

7.8.4. Determination of BLG

Human milk samples were collected by manual expression or with a breast pump before ($n=12$) and at 1, 2, 3, and 4 hours after ($n=24$) the commencement of cow's milk challenge via human milk after an at least 1-week cow's milk elimination period. The milk samples were stored at -20°C for later determination of BLG levels with ELISA as described in detail previously (Mäkinen-Kiljunen and Palosuo 1992). Briefly, affinity-purified rabbit anti-BLG in carbonate buffer was used to coat microtitre plate wells (Immunoplate I, Nunc, Roskilde, Denmark). Samples and standards (0.015 to 10 $\mu\text{g/l}$) were tested in triplicate in phosphate buffer containing 3% polyethylene glycol (PEG) 6000 (Macrogol; Ph. Nord. NOMEKO SA, Copenhagen, Denmark). The BLG bound was detected with alkaline phosphatase-conjugated immunoglobulin and *p*-nitrophenol phosphate. The detection limit of the assay was 0.002 $\mu\text{g/l}$, and recovery was 93% to 127%. The coefficient of variation was 5% to 15% within each series and 10% between series. No other cow's milk proteins were detected with the antibodies to BLG (Mäkinen-Kiljunen and Palosuo 1992).

7.8.5 ELISPOT-assay

The total number of immunoglobulin-secreting cells (ISCs) and the number of specific antibody-secreting cells (sASC) against BLG, casein, and gliadin were measured by the ELISPOT method on day 1 and day 8 of the cow's milk challenge via mother's milk, as described previously (Isolauri *et al.* 1992, Suomalainen *et al.* 1992). In brief, isolated and washed PBMCs were suspended in culture media and adjusted to a final

concentration of 2×10^6 cells/ml. The cells were incubated on antigen-coated, flat-bottomed 96-well microtitre plates (Immunoplate RI, Nunc A/S) so that the antibodies they secreted could react with the antigen nearby. The antibodies were visualised by application of enzyme-labelled antisera, followed by a substrate agarose overlay. The counting of coloured spots, each representing one cell, was done with a stereo microscope after variable periods of storage at 4°C. For determination of ISCs, the wells were coated with anti-human IgA, IgG, and IgM, and to determine the number of sASCs, BLG (90% of protein), casein (70% of protein), and gliadin were used as coating antigens (Sigma, St. Louis, MO). An immune response occurred when there were >0.5 sASCs/ 10^6 cells.

7.8.6 Determination of total and cow's milk-specific IgA

After thawing, human milk samples were diluted 1:5 in phosphate-buffered saline (PBS) solution (pH 7.4) including 0.05% Tween 20. The rest of the human milk fatty layer was removed by centrifugation at $17000 \times g$ for 10 minutes at 4°C. Supernatants were used for the determination of IgA-specific antibodies to cow's milk and 1:10 dilutions for total IgA measurement. Breast-milk total IgA was determined by radial immunodiffusion by use of LC-Partigen IgA immunodiffusion plates and N Protein Standard SL as a calibrator according to manufacturer's instructions (Dade Behring, Marburg, Germany). Results are expressed as g/l.

Specific IgA antibodies to cow's milk were determined by a modification of methods described by Knoflach *et al.* (1987). Dried whole cow's milk powder (Valio Ltd.) was dissolved in sodium carbonate buffer (0.1 mol/l Na_2CO_3 and 1 mmol/l MgCl_2 , pH 9.8) to give a stock solution of 1 mg protein per ml. Microtiter plates (Nunc-Immuno Plate MaxiSorp™, Nunc A/S) were coated with 100 μl of antigen solution diluted to a protein concentration of 0.3 $\mu\text{l}/\text{ml}$. The plates were incubated at 37°C for 3 hours. The wells were washed three times with PBS-Tween 20. One hundred microlitres of test milk supernatant and a serial dilutions of a cow's milk antibody-positive patient's serum forming a standard curve were added to the wells and incubated at room temperature for 16 hours. After the wells had been washed three times with PBS-Tween 20, 100 μl of alkaline phosphatase-conjugated anti-human IgA antiserum (Dako A/S, Glostrup, Denmark) diluted 1:1000 in PBS-Tween 20 was added to the wells and incubated at 37°C for 2 hours. After incubation, the wells were washed with PBS-Tween 20 and 100 μl of fresh substrate solution (para-nitrophenylphosphatase 1mg/ml in diethanolamine- MgCl_2 buffer, pH 9.8) was added to each well and incubated at 37°C for 30 minutes. After the end of incubation, 100 μl of 1 mol/l NaOH was added to the wells to stop the reaction. Absorbances were

determined at a wavelength of 405 nm and specific IgA concentrations as EU/ml were calculated by use of a standard curve.

7.9. Statistical analyses

Analysis of variance (ANOVA), the Mann-Whitney U test and the Kruskal-Wallis test were employed to determine the statistical significance of differences between continuous variables (I-V). Because of the skewed distributions, logarithmic transformations were used (I-V). The repeated observations were studied by ANOVA for repeated measures and a paired t -test (III-V). A χ^2 test was applied to determine differences in proportions (I, III-V). We sought associations between data with simple regression and Spearman's rank correlation test: results are given as correlation coefficient ρ and probability value (I, II).

The results for IgA determinations and breast milk cellular composition were analysed by an experienced biostatistician (V). For breast milk IgA, the data was analysed by analysis of variance for repeated measurements, with mothers as clusters. Since the content of IgA was skewed, the logarithmic transformation was applied to the values for IgA prior to model fitting. Since the content of IgA drops faster in the beginning of breastfeeding and then almost levels out, the logarithm of the baby's age was used, as such, as an explanatory variable. In the analysis, the starting model was: $\ln(\text{age})$ as the random part and the systemic one: $\ln(\text{IgA}) \sim \ln(\text{age}) * (1_{\text{allergy}} + 1_{\text{mother's atopy}} + 1_{\text{symptoms}})$, where $1_{\text{character}}$ is an indicator variable for character. The unimportant terms (significance $>5\%$) were excluded. The models were compared by analysis of variance.

The comparison of cellular compositions of breast milk between groups of mothers was also performed with the help of biostatistician (I). Since the distributions of the counts of leucocytes were skewed, and the variances could not therefore be stabilised by logarithmic transformations, the differences between the groups were studied by Kruskal-Wallis test. The cut points for the counts of leucocytes were sought by attempts at various possibilities. Fischer's exact test was used to study the independence of each two grouping variables.

Means are presented with 95% confidence intervals (CI) and medians with ranges. Statistical significance is defined as $p \leq 0.05$. The analyses were carried out with S plus 4 software (MathSoft, Inc., Seattle, WA, USA) (I, V) and Statview 4.0 software (Abacus Concepts, Inc., Berkeley, CA, USA) (I-V).

7.10. Ethical aspects

The study protocol was approved by the ethics committees of the Skin and Allergy Hospital of the Helsinki University Central Hospital and the City of Helsinki. Informed consent was obtained from the mothers for breast-milk sample collection and from the parents for longitudinal follow-up of their children.

8. RESULTS

8.1. Clinical features of subjects (I-V)

By the end of the 1-year follow-up, a total of 54 mothers (70% with atopic constitutions) had infants who had CMA, as shown by clinical cow's milk challenge, and 46 (29% with atopic constitutions) had a healthy infant. Of the 46 healthy infants, 10 (40% with atopic constitutions) had atopic dermatitis but a negative milk challenge (disease control group A), and 8 (38% with atopic constitutions) had had episodes of protracted infant colic but were also negative to a milk challenge (disease control group B). Of the 61 newborns followed up from birth, 30 had an older sibling with food allergy, and 24 (80%) of those developed CMA. In comparison, of those 31 infants with a moderate risk of allergy, only two developed CMA. The duration of breastfeeding was comparable in the infants with CMA, atopic eczema, and colic with that of healthy infants (*Table 2*). Of the infants with CMA, 35 had been exclusively breast-fed at the time of the appearance of symptoms of CMA. Positive maternal history of atopy correlated with the development of CMA (*Table 2*). Mean age of onset of symptoms in children with CMA was 1.2 months (95% CI, 0.9 to 1.5), and mean age at diagnosis was 5.1 months (4.2 to 6.1). Of the allergic infants, 19 (35%) continued to have symptoms of CMA, despite the fact that the mothers were on a very restricted diet. Of them, 6 had symptoms that were ameliorated just after discontinuation of breastfeeding, and in most of the remaining children, symptoms diminished markedly. Of the infants with CMA, 41 (76%) had hypersensitivity to cereals (rye, wheat, oats, and barley), as verified by open challenge. The SPT to cow's milk was positive in 18 (33%) of the children with CMA. In the children with an immediate reaction, SPT to cow's milk was positive in 14 (47%) of 30 children, but in only 4 (17%) of the 24 children with a delayed reaction.

Table 2. Clinical characteristics of the infants studied.

	Infant with CMA n=54	Infant without CMA n=46			p value
		Colic n=8	Atopic dermatitis n=10	Healthy n=28	(Kruskal- Wallis test)
Maternal atopy	70%	38%	40%	29%	0.0018
Duration of breastfeeding †	3.4 (2.9; 3.9)	1.4 (0.2; 8.8)	2.9 (1.4; 6.1)	2.5 (1.7; 3.7)	0.13

† Expressed as geometric mean (95% confidence interval) months.

8.2. Clinical response to cow's milk challenge (I-V)

8.2.1 Cow's milk challenge performed perorally (I-V)

Cow's milk challenge was positive in 54 (75%) of the 72 infants challenged. In 8 of the infants with CMA, the challenge was performed only through mother's milk (see 2.2). In the other 28 of the 100 infants, elimination of cow's milk was not required because those infants were free of symptoms. In the cow's milk challenge, 30 (56%) infants manifested CMA with immediate and 24 (44%) with delayed reactions; 15 children showed local urticaria immediately after introduction of a drop of cow's milk on their skin or lips. Of the rest, 22 children had cutaneous symptoms (urticaria, n=4; atopic dermatitis, n=18), 5 children gastrointestinal symptoms (loose stools or diarrhea, n=4; vomiting, n=1), and 12 children reacted both cutaneously and gastrointestinally (atopic dermatitis and loose stools, diarrhea, vomiting and/or abdominal pain) to administration of cow's milk. Three children showed respiratory symptoms (acute middle ear infection, n=2; allergic rhinitis, n=1) in addition to other symptoms of CMA. *Table 3* gives the volumes of cow's milk eliciting symptoms and the time of reaction in patients with immediate or delayed reactions.

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

	Cow's milk challenge		p value
	Immediate reaction	Delayed reaction	
Time of onset of reaction †			
From last dose	0.1 (0.1 to 0.5)	25 (1 to 7)	
From beginning	0.1 (0.1 to 3)	60 (3 to 168)	
Dose eliciting symptoms ††			
Last dose	15 (1 to 200)	100 (5 to 200)	0.017
Cumulated dose	22 (1 to 360)	312 (7 to 3500)	0.012

† Median (range) expressed as hour. †† Median (range) expressed as ml (cow's milk).

Immediate reaction is defined as occurring within 1 hour and a delayed reaction more than 1 hour after the last dose ingested.

8.2.2 Cow's milk challenge via mother's milk (IV)

Cow's milk challenge through human milk was performed for 27 infants and proved to be positive in 16 (60%) of them (*Table 4*). These 16 are included in the total number of cow's milk allergic infants (see 2.1). At the time of the challenge, the

infants were aged from 1.8 to 9.4 months. All but one of the infants with CMA showed symptoms of CMA during the cow's milk challenge via human milk. In 9 of 17 infants with CMA the challenge was continued, however, with subsequent peroral cow's milk provocation to confirm the diagnosis, because these infants reacted with mild symptoms to their mother's milk. Of the infants with CMA, every one reacted cutaneously (*Study IV: Figure 1*), 5 children also showed gastrointestinal symptoms, and three showed additional respiratory symptoms (*Table 4*). The median total dose of cow's milk ingested by the mother that elicited symptoms was 700 ml (range, 100 to 2300), and the mean time of onset of the reactions was 21 h (range, 2 to 80). All the control infants had a negative cow's milk challenge through human milk, and no reaction with peroral cow's milk.

Table 4. Findings in infants with cow's milk allergy (CMA) and in their mother's milk during cow's milk challenge performed through human milk.

Reaction time (hours)	Volume of milk ingested by mother eliciting symptoms (ml)	Age of infant (months)	Symptoms	BLG level in breast milk before challenge ($\mu\text{g/l}$)	BLG level in breast milk at time of appearance of symptoms ($\mu\text{g/l}$)
2	300	6.0	Eczema	-	<0.01
2.5	300	7.0	Eczema, diarrhoea	0.26	0.65
2.5	300	6.4	Eczema	0.52	6.93
2.5	100	4.7	Eczema	<0.01	<0.01
2.5	100	2.4	Eczema	<0.01	<0.01
4.5	700	6.6	Exanthema	-	<0.01
5	650	8.3	Exanthema	0.29	0.30
5.5	700	9.1	Eczema	-	0.01
6	700	5.0	Eczema	0.62	11.54
8	700	4.4	Exanthema	<0.01	-
9	700	9.4	Eczema, loose stools, acute media otitis	<0.01	0.03
26	1050	3.0	Eczema, abdominal pain, rhinitis	0.52	-
30	900	2.9	Eczema, diarrhoea, abdominal pain	<0.01	6.12
72	2100	4.2	Eczema, abdominal pain, regurgitation	-	4.38
74	1500	8.0	Eczema, rhinitis, acute media otitis	<0.01	0.04
80	2300	7.0	Eczema	<0.01	0.29
-	-	4.3	No reaction to cow's milk challenge through mother's milk	<0.01	<0.01

BLG= beta-lactoglobulin

8.3. Cellular, cytokine, and immunoglobulin composition of human milk (I-III, V)

8.3.1 Differential cell counts of milk leucocytes (I)

Differential cell counts of milk leucocytes were performed for 61 mothers (*Figure 1*). After careful removal of any visible fat layer in the milk specimens and several washes, there were hardly any detectable membrane/fat globules on the slides. The results from differential cell counts were comparable between two independent investigators. The predominant leucocyte in the milk of mothers with healthy infants was the macrophage (*Study I: Figure 1a*). In the milk of those mothers whose infant had CMA, the proportion of macrophages was significantly smaller than in those with healthy infants (*Table 5*). Mothers with high proportions of neutrophils in their milk (>20%) had significantly more often infants with CMA than did those with low proportions of neutrophils, $p=0.02$ (Fischer's exact test) (*Table 5; Study I: Figure 1c*). More than 1% eosinophils of milk cells was detected only in the mothers of infants with CMA (*Table 5; Study I: Figure 1d*). The risk for the breast-fed infant of developing CMA was significantly higher if the mother's milk fulfilled at least two of the following features of leucocyte differential count: <91% macrophages, further <52% macrophages, >5% neutrophils or >0% eosinophils (sensitivity 0.72, specificity 0.64, odds ratio 4.5 [95% CI, 1.5 to 13.5], $p=0.01$). As for mothers with infants with atopic dermatitis but no challenge-proven CMA, their milk contained proportionally fewer macrophages and more lymphocytes than did the milk of mothers of healthy infants (*Table 5; Study I: Figure 1b*).

Table 5. Percentages of various leucocytes in light microscopy and total leucocyte count in the milk of mothers with infants showing cow's milk allergy (CMA), or atopic dermatitis, or in the mothers with healthy infants.

Leucocyte	Milk of mothers with			<i>p</i> value
	infants with CMA	infants with atopic dermatitis	healthy infants	
Macrophages (%)	71 (13-100)	69 (52-89)	91 (63-97)	0.014
Monocytes (%)	0 (0-4)	0 (0-2)	0 (0-4)	0.78
Lymphocytes (%)	8 (0-78)	22 (5-42)	7.5 (0-24)	0.01
Neutrophils (%)	8 (0-80)	5.5 (1-19)	3.5 (0-28)	0.09
Eosinophils (%)	0 (0-72)	0 (0-1)	0 (0-1)	0.17
Total cell count (x10E6/mL)	0.32 (0.03-2.5)	0.16 (0.05-0.4)	0.4 (0.05-1.5)	0.058

Values expressed as median (range).

8.3.2 HLA-DR expression of milk leucocytes (II)

Cells were analysed by flow cytometry in the milk of 60 mothers (*Figure 1*). HLA-DR expression (geometric mean) on human milk macrophages was significantly less frequent in mothers whose infants had CMA, 58.3% (95% CI, 44.9 to 75.6), than in mothers of healthy infants, 86.9% (78.7 to 96.1) (*Figure 2*). The less frequent HLA-DR expression of breast milk macrophages did not correlate with maternal atopy. The HLA-DR expression on mothers' peripheral blood monocytes was comparable in mothers of allergic children and in those of healthy ones (100% vs. 98.5%).

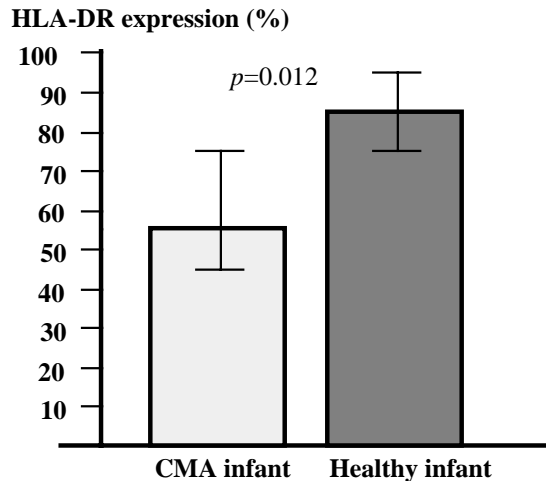


Figure 2. HLA-DR expression on human milk macrophages in mothers with infants with cow's milk allergy (CMA) and in those with healthy infants. Bars denote geometric mean with 95% CI depicted.

8.3.3 Comparison of differential cell counts and expression of human leucocyte differentiation antigens of milk cells (I)

The expression of surface markers was examined among the milk leucocytes in 14 milk samples to confirm the findings of light microscopy (*Study I: Figure 3*). The proportion of cells expressing CD14 was consistent with the proportion of macrophages verified by differential cell counts ($p=0.02$, $\rho=0.63$, Spearman rank correlation test). Similarly, the percentages of cells expressing CD3 or CD19 and those expressing CD16 correlated with proportions of lymphocytes ($p=0.04$, $\rho=0.6$) and neutrophils ($p=0.04$, $\rho=0.59$), respectively (Spearman's rank correlation test).

8.3.4 Cytokine production of milk cells (III)

Cytokine production of mothers' milk was assessed in 43 mothers (*Figure 1*). Spontaneous TNF- α production of human milk, measured from unstimulated cultures, was significantly lower in the mothers of infants with CMA than in those with healthy infants, geometric mean 4.3 pg/ml (95% CI, 2.0 to 9.5) and 22.4 pg/ml (8.2 to 60.7), respectively ($p=0.02$, Mann-Whitney *U* test) (*Figure 3*). Con A-induced TNF- α production was significantly lower in the milk of mothers whose infants were allergic than in milk of the mothers with healthy infants, geometric mean 7.9 pg/ml (95% CI, 3.9 to 15.7) and 46.6 pg/ml (20.6 to 105), respectively ($p=0.004$, Mann-Whitney *U* test) (*Figure 3*). Spontaneous IFN- γ production was very low and comparable in the milk of mothers with allergic infants to the milk of those with healthy ones. The Con A-stimulated IFN- γ production was comparable in the milk of mothers with allergic infants and in those with healthy ones, geometric mean 4.6 pg/ml (95% CI, 1.4 to 15.4) and 10.6 pg/ml (2.5 to 44.8), respectively ($p=0.14$, Mann-Whitney *U* test) (*Study III: Table 1*). Unstimulated and stimulated IL-4 production was undetectable in most samples in these two groups (data not shown).

8.3.5 Total and cow's milk specific IgA in human milk (V)

Total and cow's milk-specific IgA levels in milk were measured by radial immunodiffusion and enzyme immunoassay, respectively, in 60 mothers (*Figure 1*). Total IgA level in colostrum and in human milk was significantly lower in those mothers whose babies later developed CMA (estimated third day value 0.38 g/l) than in those whose babies remained healthy or had infant colic but not CMA (0.82 g/l, $p<0.05$) (*Study V: Figure 1*). The level of IgA dropped dramatically during the first few weeks in both groups of mothers. When total IgA in milk was measured between 6 days and 4 weeks postpartum, those infants whose mothers' IgA level was below 0.25 g/l developed CMA significantly more often (sensitivity 0.55, specificity 0.92, odds ratio 14.7 [95% CI, 3.1 to 70.2], $p<0.001$). The cow's milk-specific IgA antibody content of milk correlated with total IgA levels, $r=0.39$, $p<0.0001$. The estimated (geometric mean) value for cow's milk-specific IgA on the first day was 14.0 EU/ml (95% CI, 7.7 to 25.3) and the values decayed in 1.5 months (*Study V: Figure 2*). However, in milk from mothers of infants with CMA and from healthy controls, levels of IgA antibodies to cow's milk were comparable. Total and cow's milk-specific IgA levels did not correlate with maternal atopy.

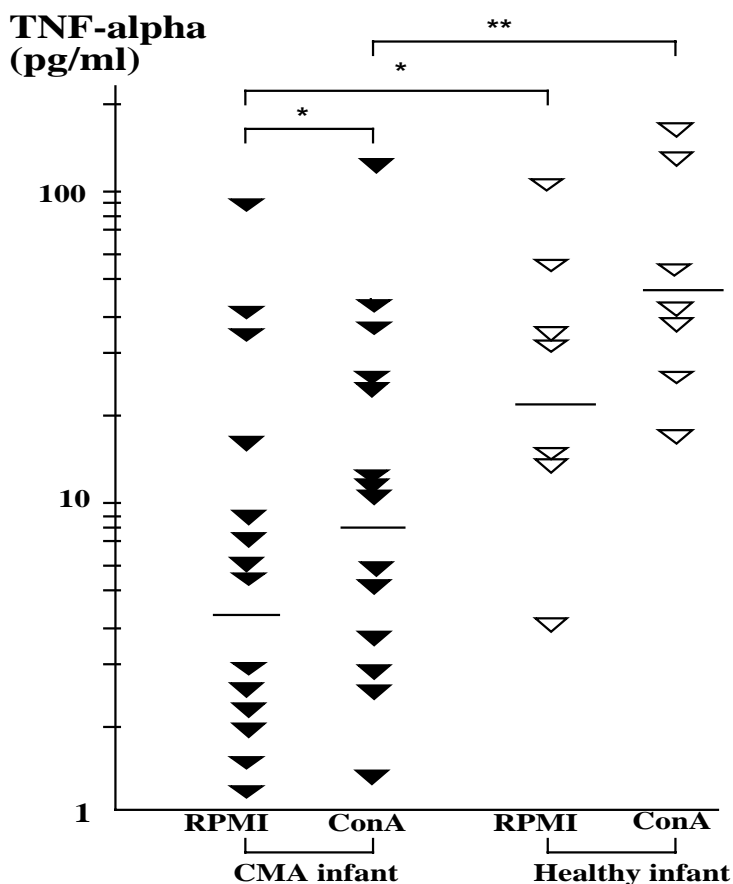


Figure 3. *In vitro* production (pg/ml) of tumour necrosis factor (TNF)-alpha of breast milk cells from mothers whose infants had cow's milk allergy (CMA) and from those with healthy infants when incubated in plain medium (RPMI) or induced by Concanavalin A (Con A). Horizontal bars denote geometric means and 95% confidence intervals. *) $p < 0.05$, **) $p < 0.005$

8.4. Lymphocyte subsets in infants and their association with cellular composition of human milk (I)

Venous blood samples were obtained from 22 infants with CMA and 7 infants without CMA. The former had a significantly smaller proportion of CD8⁺ T cells ($p=0.049$, Mann-Whitney U test) and a higher proportion of cells bearing CD19 ($p=0.036$) and CD23 (low-affinity IgE-receptor) ($p=0.0099$) than the latter ones (Study I: Table I). Further, proportions of CD4⁺ T cells and CD4/CD8 ratio were positively correlated with proportions of milk macrophages ($p=0.004$ and $p=0.002$,

respectively) (Spearman's rank correlation test), and proportions of CD4+ T cells were negatively correlated with percentage of milk neutrophils and eosinophils ($p=0.003$ and $p=0.014$, respectively) (*Study I: Table II*). In addition, total proportions of B cells and those expressing CD23 positively correlated with proportions of milk neutrophils ($p=0.002$ and $p=0.022$, respectively), and of milk eosinophils ($p=0.001$ and $p=0.044$, respectively), whereas the proportion of CD19+ B cells was negatively correlated with percentage of milk macrophages ($p=0.017$) (*Study I: Table II*).

8.5. Immune responses evoked during cow's milk challenge through mother's milk (V)

8.5.1 Transfer of BLG in human milk

BLG in human milk was assessed in 16 of 17 mothers with infants with CMA and in each of the 10 mothers with healthy infants (*Figure 1*). During cow's milk challenge via human milk, BLG levels in human milk were comparable in mothers with infants with CMA and in those with healthy infants (*Figure 4*).

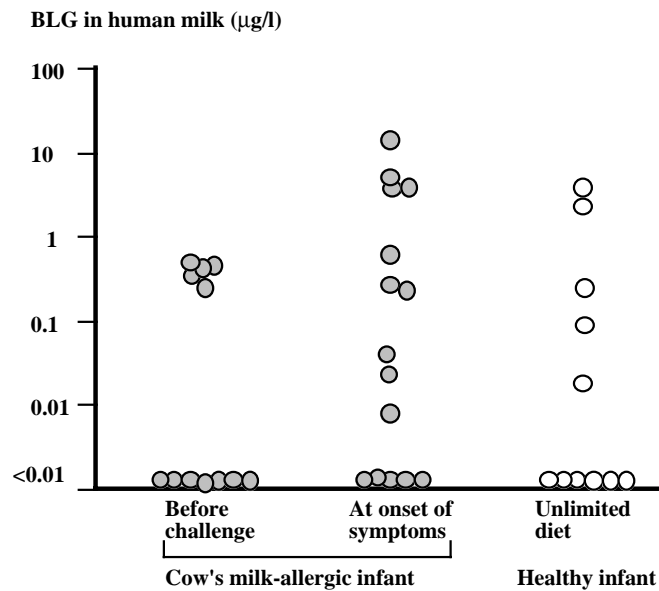


Figure 4. Secretion of beta-lactoglobulin (BLG) in human milk measured by ELISA in mothers of cow's milk-allergic infants (grey circles) before commencement of cow's milk challenge performed through mother's milk to the infant, and in mothers of healthy infants (open circles). The latter mothers were on an unlimited diet.

8.5.2 Immunoglobulin-secreting cells

Infants with CMA, showed a statistically significant increase in total number of ISCs in the IgG class from day 1, geometric mean 464 (95% CI, 242 to 887), to day 8, geometric mean 1408 (889 to 2231), $p=0.03$ (paired t -test), (Figure 5). This increase in number of ISCs was statistically significant in the IgA and IgG class in infants with CMA when compared with healthy infants, $p=0.038$ and $p=0.023$ (repeat analysis of variance) (Figure 5). In contrast, in those with a negative reaction to the challenge, the mean number of ISCs slightly increased in the IgA and IgG classes, and decreased in the IgM class during the challenge period, but no changes were statistically significant (Figure 5). Numbers of sASCs in both study groups on day 1 and day 8 of the challenge were comparable.

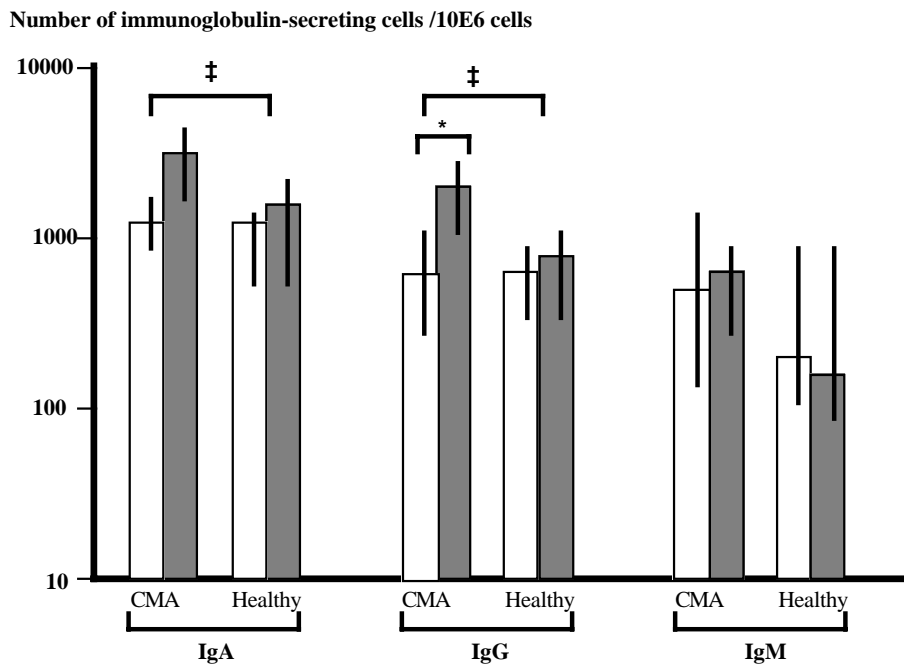


Figure 5. Total numbers of immunoglobulin-secreting cells in IgA, IgG, and IgM subclasses during cow's milk challenge through mother's milk in peripheral blood of cow's milk-allergic (CMA) or healthy infants. Prechallenge values (empty bars) and values 8 days after the commencement of the challenge (shaded bars). Bars represent the geometric mean and vertical lines the 95% confidence intervals. *) $p < 0.05$ (paired t test). ‡) $p < 0.05$ when changes in prechallenge and postchallenge values are compared between CMA and healthy infants (repeat analysis of variance).

8.5.3 Peripheral blood lymphocytes

On days 1 and day 8, the proportions of peripheral blood B cells bearing CD19, CD23, CD19 and 23 (double-positive), CD 5, or CD19 and CD5 (double-positive) and bearing CD4 or CD8 T cells were comparable in healthy infants and in those with a positive reaction to cow's milk challenge via human milk (Study IV: *Table II*).

9. DISCUSSION

9.1. Novel aspects of CMA

Cow's milk and cow's milk-based formulas are major causes of food allergy during the first years of life (Savilahti *et al.* 1992), with 1.9% of newborns in Finland experiencing allergic reactions to cow's milk during this time (Saarinen *et al.* 1999b). The symptoms of CMA commonly appear during the first months of life, within days or weeks of commencing feeding with a cow's milk-based formula, or even during breastfeeding (Jakobsson and Lindberg 1978, Gerrard 1979, Mactinger and Moss 1986, Sorva and Mäkinen-Kiljunen 1994, Isolauri *et al.* 1999). The recent findings suggest that the symptoms of CMA may already begin to appear during exclusive breastfeeding (Isolauri *et al.* 1999, I-V). This may be explained by the prospective nature of these studies and by meticulous screening for even minor symptoms of food allergy since birth, and suggests that intrauterine sensitisation to food antigens or sensitisation via mother's milk is probably more common than has previously been thought. It may also mirror a shift in modern times to an earlier age of onset of CMA. Further, the majority of infants in the present study showed food allergy against a wide variety of different foods including cereals, suggesting that severity and extent of food allergies may be increasing.

The amount of potential allergen to which the gut is exposed during early life is particularly important in directing immune responses, since large amounts of antigen are thought to induce oral tolerance while smaller amounts may prime an immune response (Hanson 1981). In animal studies, small antigen amounts ingested have been shown to induce IgE antibody responses preferentially (Jarrett 1984). Similar low-dose sensitisation may theoretically occur via the intestinal route in breast-fed babies (Björkstén 1983). In the present study, the infants who remained healthy were exclusively breast-fed for the same period of time as the CMA infants, suggesting that low-dose exposure still failed to explain why some infants remain healthy and others do not. The present study, however, confirms the finding from earlier studies and from clinical practice that even minimal quantities of food proteins to which an infant is already sensitised may provoke hypersensitivity reactions (IV). It further shows that an abnormal immune response, can also be measured in the peripheral blood in a cow's milk-allergic breast-fed infant after ingestion of mother's milk containing milk protein (IV).

IgE- and non-IgE-mediated mechanisms each account for about half the CMA in young children (Isolauri and Turjanmaa 1996). In the present study, the involvement of an IgE-mediated mechanism demonstrated as positive skin-test reactivity to cow's milk occurred in only 33% of CMA infants. Very young infants in particular showed positive SPT reactions to cow's milk strikingly seldom. At the same time, it is also known that SPT and RAST correlate poorly with a clinical challenge, even in older children. We suggest that, especially in young infants 2 to 6 months of age, SPT positivity often has not yet developed, although the child typically may have an immediate, IgE-mediated reaction against cow's milk. Many of these infants show a positive SPT reaction to cow's milk a few months later (Järvinen and Suomalainen, unpublished data).

Some studies have associated infantile colic with CMA (Jakobsson and Lindberg 1978, Iacono *et al.* 1991, Lucassen *et al.* 1998), which contradicts other views (Treem 1994). In the present study, infantile colic was strongly suggestive of cow's milk allergy in infants of 1 to 2 months of age, and these infants often responded to a cow's milk elimination diet with the disappearance of symptoms (V). This suggests that colic may represent a symptom of undetected food allergy, and should more often lead to a suspicion of food allergy than it previously has.

Genetic predisposition is an important factor in the development of allergic diseases (Kaufman and Frick 1976) and food allergy (Hide *et al.* 1991). Previous studies have focused purely on parental history of allergic diseases, with no reports on the increased risk of a newborn to develop food allergy in a family already having one food-allergic child. According to present findings, however, having a food-allergic sibling proved a good predictive factor for subsequent development of CMA in the newborn.

9.2. Human milk and development of oral tolerance

Lack of intrauterine stimulation results in the absence of any local immune response in both premature and full-term neonates. Shortly after birth, however, the gastrointestinal tract of the newborn is rapidly exposed to a wide range of microbial and food-related antigens that are potentially immunogenic. Exposure to these antigens occurs through direct oral administration or indirectly through mother's milk. Based on current data from animal studies, the main factors in developing oral tolerance are adequate local immune defense by sIgA and suppression of systemic immune responses (Strobel and Mowat 1998). In the newborn period, these factors are poorly developed.

In human milk, the most important protective factor against excessive uptake of antigens is considered to be sIgA, which provides protection at the mucosal level by preventing absorption of potentially antigenic foreign proteins from the gut. Selner *et al.* (1968) have shown that in the intestinal mucosa of a newborn, very little antibody response occurs for two weeks *post partum*, but by 28 days post partum almost 100% of infants had detectable levels of secretory antibodies. It is therefore highly likely that, in the state of relative sIgA deficiency which exists during early infancy, foreign proteins may readily gain access to the systemic circulation (Walker 1979). However, this tendency of the immature intestine of the neonate to allow excessive uptake of foreign antigens across the mucosal barrier (Kuitunen *et al.* 1994a, Kuitunen *et al.* 1994b) is offset by the passive protection of the intestinal surface which is normally afforded by ingestion of human colostrum until the time that sufficient antibody production has been switched on in the recipient (Walker 1979). Consistent with this, it has been demonstrated that the level of IgA in colostrum and human milk is high in mothers of nonallergic infants (Machtinger and Moss 1986, Savilahti *et al.* 1991, Calbi and Giacchetti 1998). This finding was confirmed in the present study (V).

In an immunologically relatively immature infant, when compared to an adult state, human milk may also compensate for insufficient production of cytokines in the network of its cellular responses. Clear evidence exists that the production of several cytokines is lower in newborns than that of T cells of adults (Garofalo *et al.* 1998), and that cytokine levels in human milk are sufficient to potentially affect the breast-fed infant (Rudloff *et al.* 1992). Numerous studies have shown that milk leucocytes are taken up into its intestinal mucosa and circulation by the offspring (Seelig and Billingham 1981, Puente *et al.* 1984, Schnorr and Pearson 1984, Seelig and Head 1987, Hughes *et al.* 1988, Jain *et al.* 1989). Transfer of tuberculin-positivity by milk T cells from a positive mother has been noted (Schlesinger and Covelli 1977), in addition to suggestions of induction of tolerance to maternal HLA on maternal milk cells taken up by the breast-fed infants' gut (Zhang *et al.* 1991). Such tolerance may allow the maternal lymphocytes to transfer immunologic information to the offspring without inducing any immune response. Consistent with this, in the breast-fed recipient, oral tolerance to food antigens present in human milk may be induced by immune factors in human milk without inducing any immune response.

In this context, an interesting study was performed in animals to investigate whether antigen administration from breast milk would have a tolerizing effect (Strobel 1996). Nursing dams were fed for 3 days with BSA at several time points after they gave birth. The kinetics of immune responses observed after administration of antigen from breast milk were comparable to those after direct administration to the offspring.

However, suckling animals received an approximate dose of antigen that was around 10^{-3} lower, which is surprising, since doses in this range have been associated with sensitisation when fed directly (Lamont *et al.* 1989). To further examine the underlying mechanism, the possibility was investigated that injection of adult spleen cells at birth, followed by an antigen, could reverse the priming effect. Neonatal mice received 10 million adult spleen cells intraperitoneally on day 1, and suckling dams were fed BSA on days 2 and 3. This was followed by an immunization 4 weeks later and measurement of immune responses *in vivo* 3 weeks after immunization. Transfer of adult spleen cells prior to antigen transfer from breast milk resulted in suppression of cell-mediated immunity during a period when neonates were otherwise not susceptible to tolerance induction (Peng *et al.* 1989). It was suggested that the inability of the neonatal rodent to be tolerized by the oral route may be related to a lack of antigen-processing capacity of the GALT, and that intestinal epithelial cells may be involved in the presentation of antigen in a suppressive way in postweaning rodents and humans (Strobel 1996).

Milk macrophages, too, have been shown to present antigens, and to induce T-cell proliferation *in vitro* (Oksenberg *et al.* 1985, Vandenplas *et al.* 1992, Mori and Hayward 1992). Expression of HLA-DR required for presentation of antigen on human milk macrophages has previously been demonstrated to be very frequent, almost 100% (Rivas *et al.* 1994). Furthermore, a significant proportion of milk macrophages has been shown to have bound casein and BLG, and a smaller percentage α -lactalbumin (Hughes *et al.* 1988). In the present study, the human milk macrophage was the predominant leucocyte in the mothers of nonallergic infants (I). In addition, their expression of HLA-DR needed for presentation of antigens, and their production of TNF- α were high in these mothers with nonallergic babies (II, III). We therefore suggest that the central role of well-functioning human milk macrophages may be the presentation of food antigens to the immunologically relatively naive infant in the gut mucosa and thereby the development in the breast-fed infant of oral tolerance.

9.3. Human milk and development of CMA

Despite the strict elimination diet of their lactating mothers, some breast-fed infants continue to show symptoms of food allergy (Isolauri *et al.* 1999; I-V). In studies I to V, these residual symptoms seemed to disappear quickly after weaning. In these infants, we cannot rule out the possibility that the symptoms were caused by hidden, contaminating amounts of the allergen in foods ingested by the mother. This may happen, although the mothers were carefully advised by the examining doctor and a

nutritionist on the performance of a meticulous cow's milk-elimination diet, and although all of the mothers recruited into the study were rather well-educated and had strong motivation to adhere to strenuous diets. Although, we detected low but measurable levels of BLG in the milk of some mothers who were on a cow's milk-elimination diet (IV), interestingly, their infants were free of symptoms despite their ingesting measurable concentrations of milk protein; on the other hand, some of the infants of mothers with virtually undetectable amounts of cow's milk transferred in their milk showed positive hypersensitivity reactions during milk challenge via mother's milk. These results support the clinical finding although the amount eliciting hypersensitivity reactions varies enormously among individuals, that the quantity of cow's milk protein in human milk is enough to evoke symptoms. However, the question of the possible ability of small amounts of cow's milk protein present in mother's milk to sensitise the breast-fed still remains unanswered. Two recent papers report a possible cross-reactivity between human milk proteins and dietary antigens (Neuteboom *et al.* 1992, Cantisani *et al.* 1997) which could explain the persistence of symptoms even with the nursing mother's being on a meticulous cow's milk elimination diet (or even sensitisation during exclusive breastfeeding) in some cases.

The composition of human milk of atopic and nonatopic mothers has been compared in only a few studies (Vassella *et al.* 1992, Businco *et al.* 1993, Duchén *et al.* 1999, Rudloff *et al.* 1999, Böttcher *et al.* 2000a; 2000b). Even fewer reports offer any correlation between the composition of mother's milk and the health status of the breast-fed infant (Machtinger and Moss 1986, Savilahti *et al.* 1991, Kalliomäki *et al.* 1999, Saarinen *et al.* 1999a). Vassella *et al.* (1992) have shown that the milk of atopic mothers contains a significantly higher number of eosinophils than the milk of nonatopic ones. Rudloff *et al.* (1999) have recently studied the presence of different cytokines in milk of atopic and nonatopic mothers. IL-10 was more often found in the milk of atopic mothers. IL-10 deactivates macrophage functions and thus exerts an immunosuppressive function (Baglioni and Dahinden 1994). Since it was found more often in atopic mothers, it might be speculated that its counteractive functions in human milk, such as activation of macrophages, may be beneficial. Milk from atopic mothers also has aberrations in its fatty acid content. Atopic mothers' milk showed lower relative levels of certain long-chain polyunsaturated fatty acids at one month of lactation, and higher ratios of n-6/n-3 fatty acids than that of the nonatopic (Businco *et al.* 1993). Further, Duchén *et al.* (1999) reported that low levels of α -linolenic acid and total n-3 long-chain polyunsaturated fatty acids in maternal milk appeared to relate to atopic sensitisation of the infants. Machtinger and Moss (1986) suggested that infants with symptom scores highly suggestive of allergic disease were receiving mother's milk with smaller quantities of IgA antibodies to whole cow's milk and casein than did infants without clinical manifestations. This was

supported by the finding of Savilahti *et al.* (1991) demonstrating a lower level of total IgA in milk of mothers whose infants showed CMA. Most recently, two papers have focused on the association between low levels of TGF- β in mother's milk and development of IgE-mediated CMA or atopic eczema in the breast-fed (Kalliomäki *et al.* 1999, Saarinen *et al.* 1999a). Although concentrations of different cytokines are low in milk and during lactation even decrease, it is known that for most *in vitro* applications, cytokines exert their biologic activity in the concentration range of 1 pg/ml to 10 ng/ml, suggesting that the levels found in human milk are sufficiently high to exert *in vivo* effects on the recipient infant (Hawkes *et al.* 1999).

The most important protective factor in human milk at the mucosal level is considered to be sIgA. Lack of IgA in early milk, on the other hand, has been suggested to lead to increased exposure of the intestinal mucosa of the breast-fed to potential allergens and enhanced risk for development of food allergies, as mentioned above (Machtlinger and Moss 1986, Savilahti *et al.* 1991), and atopic eczema (Calbi and Giacchetti 1998). This was confirmed in the present study (V). Further, we showed that total breast-milk antibody levels were highest in colostrum, fell rapidly during a 1- to 2-week period, and remained at a stable level of 0.2 to 0.3 g/l for several months both in the mothers of healthy infants and of those with a cow's milk-allergic baby. A higher concentration of sIgA in human milk in the first couple of weeks of lactation may explain why the majority of infants with CMA showed no symptoms suggestive of CMA until the age of 2 to 4 weeks. High levels of IgA in their mothers' milk may have protected the infants from the absorption of large amounts of potential food antigens in their first days of life when the gut is most permeable.

The present study suggests that the dietary antigens or the lack of IgA in human milk may not be the only factors in milk contributing to sensitisation of the newborn to foods (I-III). We demonstrated that the milk of some of the atopic mothers having infants with CMA had a high proportion of eosinophils (I). A newborn infant consumes 400 to 500 ml of breast milk per day, amounting to the overwhelming total number of 6×10^6 to 7×10^7 eosinophils ingested each day. Although number of leucocytes may slightly decrease during lactation, the total volume of milk ingested increases to up to 800 ml per day by 3 months of age. In the gut of the breast-fed infant, eosinophils will finally break by cytolysis, and highly cytotoxic mediators will be released, including ECP, even without any activation process that would lead to degranulation. In asthma, these eosinophil-derived proteins have been shown to cause damage to airway epithelium (Venge and Peterson 1989). Similarly, they may contribute to an immunoinflammatory process in the gut of the infant. Subsequently, the permeability of the gut will be enhanced, and the antigenic load thereby

increased. This being the case during every feeding, risk of any offspring prone to develop food allergy is potentially increased.

Neutrophils, too, were found in significantly larger numbers in mothers of CMA infants (I). Human milk polymorphonuclear cells are functionally exudate cells with locomotive, adherence, microbicidal, and stimulated respiratory burst capabilities less than those of their counterparts in blood (Ho and Lawton 1978, Buescher and McIlheran 1993). They therefore do not seem to provide to the breast-fed infant significant anti-infective protection (Buescher and McIlheran 1993), despite the lactoferrin they release (Kiljstra 1991). The explanation for the presence of these cells in colostrum is obscure. In general, neutrophils are cytotoxic and potentially tissue-injuring cells participating in the destructive processes and symptoms seen in a variety of inflammatory diseases including asthma. Whether they could play a role in inflammation in gut mucosa and development of CMA, when present in big numbers in milk and in the gut of the breast-fed, remains to be seen.

A popular theory at the moment is that of the “hygiene hypothesis” as applied to hay fever and asthma (Rook and Stanford 1998). This hypothesis proposes that a reduced number of infections during infancy predisposes to allergic responses. Among environmental factors, early infections and intestinal microflora have been suggested as enhancing the skewing towards Th1-type responses and protecting against development of allergy, which has a high level Th2 response (Björkstén *et al.* 1999). The Th2 paradigm does not, however, explain late phase CMA which appears to be more of a delayed-type hypersensitivity with increased IFN- γ production by systemic blood lymphocytes after exposure to cow’s milk protein. Holt and co-workers have demonstrated that stimulated peripheral blood lymphocytes of atopic individuals have a Th2 “allergic” cytokine profile which becomes reinforced during childhood (high IL-4 and low IFN- γ at 6 months of age) whereas healthy children lose the fetal Th2 cytokine profile during the first 1 to 2 years of life (low IFN- γ at birth but higher levels at 6 months) (Yabuhara *et al.* 1997, Prescott *et al.* 1999). The mechanism of down-regulation of Th2 responses and how this process fails in allergic individuals remains unexplained. The possible effect of human milk lymphocytes on polarisation of newborn T cells has not yet been considered. Evidence exists that at least tuberculin-immunity can be transferred from the mother to the breast-fed infant (Schlesinger and Covelli 1977). We found lymphocytes in rather high numbers, especially in the milk of mothers with infants suffering from atopic dermatitis (I). It is already known that the majority of lymphocytes in human milk are T cells (Crago *et al.* 1979), half of which are of the helper subtype (Wirt *et al.* 1992); their polarisation is, however, unknown. Such a numerous population of T cells may potentially have an effect on deviation of immune responses of the infant, since each T-cell subset

produces specific cytokines which act in autocrine fashion, promoting further differentiation and maturation in that subset. One might suggest that abnormal responses such as those of the Th2 type may be transferred to the infant. If this happens, the mother's leucocytes may, as a consequence, induce IgE-production in the infant.

Macrophages were found less frequently in milk of mothers of CMA infants than in that of those with nonallergic babies (I). Furthermore, their expression of HLA-DR required for presentation of antigen was less frequent in these mothers of allergic babies (II). This finding may be relevant to the development of allergy to cow's milk protein, assuming that milk macrophages are capable of presenting foreign food antigens to the suckling. The smaller number and less frequent expression of HLA-DR of milk macrophages that was manifested in the mothers of infants with CMA may result in insufficient presentation of antigens by these macrophages, which may lead to a disturbance in the development of oral tolerance (II). This theory is further supported by our finding that defective production of TNF- α , mainly produced by macrophages in human milk, correlates with development of CMA in the breast-fed infant (III). TNF- α may modify the antigen-presenting functions of APCs in the gut. It has been suggested as likely, at least in rodent models, that mucosal dendritic cells as well as gastrointestinal epithelial cells play important roles in the antigen-presentation process (Strobel and Mowat 1998). In an experimental model, TNF- α switches the monocytic APCs to mature ones capable of effectively presenting the processed antigen to T cells. We therefore hypothesize that lack of TNF- α in breast milk may downregulate the differentiation of APCs in the infant, and this may play a role in the development of CMA. Therefore, the small number of macrophages observed, together with their deficient function and deficient production of cytokines, may cause an imbalance in the communication process of the cellular network in the infant and thus may play a role in the delay of development of oral tolerance.

The explanation for the abnormal phenomena in the milk of some mothers reported in the present study remains to be seen. Interestingly, the several abnormalities or deficiencies in human milk tended to coexist in the same mothers. The regulation of milk antibody and cytokine levels, as well as leucocyte traffic, is largely unknown. Levels of the milk IgA antibodies to food antigens have not been shown to be influenced by the antigenic load in the mother's diet (Mascart-Lemone *et al.* 1991), suggesting that unknown factors, other than the antigenic load, must be of greater importance in the regulation of milk antibody levels. Since the transfer of IgA-producing B cells to the mammary gland is dependent on lactogenic hormones, the varying levels of milk IgA might reflect variations in hormonal levels. Deficiency of

IgA at one mucosal site, e.g., the mammary gland, may perhaps reflect a more widespread condition of the mother, locally affecting a number of other mucosal sites, such as her gut. Although we do not quite know how leucocyte migration to the mammary gland is regulated, it has been shown that this migratory process is due to a selective homing process, at least for lymphocytes (Slade and Schwartz 1987). Chemoattractant factors have been suggested to be responsible for the presence of eosinophils and neutrophils in human milk (Michie *et al.* 1998, Böttcher *et al.* 2000b). The presence of high numbers of neutrophils and eosinophils in the milk from some of our mothers remains unexplained. This finding seems even more puzzling since some other mothers had large proportions of eosinophils in their peripheral blood, but virtually none in their milk. This phenomenon may reflect a defect in the regulation of active migration or reflect a passive shedding of peripheral blood leucocytes into the milk. Böttcher *et al.* (2000b) found RANTES and IL-8 in higher concentrations in milk of allergic, compared with nonallergic, mothers. The presence of higher concentrations of these or other chemoattractant factors in the milk of our mothers may explain the high numbers of polymorphonuclear cells in some samples. The presence of chemoattractant factors and of leucocyte migration to the mammary gland may be genetically determined. Such a mother would thereby transfer her allergic heredity not only in her genes, but also through her milk to her infant. This would be one possible explanation for preferential maternal inheritance of atopy.

The present study is, to the best of our knowledge, the first to report that several distinct aberrations in the cellular and cytokine composition of human milk relate to the development of CMA in the breast-fed. Further, these abnormalities in mothers' milk were associated with an imbalance in the CD4⁺ and CD8⁺ T-cell ratio and a disturbed B-cell function, which were previously (Järvinen *et al.* 1998) and in the present study both found to be related to the incidence of CMA in the breast-fed infant. We also confirmed the finding of Savilahti *et al.* (1991) that proposed a correlation between low level of maternal milk IgA and CMA in the infant in a much smaller number of cow's milk-allergic infants than our population. Human milk normally provides the breast-fed with several potentially protective, immunosuppressive, or compensatory factors that provide a mechanism for evolving immune tolerance and protection against immunologically mediated diseases. Any deficiencies in the levels or the function of those suppressive factors in mother's milk may interfere with the development of oral tolerance in an offspring, and thereby contribute to the development of food allergies. In some cases, the composition of mother's milk may even be disadvantageous for the infant. These results provide fresh insight into the etiopathogenesis of CMA. Measuring these variables in mother's milk offers a modern tool for assessing those newborns at high risk for

developing food allergies, and later becoming sensitised to airborne allergens and developing asthma.

CMA is not attributable to a single cause. The development of allergies is affected by numerous factors, including parental smoking, pets, and house dust mites. In addition, the immunological status of the infant contributes to the sensitisation to foods. However, other factors thus far unknown, intrinsic as well as extrinsic, may have an important bearing on sensitisation. Our results suggest that the risk for allergies tends to be increased by human milk with an abnormal composition. Hence, in the case of an infant with food allergy whose symptoms appear during exclusive breastfeeding and remain despite of the wide elimination diet of the lactating mother, it should be borne in mind that the cause may lie in the composition of the mother's milk, especially in its cellular, cytokine, and/or immunoglobulin components. In breast-fed infants with extremely severe symptoms of CMA, the composition of the mother's milk should be studied, with early weaning in combination with commencement of a protein-hydrolysate or amino acid-based formula recommended to prevent further sensitisation of the infant to the wide variety of food proteins ingested by the lactating mother. In our study population, the majority of the infants with CMA had been prospectively followed up from birth and carefully documented to have been exclusively breast-fed at the time of the appearance of the symptoms of CMA, without even being fed any cow's milk-based formula. We therefore conclude that with high-risk babies, even exclusive breastfeeding does not seem to protect against allergic sensitisation. Although human milk is the best source of nutrition for healthy infants, the present study questions the general recommendation of prolonged breast-feeding in the prevention of development of food allergies in allergic families.

10. TIIVISTELMÄ

Pitkää rintaruokintaa suositellaan yleisesti mm. sen allergioilta suojaavan vaikutuksen vuoksi. Näyttö sen suojaavasta tehosta ei kuitenkaan ole kiistatonta. Kliininen kokemus sekä viimeaikaiset tutkimukset ovat osoittaneet, että lehmänmaitoallergian oireet alkavat usein jo pelkän rintaruokinnan aikana. Lisäksi osalla pelkkää äidinmaitoa saavista lapsista esiintyy vaikeita allergiaoireita äidin tarkasta välttämisruokavaliosta huolimatta. Nämä seikat viittaavat siihen, että äidinmaidon mukana imeväiselle kulkeutuvat ruokaperäiset proteiinit voivat toimia herkistäjänä, tai äidinmaito, sen komponentit tai niiden puutos voivat olla myötävaikuttavana tekijänä allergioiden synnyssä.

Tässä väitöskirjatyössä selvitettiin rintamaidossa esiintyvien immunologisten tekijöiden vaikutusta rintaruokituksen imeväisen riskiin sairastua lehmänmaitoallergiaan. Seurasimme 100 äiti-lapsiparia vuoden ikään asti. Näistä lapsista osa valittiin atooppisista, korkean allergiariskin perheistä ja loput ei-atooppisista perheistä. Seurannan aikana osalle kehittyi lehmänmaitoallergiaan viittaavia oireita, ja diagnoosi varmistettiin välttämis-altistuskokeella. Selvitimme rintamaidon solu- ja välittäjäainekoostumusta suhteessa imeväisen terveydentilaan tai allergian puhkeamiseen. Kliinisten oireiden lisäksi tutkimme imeväisen puolustusjärjestelmän vasteita äidinmaidon kautta tehtävän lehmänmaitoaltistuksen aikana.

Äidinmaidossa esiintyvien valkosolujen jakaumaa tutkittiin valkosolujen erittelylaskennalla valomikroskooppisesti, ja osassa näytteistä tulos varmistettiin virtausytometrialla solutyypeille spesifisiä pinta-antigeeneja kohtaan olemassaolevia vasta-aineita käyttäen. Maitoallergisten lasten äitien maidossa todettiin merkitsevästi vähemmän makrofaageja. Toisaalta lapsille, joiden äitien maidossa todettiin enemmän neutrofiilejä, kehittyi useammin maitoallergia. Huomattavia määriä eosinofiilejä havaittiin vain lehmänmaitoallergisten lasten äitien maidossa. Nämä solukoostumuksessa havaitut poikkeavuudet korreloituivat imeväisen poikkeaviin puolustusjärjestelmän vasteisiin, matalaan CD8+ T-solujen määrään ja korkeaan CD19+ ja CD23+ B-solujen määrään, joita todettiin maitoallergisilla lapsilla. Suuri lymfosyyttien osuus rintamaidossa liittyi atooppisen ihottuman esiintymiseen imeväisellä ilman maitoallergiaa.

Rintamaidon valkosolujen tehtävä on tuntematon. Eosinofiilien suuri määrä rintamaidossa saattaa lisätä imeväisen riskiä allergisoitua useille ravinnon

antigeeneille, sillä niiden mekaaninen hajoaminen imeväisen suolistossa voi sellaisenaan vapauttaa suuria määriä solutoksia välittäjäaineita, kuten eosinofiilien kationista proteiinia ECP:ä. Tämä saattaa aiheuttaa kudostuhoa limakalvoilla, kuten on havaittu astmassa, ja lisätä suolen läpäisevyyttä. Myös neutrofiilit ovat mahdollisesti kudostuhoa aiheuttavia soluja, joita havaitaan tulehduksellisissa sairauksissa. Lymfosyyttialuokkien jakaumaa rintamaidossa ei määritetty, mutta mikäli havaitut korkeat osuudet olisivat Th2-tyyppisiä eli allergiasuuntaan johdattavia, ne voisivat altistaa lasta atooppisten sairauksien puhkeamiselle.

Äidinmaidon makrofaagin toiminnan tutkimiseksi määritimme antigeenien esittelyssä tarvittavan solujen kudossopivuusantigeenin, HLA-DR:n, esiintymistä äidinmaidon makrofaageissa virtausytometrialla. HLA-DR:n on aiemmin todettu ekspressoituvan hyvin yleisesti rintamaidon soluissa, joiden on lisäksi todettu kykenevän esittelemään antigeeneja. Tässä tutkimuksessa HLA-DR-ekspression todettiin olevan merkitsevästi vähäisempää äideillä, joilla oli maitoallerginen lapsi. Tulosten pohjalta voidaan ajatella, että äidinmaidon makrofaagien tehtävänä saattaisi olla normaalitilanteessa ravinnon antigeenien esittely imeväisen kokemattomalle puolustusjärjestelmälle. Siten vähäinen HLA-DR-antigeenin esiintyminen viittaa siihen, että rintamaidon makrofaagien kyky esitellä vieraita antigeeneja T-soluille olisi alentunut. Tämä saattaa johtaa häiriöön oraalisen toleranssin kehittämisessä imeväisellä.

Äidinmaidossa esiintyvien eräiden välittäjäaineiden pitoisuuksia tutkittiin soluviljelmässä ELISA-menetelmällä. Tuumorinekroositekijä (TNF)- α :n tuotanto oli merkitsevästi vähäisempää äideillä, joiden lapsilla todettiin maitoallergia. Tämä tukee havaintoa matalasta makrofaagimäärästä ja vähäisestä HLA-DR:n esiintymisestä samojen äitien rintamaidossa. Tiedetään, että TNF- α on antigeenia esittelevien dendriittisolujen kypsymistä stimuloiva tekijä, jolla täten saattaisi olla tärkeä merkitys oraalisen toleranssin kehittämisessä imeväisen suolen limakalvolla. Täten TNF- α :n puutos rintamaidossa saattaisi johtaa dendriittisolujen kypsymisen estymiseen, mikä saattaisi altistaa imeväistä maitoallergian kehittämiselle.

Rintamaidon immunoglobuliini (IgA)- vasta-aineiden määrää mitattiin RIA-menetelmällä ja lehmänmaitospesifisiä IgA-vasta-aineita EIA-menetelmällä. Havaitimme, että IgA-pitoisuus oli merkitsevästi matalampi varhaisessa rintamaitonäytteessä äideillä, joiden lapselle kehittyi myöhemmin maitoallergia, mutta lehmänmaitospesifisen IgA:n tasoissa ei ollut eroa terveiden ja maitoallergisten lasten äitien maidoissa. Koska IgA:n ajatellaan estävän voimakasta antigeenien imeytymistä limakalvoilla, voidaan rintamaidossa olevan IgA-puutoksen ajatella lisäävän imeväisen riskiä altistua ruoka-antigeeneille ja täten ruoka-aineallergioiden kehittämiselle.

Äidinmaidon kautta tehtävän lehmänmaitoaltistuksen aikana tarkasteltiin imeväisen kliinisiä oireita ja perifeerisen veren puolustusvasteita sekä virtausytometrisesti että ELISPOT-menetelmällä. Viikon kestäneen sekä äidin ja lapsen maidottoman ruokavalion jälkeen lapsi altistettiin lehmänmaidolle siten, että äidit nauttivat suurenväisiä määriä maitotuotteita ja imettivät lasta. Tutkimme samanaikaisesti yhden maitoallergian kannalta tärkeän lehmänmaitoproteiinin, β -laktoglobuliinin (BLG), kulkeutumista ravinnosta rintamaitoon ELISA-menetelmällä. Osoitimme, että varhain puhjenneessa lehmänmaitoallergiassa oireiston voi usein aiheuttaa rintamaitoon kulkeutuvat vähäiset lehmänmaitoallergeenimäärät. Kliinisen reaktion yhteydessä havaitsimme imeväisten veren immunoglobuliineja A- ja G-tuottavien lymfosyyttien määrässä merkitsevän nousun. Tämän vuoksi useimmissa tapauksissa on perusteltua suositella ruoka-aineallergisen lapsen imettävälle äidille tarkkaa välttämisruokavaliota.

Äidinmaito on kiistatta terveen lapsen parasta ravintoa. Tässä tutkimuksessa esitetään kuitenkin ensimmäistä kertaa, että useat rintamaidossa esiintyvät poikkeavuudet ja puutteet solu-, välittäjäaine- ja immunoglobuliinikoostumuksessa saattavat olla yhteydessä rintaruokitun lapsen lehmänmaitoallergian puhkeamiseen. Edellämainittujen tekijöiden mittaaminen äidinmaidossa saattaa olla hyödyllistä etsittäessä korkean allergiariskin vastasyntyneitä ennaltaehkäisevien toimenpiteiden aloittamiseksi. Tämä uusi näkökulma maitoallergian etiopatogeneesiin antaa myös aiheen pohtia, kuinka perusteltu on yleinen suositus pitkistä rintaruokinnasta erityisesti allergiaperheissä.

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