RISK FACTORS AND CHARACTERISTICS OF COW'S MILK ALLERGY

Kristiina M. Saarinen

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Hospital for Children and Adolescents
University of Helsinki
Helsinki, Finland

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by

Kristiina M. Saarinen

ACADEMIC DISSERTATION

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Supervised by:

Erkki Savilahti, MD, Professor
Hospital for Children and Adolescents
University of Helsinki
Helsinki, Finland

Reviewed by:

Timo Reunala, MD, Professor
Department of Dermatology
University of Tampere
Tampere, Finland

Timo Vanto, MD, Docent
Department of Pediatrics
University of Turku
Turku, Finland

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1. SUMMARY

Early feeding with cow's milk (CM) is incriminated as a major environmental factor in the development of cow's milk allergy (CMA). Breast milk contains many immune factors which compensate for the undeveloped defence mechanisms of the gut of the newborn infant. Little is known about the factors affecting the development of the different reaction types in CMA. The diagnosis of CMA is based on a laborious CM elimination-challenge test. The present study was designed to study the effect of early exposure to different supplementary feeds on the subsequent incidence of CMA. The effects of exclusive breast-feeding and the immune factors in colostrum were also evaluated. The course of development of the different immunological and clinical features of CMA with respect to different infant-feeding patterns was analysed. The diagnostic values of 4 immunological tests in CMA were assessed.

We prospectively followed 6209 full-term, healthy newborn infants from birth up to 12 months of age for the development of CMA. The CM elimination-challenge test was positive in 118 (1.9%) infants. The cumulative incidence of CMA was 2.4% in the infants fed CM at the maternity hospital, 1.5% in those fed extensively hydrolysed whey formula and 1.7% in those fed pasteurised human milk. Of the infants exclusively breast-fed at hospital, 2.1% developed CMA. Among the infants who required supplementary feedings at hospital, both exposure to CM while in the hospital (odds ratio [OR] 1.54, 95% confidence interval [CI] 1.04-2.30) and obvious parental atopy (OR 2.32, 95% CI 1.53-3.52) increased the risk of CMA. The contents of total IgA, IgM and CM-specific IgA, TGF-β1, IFN-γ and IL-6 were similar in maternal colostrum of infants with CMA and in control mothers. In the infants with CMA, the CM-specific IgE response was measured as specific antibodies and by a skin-prick test. Risk factors (OR, 95% CI) for the IgE-positivity were long breast-feeding (3.9, 1.6-9.8), exposure to CM while in the hospital (3.5, 1.2-10.1) and breast-feeding during the first 8 weeks of life either exclusively (5.1, 1.6-16.4) or combined with infrequent exposure to small amounts of CM (5.7, 1.5-21.6). The mean (95% CI) content of TGF-β1 in maternal colostrum was lower in the IgE-positive (589 pg/ml, 413-840) than in the IgE-negative group (1162 pg/ml, 881-1531). The allergic infants' IgA and IgG antibodies to CM proteins correlated positively and their cellular responses to CM negatively with the level of TGF-β1 in colostrum. The parallel use of skin-prick and patch tests and serum ECP and CM-specific IgE detected 76% of infants with CMA with a specificity of 0.67. Of the infants with an immediate type reaction at challenge (<2 h), the same combination detected 94% with a specificity of 0.55.

In conclusion, early exposure to CM increases the risk of CMA, but exclusive breast-feeding does not eliminate the risk. Prolonged breast-feeding exclusively or combined with infrequent exposure to small amounts of CM during the first 8 weeks of life induces the development of the CM-specific IgE response in infants prone to developing CMA. Furthermore, TGF-β1 in colostrum may inhibit IgE- and cell-mediated reactions and promote IgA-IgG antibody production in these infants. The diagnosis of CMA requires confirmation by a challenge test.

2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals:

I Saarinen KM, Juntunen-Backman K, Järvenpää A-L, Kuitunen P, Lope L, Renlund M, Siivola M, Savilahti E. Supplementary feeding in maternity hospitals and the risk of cow's milk allergy: A prospective study of 6209 infants. J Allergy Clin Immunol 104:457-461, 1999.

II Saarinen KM, Vaarala O, Klemetti P, Savilahti E. Transforming growth factor-β1 in mothers' colostrum and immune responses to cows' milk proteins in infants with cows' milk allergy. J Allergy Clin Immunol 104:1093-1098, 1999.

III Saarinen KM, Savilahti E. Infant feeding patterns affect the subsequent immunological features in cow's milk allergy. Clin Exp Allergy 30:400-406, 2000.

IV Saarinen KM, Suomalainen H, Savilahti E. Diagnostic value of skin-prick and patch tests and serum eosinophil cationic protein and cow's milk-specific IgE in infants with cow's milk allergy. Submitted.

Some previously unpublished data are also presented.

3. ABBREVIATIONS

Ab	antibody
ANOVA	analysis of variance
BLG	β-lactoglobulin
BSA	bovine serum albumin
CI	confidence interval
CD	cluster of differentiation
CM	cow's milk
CMA	cow's milk allergy
ECP	eosinophil cationic protein
ELISA	enzyme linked immunosorbent assay
ESPGAN	the European Society for Paediatric Gastroenterology and Nutrition
FIM	Finnish mark
h	hour
HLA	human leucocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
SI	stimulation index
SPT	skin-prick test
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor

4. INTRODUCTION

Cow's milk allergy (CMA) is usually the first allergic disease in the "atopic march", because cow's milk (CM) proteins are the first foreign antigens encountered by an infant (Zeiger 2000). The competence of a newborn infant's immature immune system to develop oral tolerance to ingested antigens may be defective, especially in those who are genetically prone to atopy (Björkstén 1999). It has been claimed that the principal environmental risk factor for CMA is feeding with CM at the maternity hospital, "the dangerous" or "hidden bottle". In a Danish cohort of 1749 newborn infants (Høst et al. 1988), 88% had been given CM at the maternity ward. Of these infants, 2.2% developed CMA, all of them had been exposed to CM at the hospital. However, CMA may also develop in exclusively breast-fed infants (Isolauri et al. 1999). The minute amounts of CM present in breast milk may trigger the sensitisation (Duchén and Björkstén 1991), or differences in the composition of breast milk may affect the risk of developing CMA (Savilahti et al. 1991, Björkstén 1999).

The clinical and immunological picture of CMA is diverse: symptoms in the skin and in the gastrointestinal and respiratory tracts may occur separately or simultaneously, the reaction time between exposure to the antigen and development of symptoms varies from minutes to days, and the adverse symptoms probably are due to more than one immunological mechanism (Hill et al. 1986). These heterogeneous features constitute a great challenge for the diagnostic procedure, which is still a clinical elimination-challenge test.

After breast-feeding, the nutrition of infants with CMA is provided by soy or hydrolysed or amino acid-based formulas usually given until the age of 2 years (Isolauri et al. 1995, Tiainen et al. 1995). The cost of these formulas is high: from about 13.000 FIM to 58.000 FIM per infant (daily use for 2 years), depending on the product. In addition, although most infants recover from CMA, the risk of other atopic diseases, such as asthma, is increased in these children, especially in those with IgE-mediated CMA (Hill et al. 1994, Zeiger 2000).

Since the genetic inheritance cannot be modulated, at least not at present, preventive measures must concentrate on environmental factors. Several prospective studies on high-risk infants have evaluated the extent to which avoidance of dietary allergens is reflected by subsequent prevention of atopy (Businco et al. 1987, Chandra et al. 1989b, Halken et al. 1992, Zeiger and Heller 1995, Hide 1997). However, no large population-based, prospective studies had been published by 1994, when we initiated the present study of over 6200 infants. We aimed to study, whether the incidence of CMA could be reduced by feeding newborn infants with extensively hydrolysed formula or banked human milk as compared to CM formula. We also studied the effect of breast-feeding on

the incidence of CMA and whether differences in the quality of breast milk would affect the risk. The clinical and immune responses of infants with CMA in relation to the infant feeding practices were evaluated. Furthermore, we examined the diagnostic value of several immunological tests in CMA.

5. REVIEW OF THE LITERATURE

5.1 Definition and pathogenesis of CMA

CMA is an adverse clinical reaction to ingested CM proteins based on an immunologically mediated adverse reaction to the provoking proteins (The ESPGAN Working Group for the Diagnostic Criteria for Food Allergy 1992). Although exposure to CM proteins always provokes an immune response in an infant (Tainio et al. 1988, Kaila et al. 1994), the immunological reaction in CMA is abnormally strong or qualitatively altered (Savilahti et al. 1989). Only the IgE-mediated reaction, usually associated with an immediate-type reaction, is well characterised; the immunological mechanisms associated with a delayed-type reaction are still poorly understood (Sampson 1997). Antigen-antibody complex formation and a cell-mediated immune response are suggested to be responsible for other than IgE-mediated reactions but the evidence is lacking (The ESPGAN Working Group for the Diagnostic Criteria for Food Allergy 1992). More than one immunological mechanism may operate at the same time (Saalman et al. 1991). Reaginic antibodies to CM protein fractions, such as casein, β-lactoglobulin (BLG), α-lactalbumin, bovine serum albumin (BSA) and lactoferrin can be detected in patients with CMA (Wal et al. 1995). These proteins are capable of provoking the symptoms (Savilahti and Kuitunen 1992). The fraction that triggers the symptoms may vary between individuals; most patients react to more than one fraction and no relationship has been found between a given symptom and a given protein fraction (Goldman et al. 1963, Kuitunen et al. 1975).

5.2 Incidence and prognosis

CMA usually develops during the first year of life (Bock 1987). The incidence in population-based studies has varied from 1.9% to 7.5% (Gerrard et al. 1973, Jakobsson and Lindberg 1979, Bock 1987, Høst et al. 1988, Schrander et al. 1993). The prognosis is good: recovery rates of 45-56% at 1 year, 65-77% at 2 years, and 87% at 3 years have been reported (Jakobsson and Lindberg 1979, Høst and Halken 1990). Infants with IgE-mediated CMA recover more slowly than those with non-IgE-mediated CMA (Hill et al. 1993, Schrander et al. 1993).

5.3 Aetiological Factors

The aetiology of CMA is only partially understood. Atopic heredity is an important risk factor for CMA (Høst 1994). The time of introduction of CM affects the ensuing immune response (Koning et al. 1996). The gastrointestinal tract of newborn infants is deficient in both non-specific and specific defence factors, and its digestive capacity is not fully developed (Udall 1990). The permeability of the gut to macromolecules is greatest during first 2 months of life (Kuitunen et al. 1994), the immune system of the neonate is immature (Hanson et al. 1996) and specific T cell responses are mostly of T helper (Th) 2-type (Prescott et al. 1999). In animal experiments, early exposure to CM proteins retards gut closure (Arvola et al. 1993). In infants with CMA, the reactive T cells were found to express a membrane protein ($\alpha 4\beta 7$) which directs the migration of the memory T cells to the gut mucosa independently of the symptoms, strengthening the hypothesis that the site of sensitisation is the gut (Eigenmann et al. 1999).

5.3.1 Family history of atopy

A positive family history of atopy — usually defined as the occurrence of asthma, atopic dermatitis or allergic rhinitis in 1 or more first-degree relatives — is a known risk factor for atopic diseases (Casolaro et al. 1996). In population-based studies on infants with CMA, the prevalence of a positive family history of atopy has varied between 41% and 70% as compared with that of 29% to 35% in non-allergic controls (Gerrard et al. 1973, Jakobsson and Lindberg 1979, Ventura and Greco 1988, Høst and Halken 1990, Schrander et al. 1993). Correlations have been found between an immediate-type reaction to CM (Dannaeus and Johansson 1979) and extra-intestinal symptoms of CMA (Ventura and Greco 1988) and atopic heredity.

Several, as yet unknown genes are probably responsible for the inheritance of atopic diseases (Casolaro et al. 1996). There have been only a few studies on genetic predisposition and CMA. Recently, a correlation between the HLA-DQ7 antigen and CMA was reported (Camponeschi et al. 1997). In an earlier study, no association was found to exist between HLA-A, -B, -C or -DR locus antigens and intestinal CMA (Verkasalo et al. 1983).

5.3.2 Early formula feeding

Early exposure to CM has been incriminated as an important factor in the development of CMA. In the Danish study, of the 39 infants developing CMA, 9 were exclusively breast-fed at the time of diagnosis (Høst et al. 1988). However,

retrospective examination of the medical files revealed that all these 9 infants had been exposed to CM formula at the maternity hospital during the first 3 days of life, whereas none of the 210 infants exclusively breast-fed at hospital developed CMA (Høst et al. 1988). Similarly, feeding of pre-term infants with a positive atopic heredity with CM-based formula increased the cumulative incidence of allergic symptoms, notably eczema, at 18 months of age as compared with feeding of human milk (Lucas et al. 1990).

In contrast, in a Swedish study of 207 term infants, feeding of CM formula before the commencement of breast-feeding reduced the appearance of allergic symptoms during the first 18 months as compared with exclusive breast-feeding (Lindfors and Enocksson 1988). The infants were reinvestigated at a mean age of 5 years: the formula-fed infants with a double family history of atopy still had a lower frequency of mild allergic symptoms than those exclusively breast-fed with similar atopic heredity (Lindfors et al. 1992). Similarly, in a Finnish study on 69 pre-term infants given either CM formula or human milk from birth, feeding of CM reduced the cumulative incidence of allergic symptoms at 11 year of age (Savilahti et al. 1993b).

No difference was found in the cumulative incidence of atopic diseases between 736 term infants given either CM formula or human milk at the maternity hospital (Gustafsson et al. 1992) or between 1533 infants exposed to either CM or placebo during the first 3 days of life (de Jong et al. 1998). Yet 2 studies have failed to show any differences in atopic manifestations between infants given either CM formula or other supplements at the maternity hospital (Schmitz et al. 1992, Juvonen et al. 1996).

Extensively hydrolysed formulas, processed from CM whey or casein by heat treatment and/or enzymatic hydrolysis, are used for the management of CMA (Businco et al. 1993). The concentration of BLG in these formulas is at lowest 1/4 800 000 that of CM (Mäkinen-Kiljunen and Sorva 1993). In many studies on infants at high risk of atopic disease, the incidence of food allergy was reduced by feeding the infants on extensively hydrolysed formula for several months after birth (Chandra et al. 1989a, Halken et al. 1992, Zeiger and Heller 1995, Oldaeus et al. 1997), but comprehensive, population-based studies are lacking. In a small Swedish study on 129 unselected infants, of the 37 infants exclusively fed with casein hydrolysate formula during the first 3 days of life, 2 developed CMA, whereas none of the 53 and 39 infants given human milk and CM formula developed CMA (Juvonen et al. 1996). Moreover, adverse clinical and immunological reactions caused by these formulas have been detected in infants with CMA (Saylor and Bahna 1991, Hill et al. 1995, van Beresteijn et al. 1995, de Boissieu et al. 1997a), suggesting that some residual allergenicity is still present in the formulas. As a substitute for extensively hydrolysed formulas, amino acid-based formulas have been introduced for the management of CMA (Isolauri et al. 1995, Vanderhoof et al. 1997).

5.3.3 Breast-feeding

Breast-feeding may reduce the incidence of food allergy (Saarinen et al. 1979, Høst et al. 1988) as well as the risk of other atopic diseases (Chandra et al. 1985, Saarinen and Kajosaari 1995, Tariq et al. 1998). The protective effect of breast-feeding, however, is controversial (Van Asperen et al. 1984, Cogswell et al. 1987, Savilahti et al. 1987). Several studies have reported symptoms of CMA in exclusively breast-fed infants (Jakobsson and Lindberg 1978, de Boissieu et al. 1997b, Isolauri et al. 1999, Järvinen et al. 1999b). Breast milk contains very small amounts of food antigens ingested by the mother (Axelsson et al. 1986, Sorva et al. 1994, Fukushima et al. 1997) and the specific IgE-response to CM has been detected in exclusively breast-fed infants (Hattevig et al. 1990). Adverse clinical symptoms developed in breast-fed infants when challenged with CM via breast milk (de Boissieu et al. 1997b, Järvinen et al. 1999b). In studies on high-risk infants, maternal avoidance of CM and other allergenic foods has reduced the emergence of CMA and other atopic diseases (Chandra et al. 1989a, Lovegrove et al. 1994, Zeiger and Heller 1995). Recently, the effect of breast-feeding on infants' IgE levels was shown to depend on maternal atopy (Wright et al. 1999). If maternal IgE was low, the breast-fed infants had a lower serum total IgE at 6 years of age than infants who were never breast-fed. In contrast, if the maternal IgE was high, breast-feeding for over 4 months resulted in an increased IgE level in the infant as compared with those who had no or less than 4 months breast-feeding.

5.3.4 Immune factors in breast milk

Human milk contains numerous specific and non-specific defence factors (Hanson et al. 1985, Goldman 1993) and digestive enzymes (Hernell and Bläckberg 1994) which compensate for the immaturity of the gastrointestinal tract and the immune system of newborn infants (Udall 1990). In exclusively breast-fed infants the rate of subsequent infectious diseases is lower (Wright et al. 1998), as well as the incidence of autoimmune diseases, such as insulin-dependent diabetes mellitus (Åkerblom and Knip 1998) and Crohn's disease (Koletzko et al. 1989).

Secretory IgA is the predominant immunoglobulin in breast milk (90%), the excretion rate reaching up to 0.5 g/day (Groër and Walker 1996) and the highest concentrations are detected in colostrum (Machtinger and Moss 1986, Savilahti et al. 1991). Secretory IgA protects the infant from ingested antigens by binding to them in the gut lumen and preventing their absorption (Hanson et al. 1977, Groër and Walker 1996). Low concentrations of total and specific IgA in mature breast milk have been associated with symptoms of CMA (Machtinger

and Moss 1986), and a low content of total IgA in colostrum with the development of CMA (Savilahti et al. 1991).

In recent years, much interest has been focused on immunoregulatory agents, such as cytokines, and living cells secreting these factors in breast milk (Michie et al. 1998) because of their potent role in the development of the infant's immune system (Goldman 1993). Many cytokines have been detected in human milk: interleukins (IL)-1,-4,-5,-6,-10 and -13, transforming growth factors (TGF)-β1 and-β2, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, epidermal growth factor and granulocyte-macrophage colony-stimulating factor (Saito et al. 1993, Eglinton et al. 1994, Hawkes et al. 1999, Böttcher et al. 2000). TGF-β has been detected in 100% of colostrum samples, whereas the dection rate of IL-6 has varied from 17 to 86% and that of IFN-γ from 8 to 97% (Eglinton et al. 1994, Hawkes et al. 1999, Böttcher et al. 2000). The concentrations of TGF-β and IL-6 are higher in colostrum than in mature milk (Kalliomäki et al. 1999, Böttcher et al. 2000).

Regulatory T cells of Th3 type produce TGF-β (MacDonald 1998), which is crucial for maintaining immunological homeostasis (Letterio and Roberts 1997). Development of oral tolerance by feeding low doses of antigen is characterised by generation of specific Th2 and Th3 T cells that produce TGF-β and various amounts of IL-4 and IL-10 (Chen et al. 1994, Weiner 1997). IL-4 is a differentiation factor for TGF-β (Inobe et al. 1998). TGF-β down-regulates Th1 and Th2 responses (Weiner 1997), inhibits T-cell (Kehrl et al. 1986) and B-cell proliferation (Stavnezer 1995), suppresses allergic inflammation (Inobe et al. 1998), inhibits IgE synthesis (Borish and Rosenwasser 1996) and helps at mucosal sites by promoting IgA switching (Inobe et al. 1998).

IL-6 stimulates B lymphocyte maturation, regulates T cell activation and differentiation, increases IgE production in synergy with IL-4 and IL-13 and enhances IgA production (Goldman 1993, Borish and Rosenwasser 1996). IFN-γ, the most important cytokine for cell-mediated immunity, inhibits IgE synthesis by inhibiting IL-4 and IL-13 (Borish and Rosenwasser 1996). TGF-β can inhibit IFN-γ-mediated intestinal inflammation (Strober et al. 1997). However, the precise effects of these immunoregulatory agents on an infant's immune responses through human milk are unknown.

The leucocytes detected in breast milk are macrophages, neutrophils and lymphocytes. The phenotypic distribution of T cells differs from that of blood and both helper and suppressor T cells are more activated (Goldman 1993, Eglinton et al. 1994) When stimulated *in vitro*, breast milk-derived mononuclear cells are able to secrete cytokines (Skansén-Saphir et al. 1993). Because of the relatively neutral pH of the stomach in newborn infants, T cells and cytokines are thought to survive in the gut of the neonate and to transfer immune responses (Eglinton et al. 1994). Lower expression of the HLA-DR antigen on breast-milk macrophages has been detected in mothers of infants with CMA than in those of healthy infants, suggesting defective function and antigen presentation of the

maternal macrophages of allergic infants (Järvinen et al. 1999a). Furthermore, variations in the lipid composition of human milk have been suggested to explain the controversy of protective effects of breast-feeding against allergy (Duchén et al. 1998).

5.3.5 Other environmental factors

In addition to differences in infant feeding, several other environmental factors may affect the development of CMA. In a prevention study on high-risk infants evaluated at 12 months of age, reduced exposure to ingested and inhaled allergens decreased the incidence of food intolerance, atopic eczema and asthma, whereas exposure to parental smoking increased the risk of food intolerance (Arshad et al. 1992). Measurable amounts of BLG have been detected in house dust (Witteman et al. 1995) and a case of adult-onset sensitisation to casein inhaled in a laboratory has been described (Vaswani et al. 1999). Infants born during autumn and winter have more frequently had specific IgE responses to CM, egg and wheat (Aalberse et al. 1992, Nilsson and Kjellman 1996, Nilsson et al. 1997). The more older siblings an infant has, the less frequent is the rate of atopic sensitisation (Strachan 1989, Räsänen et al. 1997) suggesting that transmission of infections from older siblings drives the infant's immune system towards a Th1 response.

5.4 Clinical features of CMA

5.4.1 Appearance of symptoms

The first symptoms of CMA are reported to develop within 1-36 weeks after the introduction of CM formula, in 75-90% of patients within 4 weeks (Jakobsson and Lindberg 1979, Høst and Halken 1990). Some infants react to CM formula at their first exposure (Jakobsson and Lindberg 1979) and 2-23% have their first symptoms during exclusive breast-feeding (Gerrard et al. 1973, Jakobsson and Lindberg 1979, Høst and Halken 1990).

The reaction time at CM challenge varies: 2 clinically distinct groups of patients, immediate and delayed reactors, have been discerned (Dannaeus and Johansson 1979, Ford et al. 1983, Isolauri and Turjanmaa 1996). However, the time limit between the 2 groups is not exactly defined: some authors have used 1 h (Dannaeus and Johansson 1979, Ford et al. 1983, Tainio and Savilahti 1990), others 2 h (Räsänen et al. 1992, Baehler et al. 1996) or 8 h (Høst and Halken 1990). In one study, all the positive responses occurring within 2 h after the last (4^{th}) dose were considered to be immediate reactions (Vanto et al. 1999). Hill et

colleagues (1986) even described 3 patient groups: immediate reactors showing adverse symptoms within 45 min, intermediate reactors (with symptoms between 45 min to 20 h) and delayed reactors (with symptoms over 20 h after the start of the challenge). This inconsistency in defining the time limits for the different clinical reaction types is highly dependent on the challenge protocol: in the different studies, there has been variation in the volume of the first challenge dose, in the volumes of consecutive doses and in the time interval between the doses, and also in whether the reaction time is counted from the start of the challenge or from the last dose (Bock and Atkins 1990). According to the ESPGAN (1992), immediate reactions appear within 1 h of the start of the challenge, and later emerging symptoms are of delayed type. However, the percentage of patients reacting within 1 h at challenge has varied from 25% to 74% (Goldman et al. 1963, Dannaeus and Johansson 1979, Jakobsson and Lindberg 1979, Ford et al. 1983, Hill et al. 1986, Tainio and Savilahti 1990). In studies using other limits for reaction types, 40% to 57% of patients have been classified as immediate reactors (Høst and Halken 1990, Baehler et al. 1996, Isolauri and Turjanmaa 1996, Vanto et al. 1999).

5.4.2 Symptoms

The manifestations most commonly seen in CMA are cutaneous, gastrointestinal and respiratory symptoms (Bock and Sampson 1994). The majority of infants with CMA present with more than one symptom (Høst 1994) and multiple symptoms are reported in 58-92% of patients (Goldman et al. 1963, Gerrard et al. 1973, Jakobsson and Lindberg 1979, Høst and Halken 1990). According to Bock and Sampson (1994), confirmed symptoms of CMA are anaphylaxis, atopic dermatitis, urticaria, angioedema, rhinitis, conjunctivitis, wheezing, cough, vomiting, diarrhoea, bloody stools and growth retardation (Bock and Sampson 1994). The prevalence of the different symptoms varies between studies, most of which report the symptoms recorded after the challenge with CM.

Of the cutaneous symptoms, urticaria and/or exanthema are reported in 11-35% of patients (Goldman et al. 1963, Jakobsson and Lindberg 1979, Ford et al. 1983, Hill et al. 1986, Høst and Halken 1990, Tainio and Savilahti 1990, Baehler et al. 1996), angioedema in 12-28% (Ford et al. 1983, Baehler et al. 1996) and atopic dermatitis in 17-67% of patients (Goldman et al. 1963, Gerrard et al. 1973, Jakobsson and Lindberg 1979, Ford et al. 1983, Hill et al. 1986, Ventura and Greco 1988, Høst and Halken 1990, Baehler et al. 1996). Urticaria, exanthema and angioedema usually appear within minutes after ingestion of a provoking dose of CM, whereas the emergence of symptoms of atopic dermatitis, such as erythema, pruritus, oedema, papules, oozing, crusting and excoriation vary from hours to days (Hill et al. 1986, Stalder and Taïeb 1993, Bock and Sampson 1994, Isolauri and Turjanmaa 1996).

The most frequently reported gastrointestinal symptoms are diarrhoea and vomiting, which are seen in 8-48% and 11-55% of patients, respectively (Goldman et al. 1963, Gerrard et al. 1973, Jakobsson and Lindberg 1979, Hill et al. 1986, Ventura and Greco 1988, Høst and Halken 1990, Tainio and Savilahti 1990, Baehler et al. 1996). Ford et al. (1983) reported incidences of 57% for diarrhoea and 58% for vomiting, but infants with gastrointestinal CMA were over-represented in their study (Ford et al. 1983). Haematochezia (gross or occult) (Kokkonen and Similä 1980) has been reported in 5% (Gerrard et al. 1973) and 35% (Ventura and Greco 1988) of patients. Gastrointestinal symptoms appear within hours or days after commencement of ingestion of CM, though immediate projectile vomiting usually starts within 1-2 h (Jakobsson and Lindberg 1979, Ford et al. 1983, Hill et al. 1986, Ventura and Greco 1988, Baehler et al. 1996). Several symptoms and findings, not confirmed by population-based studies, may be related to hypersensitivity to CM: chronic constipation (Iacono et al. 1998), gastro-oesophageal reflux (Iacono et al. 1996), abdominal pain with lymphonodular hyperplasia of the duodenum or colon (Kokkonen et al. 1999), infantile colic (Jakobsson and Lindberg 1978, Forsyth 1989), eosinophilic gastroenteritis (Kelly 2000) and proctocolitis (Lake 2000), defective gastric function with changes in the gastric or jejunal mucosa (Kokkonen et al. 1979) and enterocolitis syndrome (Sicherer et al. 1998). Reports on the CM-induced malabsorption syndrome with a damaged jejunal mucosa and poor weight gain (Kuitunen et al. 1975) are nowadays hardly seen (Savilahti 2000).

CM may also induce respiratory symptoms, such as allergic rhinitis, wheezing and cough. Symptoms from the upper respiratory tract are seen in 11-36% and those from the lower respiratory tract in 12-44% of patients with CMA (Goldman et al. 1963, Gerrard et al. 1973, Ford et al. 1983, Hill et al. 1986, Høst and Halken 1990, Tainio and Savilahti 1990, Baehler et al. 1996). Respiratory symptoms are seen in infants with both the immediate and the delayed type of reaction (Ford et al. 1983, Hill et al. 1986, Høst and Halken 1990, Baehler et al. 1996).

The most severe symptom of CMA is anaphylaxis (Sampson 1997). Goldman et al. (1963) reported an incidence of 9% and Ventura et al. (1988) that of 5% in patients challenged with CM. In the former study, the symptoms were induced by a small amount of CM and described as ”sudden alarming reactions with profound weakness and seizures or signs of shock, 1 infant having tonic-clonic seizures and diarrhoea”. In the latter, the reactions were described as ”acute, self-limited, anaphylactic-like”. A history of generalised urticaria was reported in 4/8 patients with CM-induced anaphylactic reactions (Goldman et al. 1963) and a history of asthma in 2/2 patients (Sampson et al. 1992). In a series of 54 anaphylactic reactions to foods, 22% of the reactions were attributed to CM (Novembre et al. 1998).

The concordance between the symptoms reported by the parents and those observed at challenge has varied from 45% (Ventura and Greco 1988) to 78% (Goldman et al. 1963). Baehler et al. (1996) studied the concordance of the reaction time and found a 100% correlation between the history reported by the parents and the challenge outcome.

5.5 Immune responses of infants with CMA

5.5.1 IgE CM antibodies

Type I hypersensitivity, characterised by onset of clinical symptoms within minutes after contact with the allergen, is mediated by IgE antibodies bound to mast cells and basophils (Ishizaka and Ishizaka 1967, Johansson 1967, Sampson 1997). When an antigen is bound to the specific IgE antibody, an antigen-antibody complex is generated and mast cell degranulation and mediator release (e.g. histamine) occur (Leung 1995). In addition, mast cells may release cytokines, such as IL-4, IL-6, TNF-α and platelet-activating factor, that may induce late-phase reactions, such as exacerbation of atopic dermatitis (Leung 1995).

In infants with an immediate-type reaction to CM, specific IgE antibodies can be measured more often than in those with a delayed reaction (Dannaeus and Johansson 1979, Hill et al. 1986, Räsänen et al. 1992, Vanto et al. 1999). The symptoms of CMA most frequently associated with IgE-mediated hypersensitivity are urticaria and anaphylaxis, but specific IgE antibodies are also detected in infants with atopic dermatitis and gastrointestinal and respiratory symptoms (Hill et al. 1986, Räsänen et al. 1992, Baehler et al. 1996).

The amount of antigen, the frequency of doses and the age at introduction all influence the type and magnitude of the immune response that an infant develops (Hanson et al. 1993, Vaarala et al. 1995). Studies on animals have shown that very early exposure to antigen primes development of IgE (Hanson 1981, Strobel and Ferguson 1984), and the larger the antigen dose and the more frequent the feeding, the more the production of IgE is suppressed (Jarrett 1984, Fritsché et al. 1997). Conversely, low doses of antigen prime the Th2 T cells that produce IL-4, a key cytokine in IgE production (Koning et al. 1996). Transient low-level IgE responses to food antigens may be seen in young infants without clinical disease (Hattevig et al. 1984), but in those developing an allergic disease, the response is stronger and more persistent (Koning et al. 1996). In infants with CMA, the level of CM-specific IgE is inversely correlated with the volume of CM ingested prior to the diagnosis (Firer et al. 1981).

5.5.2 IgA, IgG and IgM CM antibodies

In type III hypersensitivity, antigen-antibody complexes precipitate in tissue spaces, causing inflammation and complement activation. The pathogenic role of antibodies of the IgA, G and M isotypes is undetermined. The CM-specific plasma levels of these immunoglobulins have been found to be similar (Burks et al. 1990, Savilahti et al. 1991, Høst et al. 1992) or higher (May et al. 1980) in infants with CMA than in controls. Low levels of CM-specific IgG have been related to acute-onset CMA (Firer et al. 1982) and high levels of IgG to BLG to persistent CMA (Høst et al. 1992).

IgG antibodies to CM are already present in plasma at birth (Tainio et al. 1988). The later increase in specific IgG antibodies depends on the type of feeding (Vaarala et al. 1995), and the earlier CM formula is introduced, the stronger is the response (Tainio et al. 1988, Harris et al. 1989). Exclusive feeding of CM formula during the first 3 days of life was associated with higher IgG CM antibody levels even at 2 years of age than after early exclusive feeding of casein hydrolysate formula (Juvonen et al. 1999). Specific IgA and IgM antibodies increase more slowly (Tainio et al. 1988), the levels of CM-specific IgA being higher among CM formula-fed than among breast-fed infants (Kaila et al. 1994, Kuitunen and Savilahti 1995). Furthermore, during and directly after a CM elimination diet, the CM-specific IgA- and IgG-secreting cells were undetectable in most healthy infants, but after prolonged CM feeding, were detectable again (Kaila 1993). The CM-specific IgM-secreting cells persisted throughout the dietary manipulation (Kaila 1993).

5.5.3 Cellular immune responses

Type IV hypersensitivity is characterised by a cellular immune response to a specific antigen. Sensitised T lymphocytes proliferate after contact with antigen ultimately causing an inflammatory reaction. T cell responses are suggested to play a significant role in late-phase reactions to CM (Savilahti et al. 1989, Sampson 1997).

However, the role of T cell responses in food hypersensitivity is disputed. One study showed that patients with CMA could be distinguished by measuring the leucocyte migration inhibition factor from controls (Ashkenazi et al. 1980), whereas another study found that this was also positive in infants who were negative at challenge (Vanto et al. 1987). *In vitro* proliferation of peripheral blood mononuclear cells (PBMC) to specific antigen has been measured in several studies. An enhanced proliferative response of PBMCs to CM proteins was detected in patients with CMA (Albani et al. 1989, Räsänen et al. 1992), and was correlated with CM-sensitive atopic dermatitis (Kondo et al. 1990) and with the immediate type of reaction to CM (Tainio and Savilahti 1990). However,

others CMA (Baudon et al. 1987, Eigenmann et al. 1995) have failed to find any correlation between the specific proliferative responses of PBMCs and CMA. Age has been shown to correlate inversely with the magnitude of the proliferative response in infants with CMA and on a CM elimination diet the response decreased (Iida et al. 1995). In healthy infants, early introduction of CM formula induced a more vigorous specific T cell response than later introduction (Vaarala et al. 1995).

Recently, studies on the cytokine production and profile of patients with CMA have given new insights into the pathology of CMA. Some showed that the PBMCs of infants with CMA produce less IFN-γ and TNF-α (Suomalainen et al. 1993, Österlund et al. 1999), whereas others found enhanced production of TNF-α (Benlounes et al. 1999). Recently, both Th1- and Th2-type responses have been found to be enhanced in infants with CMA. The numbers of IFN-γ-, IL-4-, IL-5- and IL-10-secreting PBMCs and the numbers of IFN-γ- and IL-4-secreting cells in the duodenal lamina propria were increased in infants with CM-sensitive enteropathy (Hauer et al. 1997) and the PBMCs of infants with CM-induced atopic dermatitis expressed both CD4+ and CD8+ surface antigens and had the capacity to produce both IFN-γ and IL-4 (Reekers et al. 1996). Enhanced IL-4 production has been associated with IgE-mediated CMA (Hauer et al. 1997, Campbell et al. 1998).

Specific T cells reacting to food antigens can also be isolated from the skin (van Reijsen et al. 1998, Reekers et al. 1999). After casein stimulation, a larger proportion of T cells of infants with CM-induced atopic dermatitis expressed cutaneous lymphocyte antigen-homing receptor than of T cells of infants having gastrointestinal CMA or of those from non-atopic adults (Abernathy-Carver et al. 1995). This suggests that the different localisation of allergic symptoms may be due to differences in the memory T cells.

The skin-patch test is suggested to be a safe and inexpensive method for measuring the allergen-induced eczematous skin reaction and lymphocytic infiltration (Breneman et al. 1989, de Bruin-Weller et al. 1999). In patients with atopic dermatitis who had a positive patch test to the corresponding antigen, the specific proliferative response of PBMCs was higher than in non-reactive patients with atopic dermatitis (Wistokat-Wülfing et al. 1999), suggesting an association between allergen-specific T cells, a delayed skin reaction and a positive response to the patch test. A positive patch test response to CM has been associated with delayed-type reaction to CM (Räsänen et al. 1992, Isolauri and Turjanmaa 1996, Majamaa et al. 1999b). Others have found a positive correlation between patch test reactivity and specific IgE (Darsow et al. 1997, Vanto et al. 1999).

5.5.4 Other markers of allergic inflammation

Activated eosinophils release granular cytotoxic proteins such as eosinophil cationic protein (ECP), eosinophil protein X, eosinophil peroxidase and major basic protein (Venge et al. 1999). Degranulation of eosinophils is increased during exposure to allergens (Carlson et al. 1992). Serum ECP is increased in children with active atopic dermatitis and in those with a positive skin-prick test (SPT) (Carlsen et al. 1997, Remes et al. 1998); it correlates with the severity of the asthma (Carlsen et al. 1997) and decreases with proper treatment (Vatrella et al. 1996). In infants with CMA, an increase in serum ECP during a challenge was associated with symptoms of atopic dermatitis and urticaria (Niggemann et al. 1994, Suomalainen et al. 1994). A more accurate method may be to measure ECP in various body fluids, such as sputum, tears and nasal and bronchoalveolar lavages (Pizzichini et al. 1997, Venge et al. 1999). In infants with CMA, faecal ECP increased during a CM challenge and decreased on an elimination diet (Majamaa et al. 1996, Majamaa et al. 1999a). In adults with IgE-negative CM-related gastrointestinal symptoms, a luminal CM challenge led to increased secretion of ECP and histamine into the gut lumen (Bengtsson et al. 1997).

In addition, increased levels of other markers of allergic inflammation, such as faecal TNF-α and α_1-antitrypsin (Majamaa et al. 1996), and serum IL-5 (Matsumoto et al. 1999) have been detected in infants with CMA. An increased release of histamine from basophils has been associated with an immediate-type reaction (Sampson et al. 1989, Räsänen et al. 1992).

5.6 Diagnosis of CMA

The diagnosis of infantile CMA is based on a clinical response to the elimination of CM from the diet, followed by reappearance of symptoms during a challenge test and their disappearance on a renewed elimination diet (The ESPGAN Working Group for the Diagnostic Criteria for Food Allergy 1992, Bock 2000). Neither the clinical symptoms or any laboratory test can predict the challenge outcome satisfactorily (Burks and Sampson 1992). Of the infants reported by their parents to have symptoms suggestive of CMA, only 33% (Høst and Halken 1990), 36% (Baehler et al. 1996), 50% (Sampson and Ho 1997), 53% (Bock 1987), 54% (Isolauri and Turjanmaa 1996) and 59% (Vanto et al. 1999) have shown a positive response to a CM challenge test.

5.6.1 The CM elimination-challenge test

The recommended period of elimination of CM from the diet is at least 2 weeks (Bruijnzeel-Koomen et al. 1995). Immediate reactions usually resolve rapidly, whereas infants with chronic symptoms, such as long-lasting atopic dermatitis or diarrhoea, may require several weeks for recovery (The ESPGAN Working Group for the Diagnostic Criteria for Food Allergy 1992, Vanto et al. 1999). CM formula is usually replaced with an extensively hydrolysed or soy formula (Høst and Halken 1990). If an infant is suspected to react to the CM present in breast milk, the breast-feeding mother is also put on a CM-free diet (Järvinen et al. 1999b). Infants with CMA, especially those with gastrointestinal symptoms, may also react to soy (Merritt et al. 1990, Zeiger et al. 1999). Rarely, clinical symptoms are provoked by extensively hydrolysed formula (de Boissieu et al. 1997a, Vanderhoof et al. 1997). In that case, the least allergenic formula, based on amino acids, can be used (Isolauri et al. 1995).

For diagnosis, Goldman et al. (1963) accepted only a positive response to 3 consecutive milk challenges. However, this has been judged as time-consuming and unacceptable (Høst 1994). The proposed gold standard for diagnosis of food allergy is a double-blind placebo-controlled food challenge (Bock et al. 1988), but in infants with CMA, most of whom are challenged during the first year of life, open challenges do not seem to cause bias (Isolauri and Turjanmaa 1996). When a history of anaphylaxis is related to ingestion of a particular food and specific IgE antibodies are detected, a challenge test is not recommended (Burks and Sampson 1992).

The challenge should be performed under medical supervision, because life-threatening symptoms may appear (Bruijnzeel-Koomen et al. 1995) and challenges done at home yield a great number of false-positive results (The ESPGAN Working Group for the Diagnostic Criteria for Food Allergy 1992). The starting quantity should be less than that required to cause the symptoms (Bock et al. 1988). Many authors start with 1 ml of CM formula (Jakobsson and Lindberg 1979, Hill et al. 1986, Isolauri and Turjanmaa 1996, Vanto et al. 1999). Then, if no symptoms appear, the dose is increased stepwise at intervals of 15-60 minutes until volumes are administered *ad libitum* (Hill et al. 1986, Isolauri and Turjanmaa 1996, Sampson 1997). The patient is usually sent home at the end of the first challenge day, but should be reinvestigated for delayed symptoms after 5-7 days (Ford et al. 1983, Hill et al. 1986, Isolauri and Turjanmaa 1996, Vanto et al. 1999). Some authors start with a dermal CM challenge (Räsänen et al. 1992, Vanto et al. 1999) in which a positive response (urticaria or erythema) is a sign of a contact urticaria syndrome (Bock and Atkins 1990). This is a common phenomenon in infants with atopic dermatitis (Salo et al. 1986) and a good correlation between dermal and oral food challenges has been established (de Waard-van der Spek et al. 1998). A contact urticaria syndrome may, however,

remain positive after development of oral tolerance to the respective food (Yamada et al. 1997).

5.6.2 The value of immunological tests in CMA

Specific IgE antibodies may be found in the circulation, or those that are fixed to skin mast cells can be measured by the SPT (Sampson 1997). Recently, CM-specific IgE antibodies were found in duodenal biopsy specimens taken during a challenge from infants with delayed-type CMA in whom skin and blood tests for specific IgE were negative (Caffarelli et al. 1998). Age may also influence the test results: several infants with immediate-type CMA were IgE-negative at the initial examination at a median age of 5 months but, when reinvestigated at 1 year of age, they showed a positive specific IgE response (Høst and Halken 1990).

In different study populations subjected to a CM challenge, the sensitivity of the SPT has varied between 41 and 80% and the specificity between 86 and 97% (Sampson and Albergo 1984, Hill et al. 1986, Høst and Halken 1990, Isolauri and Turjanmaa 1996). In detecting an immediate-type reaction, the sensitivity of the SPT has ranged from 65% to 89% (Dannaeus and Johansson 1979, Ford et al. 1983, Hill et al. 1986, Räsänen et al. 1992, Isolauri and Turjanmaa 1996, Vanto et al. 1999) and the specificity from 86% to 91% (Isolauri and Turjanmaa 1996, Vanto et al. 1999). Measurement of serum IgE CM antibodies has produced lower values for sensitivity and specificity (Sampson and Albergo 1984, Hill et al. 1986, Høst and Halken 1990, Vanto et al. 1999). Better sensitivity for the SPT was achieved when fresh foods were used instead of commercial allergen extracts (Rosen et al. 1994, Rancé et al. 1997).

For non-IgE-mediated reactions, no good tests are available (Bock 2000) (see sections 5.5.2 and 5.5.3). The sensitivity of the patch test has varied from 17% (clearly positive reactions) and 48% (doubtful and clearly positive reactions) (Vanto et al. 1999) to 61% (Isolauri and Turjanmaa 1996), and the specificity from 89% and 55% to 81%, respectively.

The following values for sensitivity and specificity have been reported in studies on CMA in which 2 or more tests have been used in parallel: 80% and 71% for the SPT and serum IgE CM antibodies (Sampson and Albergo 1984), 88% and 67% for serum IgE CM antibodies and the lymphocyte proliferation test (Tainio and Savilahti 1990), 95% and 58% for the SPT, serum IgE CM antibodies, the basophil histamine release test, the lymphocyte proliferation test and the patch test (Räsänen et al. 1992) and 86% and 72% for the SPT and the patch test (Isolauri and Turjanmaa 1996).

6. AIMS OF THE STUDY

The objectives of the present study were:

1. To evaluate prospectively in a population-based group of healthy full-term infants whether supplementary feeding of CM at the maternity hospital would increase the risk of CMA as compared with feeding of pasteurised human milk or extensively hydrolysed whey formula (I).

2. To assess the effects of exclusive breast-feeding, infant feeding patterns at home and other environmental and genetic factors on the incidence of CMA (I).

3. To investigate the impact of several immune factors in colostrum on the development of CMA and on the immune responses of infants with CMA (II).

4. To evaluate the effect of different feeding patterns, and other environmental and genetic factors on the subsequent type of CMA classified by the presence or absence of IgE antibodies to CM (III).

5. To study the usefulness of 4 different immunological tests for the diagnosis of CMA as compared with the result of the CM challenge test (IV).

7. SUBJECTS

7.1 Study population and randomisation procedure

The study participants were collected by explaining the study to 15 400 mothers of healthy, full-term infants immediately after delivery between August 1994 and November 1995 in 3 maternity hospitals in the Helsinki region. The hospitals were: the Department of Obstetrics, Helsinki University Central Hospital, Helsinki City Maternity Hospital and Jorvi Hospital. Within 12 h after delivery, 6267 mothers (41%) agreed to participate. Of them, 58 (0.9%) were lost for various reasons (withdrawal, infant death or disease, no questionnaires returned), leaving a final cohort of 6209 infants.

Although breast-feeding was strongly encouraged in these hospitals, 5385 (87%) of the infants required supplementary milk at hospital because of insufficient secretion of breast milk. These infants were randomly assigned to 1 of 3 study groups according to the supplement given: liquid CM formula (Tutteli®, Valio, Finland); pasteurised human milk (a mixture of milk from multiple donors expressed 1 to 6 months after delivery); and extensively hydrolysed whey formula (Pepti-Junior®, Nutricia, The Netherlands). The supplements were supplied to the maternity wards in colour-coded bottles; only 1 of the authors (LL), who was not in contact with the ward personnel or the infants, was aware of the code. The supplements were changed monthly; the 3 hospitals each used a different supplement, and thus the supplement that an infant received depended on the month of birth and the hospital where it was born. The comparison group comprised 824 (13%) exclusively breast-fed infants. The infants were kept in the hospital for a mean of 4 days.

7.2 Definition of CMA

7.2.1 Infants with CMA diagnosed by the study protocol

The primary end-point of the study was an adverse reaction to challenge with CM. Before leaving hospital, the parents were given written information about the symptoms suggestive of CMA (urticaria, atopic dermatitis, vomiting, diarrhoea, poor gain in weight, allergic rhinitis or wheezing). If an infant had any such symptoms, the parents were asked to call the author (KMS). The well-baby clinics in the area, at which every infant is seen, on average, 8 times during the first 12 months of life, were also informed of the study. If, after a comprehensive interview, the symptoms were compatible with CMA, a CM elimination-

challenge test was performed (see section 8.1). The infants with CMA are being followed up and re-challenged every 6 to 12 months until clinical tolerance to CM has developed.

The number of contacts with the parents of infants with symptoms suggestive of CMA diminished spontaneously. The last new contact was made in mid-October 1996, and the last challenge test was performed in November 1996. The milk code was broken in December 1996.

7.2.2 Infants with CMA diagnosed elsewhere

We also sought to study the number of infants with CMA diagnosed elsewhere. Therefore, in May 1997, when the mean (range) age of participants was 27 (18 to 34) months, the number of infants diagnosed as having CMA was obtained from the Social Insurance Institution. This national organisation subsidises the treatment costs for infants under 2 years of age who are allergic to CM. For that, the Institution requires a detailed certificate of symptoms and the process of diagnosis written by a doctor. According to this register, CMA was diagnosed elsewhere in 81 of the infants. These infants are included in the results shown in Table 4, but are excluded from the other analyses because of possible heterogeneity in diagnostic criteria. Moreover, of these 81 infants, 8 were challenged by us and diagnosed as not having CMA. Because the classification of these 8 infants is uncertain, they have been excluded from all analyses, except that shown in Table 4.

7.3 Infant feeding patterns

The time and amount of every supplementary feeding was recorded in the delivery ward. When at home, the mothers were advised to follow the normal feeding recommendations given by the well-baby clinics, i.e. to supplement breast-feeding with adapted CM formula when required and to start solid foods between the ages of 4 and 6 months. The mothers were asked to record the infant-feeding regimen daily during the first 8 weeks. These data were available in all 118 infants subsequently found to react positively to a CM challenge and in the 4543 (76%) tolerant infants (similar proportions in all 4 groups). A questionnaire on the infant-feeding regimen filled in at 6 and 12 months of ages was returned by 77% and 73% of the parents of the tolerant infants. Similar data, recorded by the author during follow-up visits, were available for all 118 allergic infants.

7.4 Family history of atopy and environmental factors

While in the hospital, the parents filled in a questionnaire about the family history of atopy defined as past or present asthma, atopic dermatitis, allergic rhinitis or conjunctivitis. The data were categorised as follows: no atopy and obvious atopy (asthma or symptoms from 2 different organs in 1 or both parents and/or in 1 or more siblings). Data on parental smoking, numbers of siblings, and furred household pets were also recorded.

7.5 Ethics

The study protocol was approved by the ethics committee of each maternity hospital and of the Hospital for Children and Adolescents, University of Helsinki. Written informed consent was obtained from the mother of each infant entering the study.

8. METHODS

8.1 The CM challenge procedure

If an infant had symptoms suggestive of CMA (see section 7.2.1), CM was eliminated from the infant's diet for 2 to 4 weeks. Those who required supplementary milk were given extensively hydrolysed formula (Pepti-Junior®). If the symptoms did not subside in 2 to 4 weeks, hydrolysed formula was replaced with amino acid-based formula (Nutri-Junior®, Nutricia, The Netherlands). If the infant's symptoms had appeared during exclusive breast-feeding and the mother continued to breast-feed, she was advised to avoid CM herself.

If the symptoms had subsided, an open CM challenge was done at the out-patient clinic. Breast-fed infants were challenged when they regularly needed supplementary milk. Before the challenge, the parents were carefully interviewed about the history of the infants' symptoms. If the SPT with CM was positive (≥3 mm) the challenge was started with drops of CM placed on the volar side of the wrist, the cheek and the lips, followed by CM formula given orally in quantities of 1, 10, 50 and 100 ml at intervals of 30 to 60 min. In the infants with a negative SPT, the challenge was started with 1 ml of CM given orally. Infants who were without symptoms next day continued to receive formula at home. The parents were advised to record all adverse symptoms and the daily amounts of CM formula consumed. Every infant was examined for delayed symptoms on the fifth day of the challenge. The challenge was considered positive if 1 or more of the following symptoms had appeared in the interim: anaphylaxis, urticaria, exanthema, atopic dermatitis, vomiting, diarrhoea, wheezing or allergic rhinitis. Infants reacting adversely to the challenge were given a CM-free diet and those with no symptoms received CM formula. One month later, all the infants were re-examined to confirm the challenge result.

8.2 Skin tests

8.2.1 The SPT (I-IV)

Before the challenge, SPTs with liquid CM formula and with commercial allergen extract of egg (Soluprick®, Allergologisk Laboratorium, Hørsholm, Denmark) were performed on the volar surface of the forearm according to a standard technique (Dreborg et al. 1989). Histamine dihydrochloride (10 mg/ml) served as a positive and 50% glycerol solution (Soluprick®) as a negative control. The mean diameter of the weal was calculated by averaging the sum of

the largest diameter of the weal and the diameter perpendicular to it. A mean diameter ≥3 mm greater than the negative control was taken as a positive response (I, II, III).

8.2.2 The patch test (IV)

In skin-patch test, we used CM formula powder, bovine serum albumin (Sigma, St. Louis, MO), crystallized bovine BLG (Sigma) and bovine casein (Sigma). Each test substance was mixed with 0.9% saline solution in a separate tube in which a filter-paper disc was placed for an hour to be come saturated with the test substance and was then attached to the patient's back under an aluminium cup (Finn Chamber, Epitest Ltd, Hyrylä, Finland) fixed with a Scanpore tape. A filter paper moistened with 0.9% saline solution was used as a negative control. The occlusion time was 48 h, and the results were read 48 h after removal of the cups. Marked erythema (over half the size of the cup) and erythema with oedema were considered to be positive responses.

8.3 Blood samples

Shortly before the challenge and on day 4 after the beginning of the challenge a blood sample was drawn from the infants challenged with CM. A sample taken before the challenge was available in 116 allergic and in 117 tolerant infants and a sample taken on challenge day 4 in 103 and 53 infants, respectively. Serum samples were stored frozen at -20°C until analysed.

8.3.1 CM-specific and total IgE and ECP (II, III, IV)

Total and CM-specific IgE were measured in the pre-challenge samples. Serum ECP was measured in the pre- and post-challenge samples of those infants for whom both samples were available. We used the Pharmacia CAP system (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Values for specific IgE CM antibodies ≥0.7 kU/l were considered positive (II, III). The following cut-off values were selected for serum ECP measured 4 days after the challenge: ≥15 µg/l (suggested by the manufacturer) (Ahlstedt 1995), ≥20 µg/l (Fitch et al. 1999) and ≥24.7 µg/l (Remes et al. 1998) (IV).

8.3.2 IgA and IgG antibodies to CM and its fractions (II)

IgA and IgG antibodies to whole CM and BLG were measured with an enzyme-linked immunosorbent assay (ELISA) (Savilahti et al. 1993a). α-Casein antibodies were measured by a similar method in microtitre wells coated with 2 μg/ml of commercial α-casein (Sigma).

8.3.3 Lymphocyte proliferation assay (II)

Proliferation tests of PBMCs with CM antigens were performed randomly on 54 of the infants diagnosed as having CMA as described earlier (Vaarala et al. 1996). The CM-derived antigens, α- and β-casein and BLG, were used at concentrations of 20 and 200 μg/ml. Proliferation was expressed as a stimulation index (SI): median counts per minute of tritiated thymidine incorporated in the presence of the antigen divided by median counts per minute of tritiated thymidine incorporated in the absence of the antigen.

8.4 Breast milk samples

We had a colostrum sample from 108 of the mothers of the 118 infants with CMA. We selected 207 controls randomly from among the mothers whose infants did not develop CMA. IgA, IgM and IgA CM-specific antibodies were measured from all samples. The total concentrations of IgA and IgM were measured by an immunoturbidometric method, using monospecific antisera to human IgA and IgM. (Method for IgA CM antibodies; see section 8.3.2.) Cytokines were measured only in half randomly selected control samples because of the high cost of the antibodies and kits needed for the measurements.

Colostrum samples were collected on days 1 to 4 postpartum and were available from 90% of the participating mothers. Each sample was frozen within 12 h of collection and kept frozen at -20°C for 18 to 24 months. After thawing, the sample was centrifuged at 10 000 g for 30 min, the cellular debris and the fat layer were discarded and the clear middle layer was used for analyses.

8.4.1 IL-6 and INF-g (II)

In ELISA for IFN-γ and IL-6, 96-well Maxisorb plates (Nunc, Roskilde, Denmark) were coated with monoclonal anti-human IFN-γ antibody (M-700A, Endogen, Cambridge, MA) or rat anti-human IL-6 monoclonal antibody (18871D, Pharmingen, San Diego, CA) at a concentration of 2 μg/ml (50 μl/well)

in 0.1 M Na_2HPO_4, pH 9.0. After washing with phosphate-buffered saline (PBS), the plates were blocked with 1% BSA in PBS. Dilutions of recombinant human IFN-γ (19751N, Pharmingen) and human IL-6 (19661V, Pharmingen) were used to create a standard curve. Samples of colostrum (200 µl/well) and standards were incubated for 2 h at 37°C. After washing with PBS-0.05% Tween, biotinylated anti-human IFN-γ monoclonal antibody (M-701, Endogen, Boston, MA) or biotinylated rat anti-human IL-6 monoclonal antibody (18882D, Pharmingen) was added at a concentration of 0.25 µg/ml (50 µl/well) and the plates were incubated for 2 h at 37°C. After washing with PBS-Tween, streptavidin-alkaline phosphatase complex (Zymed, San Francisco, CA) was added and *p*-nitrophenyl phosphate was used to develop the colour, which was read at 405 nm. The detection level of the assay for detection of IFN-γ was 150 pg/ml and for detection of IL-6 50 pg/ml.

8.4.2 TGF-b1 (II)

Concentrations of TGF-β1 in breast milk samples were measured by using a Human TGF-β1 DuoSeT (Genzyme, Cambridge, MA). The plates were coated with anti-human TGF-β1 capture antibody in 0.1 M Na2HPO4, pH 9.0, and incubated overnight at 4°C. The plates were then washed with 1% BSA-0.05% Tween-PBS before adding blocking buffer. Samples were activated with 1N HCl (1/20 of the sample volume) and incubated at 4°C for 60 min. Samples were neutralised with 1N NaOH and incubated with standards (activated recombinant human TGF-β1). The second antibody was added in 1% BSA-0.05% Tween-PBS at a concentration of 1.5 µg/ml. To develop the assay, 3,3´,5,5´-Tetramethylbenzidine (Sigma) substrate reagent was used. The detection level of the assay was 60 pg/ml.

8.5 Statistical analyses

Calculations of sample size were based on prediction of a 3.5% cumulative incidence of CMA (Savilahti et al. 1991). The trial was designed to detect a 60% lower cumulative incidence in the pasteurised human milk or whey hydrolysate groups than in the CM group, with a power of 80% and α=0.05. The projected sample size for a group was 1640 infants (I).

To analyse the effect of the type of early feeding on CMA and the independent contributions of environmental and genetic factors to the risk, we used a logistic regression model that included all risk factors and tested the significance of each one with control of all other factors (I). The independent contributions of genetic and environmental factors to the risk of having an IgE-

mediated reaction to CM were studied with the same method (III). For this purpose, continuous variables were categorised dichotomously, with the median as the cut-off point.

Associations between categorical variables were examined by Pearson's χ^2 test (I, III, IV). The distributions of continuous data on infant feeding and on measurements from colostrum and blood samples were all skewed. The differences between the volumes of CM given to the infants with CMA and to tolerant infants were analysed by the Kruskal-Wallis test (I). All other comparisons were made after logarithmic transformation, and we applied analysis of variance (ANOVA) for multiple comparisons and Student's *t* test for comparisons between the 2 groups (II, III, IV). Correlations were calculated with Spearman's rank correlation test (II, IV). For comparisons between the paired measurements of serum ECP, we used the Wilcoxon signed-rank test (IV). The following formulas were used for calculations (IV): sensitivity= the number of infants with a positive challenge identified as correct by the test(s)/ the total number of infants with a positive challenge. Specificity= the number of infants with a negative challenge identified as correct by the test(s)/ the total number of infants with a negative challenge. Positive predictive value= the number of positive challenges identified as correct by the test(s)/ the total number of positive test results. Negative predictive value= the number of negative challenges identified as correct by the test(s)/ the total number of negative test results. Overall agreement = the total number of infants correctly classified by the test(s) / the total number of infants tested.

Means are presented with 95% confidence intervals (CI) and medians with 95% CI or with ranges. Statistical significance was defined as $p \leq 0.05$. Two-sided tests of significance were used throughout. Analyses were performed using SAS software (SAS Institute, Cary, NC) (I) and an SPSS (version 7.5 for Windows; SPSS, Chicago, IL) software package (I-IV).

9. RESULTS

9.1 Clinical and immunological features of infants challenged with CM

9.1.1 Symptoms suggestive of CMA (III, IV)

A total of 622 families contacted the author because their infants had symptoms suggestive of CMA at home, and 247 infants recovered on the elimination diet. These latter were challenged with CM, but 8 (3%) of them are excluded from the analyses because of uncertainty of the diagnosis (see sections 7.2.2 and 10.4). Of the remaining 239 infants, 232 recovered on extensively hydrolysed formula (Pepti-Junior®), and 7 on amino acid-based formula (Nutri-Junior®). The parents noticed their infants' first adverse symptoms to CM at mean (95% CI) ages of 2.9 (2.5-3.2) and 3.6 (3.2-4.0) months in the 118 challenge-positive and in the 121 challenge-negative infants (p=0.008). According to the parents' reports, the challenge-positive infants had had skin symptoms more often, but diarrhoea less frequently, than those with a negative challenge (*Table 1*). In the challenge-positive infants, a history of urticaria and/or exanthema (67% vs. 9%) and immediate vomiting (33% vs. 12%) was associated with the presence of CM-specific IgE antibodies, whereas continuous regurgitation (11% vs. 30%) was more common in the IgE-negative infants (p<0.01 for all comparisons).

Table 1. *Symptoms suggestive of CMA reported by parents and symptoms at challenge in infants with IgE-positive and -negative CMA and in infants with negative challenge.*

	Number (%) with symptoms					
	Suggestive of CMA			At challenge		
Symptom	Positive challenge n = 118	Negative challenge n = 121	χ^2 test p value	IgE-positive n = 75	IgE-negative n = 43	χ^2 test p value
Urticaria/exanthema	54 (46)	28 (23)	<0.001	57 (76)	4 (9)	<0.001
Atopic dermatitis	106 (90)	94 (78)	0.01	21 (28)	31 (72)	<0.001
Gastrointestinal	61 (52)	73 (60)	0.18	7 (9)	17 (40)	<0.001
- Vomiting	51 (43)	53 (44)	0.93	7 (9)	13 (30)	0.004
- Diarrhoea	24 (20)	41 (34)	0.02	0	10 (23)	<0.001
Respiratory symptoms	42 (36)	31 (26)	0.09	9 (12)	9 (21)	0.19
Allergic conjunctivitis	6 (5)	4 (3)	0.49	4 (5)	1 (2)	0.44
Anaphylactic reaction	3 (3)	0	—	1 (1)	0	—
Multiple symptoms	76 (64)	75 (61)	0.60	14 (19)	13 (30)	0.15

9.1.2 Categorisation of infants according to CM-specific IgE (II, III)

Of the 118 infants with CMA, 75 (64%) were IgE-positive (SPT ≥3 mm and/or IgE CM antibodies ≥0.7 kU/l) and 43 (36%) IgE-negative. The SPT was positive in 72 (96%) of the IgE-positive infants and 52 (69%) of them had increased serum IgE CM antibodies.

9.1.3 The CM challenge test (I, III, IV)

The challenge was performed at mean (95% CI) ages of 7.0 (6.5-7.4), 6.4 (5.6-7.2) and 7.1 (6.6-7.5) months in the IgE-positive and -negative groups and in the infants with a negative challenge. Of the challenges, 49% were positive and 51% negative. Among the infants with a positive SPT to CM, a dermal CM challenge was positive in 31/68 (46%) infants with a positive and in 4/27 (15%) infants with a negative challenge (p=0.005). The symptoms appeared earlier in the IgE-positive group (*Figure 1*). Acute skin symptoms were more frequent in the IgE-positive group, whereas, in the IgE-negative group, atopic dermatitis and gastrointestinal symptoms predominated (*Table 1*). Similar percentages of IgE-positive and -negative infants had rhinorrhoea, but the latter more often had wheezing and cough (14% vs. 1%, p<0.01).

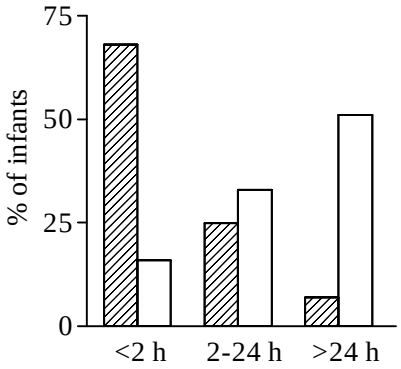

Figure 1. *Reaction time from the start of challenge until the positive reaction in relation to IgE-positivity (hatched bars) and IgE-negativity (open bars) in infants with CMA (p<0.001, c^2 test).*

9.1.4 Diagnostic value of the 4 immunological tests (III, IV)

SPT and serum CM-specific IgE antibodies were more often positive in the infants with CMA (*Figure 2*). The skin-patch test was also more often positive in the infants with CMA, both whole CM and CM protein fractions eliciting positive responses more frequently in infants with CMA (*Figure 2*). However, 29/72 (40%) of the positive patch test responses were related to a negative challenge. During the challenge, serum ECP increased significantly in the infants with CMA but not in those with a negative challenge (data not shown). Similar

percentages of infants with a positive and a negative challenge had moderately elevated serum ECP measured on challenge day 4, but the highest values were confined to the infants with a positive challenge (allergic vs. non-allergic infants: 13% vs. 2%, p=0.03) (*Figure 2*).

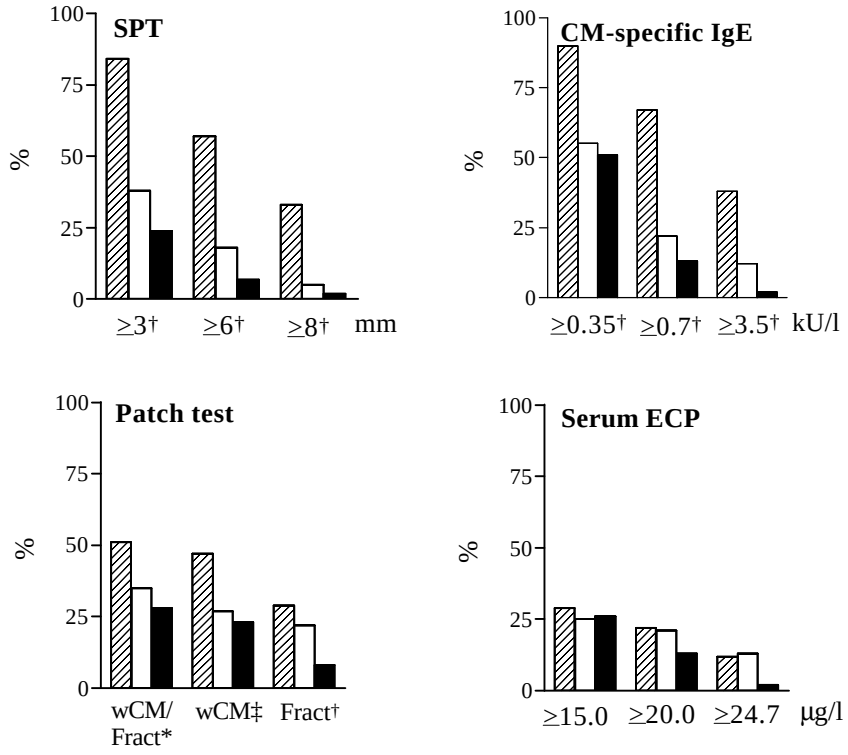

Figure 2. *Percentages of infants with an immediate (hatched bars), delayed (open bars) and a negative (black bars) response to the CM challenge having a positive response to the SPT and the patch test, increased serum CM-specific IgE antibodies and ECP at different cut-off levels. The tests were performed at the time of the challenge. wCM= whole CM, Fract= CM protein fractions: BSA, BLG and casein. *p<0.05, ‡p<0.01, †p<0.001, c^2 test.*

Using the cut-off values of each test with the best overall agreement, a combination of SPT ≥3 mm, milk-specific IgE ≥0.7 kU/l, a positive reaction to one or more CM protein fractions in the patch test and ECP ≥20 µg/l used in parallel produced the best overall agreement (0.73) with a sensitivity of 0.76 and a specificity of 0.67 (*Table 2*).

Table 2. *Sensitivity, specificity, and predictive indices at different cut-off levels for the 4 tests derived from the responses of 137* infants challenged with CM.*

Test and cut-off value						Positive	Negative	Overall
SPT (mm)	IgE CM (kU/l)	Patch test†	ECP (μg/l)	Sensi-tivity	Speci-ficity	predictive value	predictive value	agree-ment
≥3	≥0.35	wCM/Fract	≥15	0.85	0.23	0.67	0.46	0.64
≥6	≥0.7	wCM	≥20	0.66	0.65	0.78	0.51	0.66
≥8	≥3.5	Fract	≥24.7	0.54	0.94	0.94	0.52	0.68
≥3	≥0.7	Fract	≥20	0.76	0.67	0.81	0.60	0.73

Positive response: a positive reaction to 1 or more tests. Negative response: negative reaction to all 4 tests. *Number of infants on whom all 4 tests were done. †wCM: whole CM. Fract: BSA, BLG and casein.

Both the SPT and CM-specific IgE were, and the patch test tended to be, more often positive in infants with an immediate reaction at challenge, whereas serum ECP poorly distinguished infants with immediate or delayed reactions from those with a negative challenge (*Figure 2* and *Table 3*). A combination of these 4 tests used in parallel detected 94% of the immediate reactors with a specificity of 0.55, but only 58% of the delayed reactors with a specificity of 0.37 (*Table 3*; Saarinen KM, unpublished results). The challenge positive infants not detected by this combination more often had intestinal symptoms.

Table 3. *Sensitivity, specificity and predictive indices of the SPT, serum CM-specific IgE, the patch test and serum ECP in relation to the reaction time at challenge.*

Test	Cut-off value	n	Sensi-tivity	Speci-ficity	Positive predictive value	Negative predictive value	Overall agree-ment
Immediate reaction (<2 h)							
SPT (mm)	≥3	239	0.84	0.71	0.49	0.93	0.74
IgE CM (kU/l)	≥0.7	233	0.67	0.84	0.58	0.89	0.80
Patch test	Fract†	204	0.29	0.88	0.44	0.79	0.73
Serum ECP (μg/l)	≥20.0	156	0.22	0.83	0.38	0.69	0.72
4 tests combined*		137	0.94	0.55	0.51	0.94	0.68
Delayed reaction (³2 h)							
SPT (mm)	≥3	239	0.38	0.56	0.23	0.73	0.52
IgE CM (kU/l)	≥0.7	233	0.22	0.69	0.19	0.73	0.58
Patch test	Fract†	204	0.22	0.85	0.32	0.78	0.70
Serum ECP (μg/l)	≥20.0	156	0.21	0.83	0.38	0.68	0.62
4 tests combined*		137	0.58	0.37	0.30	0.66	0.44

*The 4 tests used in parallel with cut-off values shown in this table. †Fract: BSA, BLG and casein.

The patch test correlated with the SPT: of the infants with positive a patch test, 58% had a positive SPT (≥3 mm) and, of those with a negative patch test, 64% had a negative SPT (p=0.002). A positive correlation was also found between the diameter of the weal in the SPT and the amount of circulatory CM-specific IgE (Spearman's ρ=0.724, p<0.001).

9.2 Factors affecting the development of CMA

9.2.1 Type of feeding at the maternity hospital (I)

Of the infants diagnosed by the study protocol, fewer infants in the whey hydrolysate group developed CMA than of those in the CM group (*Table 4*). The exclusively breast-fed infants and those exposed to CM were at similar risk. When the all infants with CMA were included in the analysis, those exposed to CM were again at higher risk of CMA than those given either pasteurised human milk or whey hydrolysate formula (Saarinen KM, unpublished results). However, the infants with CMA diagnosed elsewhere are excluded from the other analyses because of possible heterogeneity in diagnostic criteria.

Table 4. *Cumulative incidence of CMA and odds ratios (OR) for the risk of developing CMA according to the type of feed in the hospital after delivery.*

	Infants with positive CM challenge*			All infants with CMA†		
Feeding group (n total)	n (%)	OR (95%CI)	p value	n (%)	OR (95%CI)	p value
Randomised groups						
CM formula (1789)	43(2.4)	1.0	—	74 (4.1)	1.0	—
Pasteurised human milk (1859)	32 (1.7)	0.70 (0.44-1.12)	0.14	47 (2.5)	0.60 (0.41-0.87)	0.007
Whey hydrolysate formula (1737)	26 (1.5)	0.61 (0.38-1.00)	0.05	48 (2.8)	0.66 (0.46-0.95)	0.03
Non-randomised group						
Own mother's milk = Comparison group (824)	17 (2.1)	0.85 (0.48-1.51)	0.59	30 (3.6)	0.88 (0.57-1.35)	0.55

*According to the study protocol. †Infants diagnosed as having CMA obtained from the Social Insurance Institution are included.

Supplementary feeds were given similarly to all 3 groups: they were introduced at a median age of 14 h and continued for a median of 45 h. In the CM group, the infants with CMA had been exposed to smaller volumes of CM than the tolerant infants (*Figure 3A*).

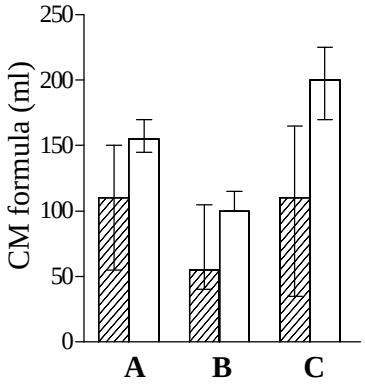

Figure 3. *Median (95% CI) volume of CM/infant given to those developing CMA (hatched bars) and to tolerant infants (open bars) in the CM formula group.* ***A.*** *Total volume at hospital (n=43 and n=1715, p=0.03).* ***B.*** *Daily volume at home <2 weeks of age (n=15 and n=542, p=0.04).* ***C.*** *Daily volume at 2-8 weeks of age (n=21 and n=605, p=0.007). Kruskall-Wallis test.*

9.2.2 Infant feeding at home during the first 8 weeks (I)

During the first 8 weeks of life, similar percentages of allergic and tolerant infants in the randomised groups had been given CM at home: 58% and 56% in the CM, 50% and 53% in the human milk, and 54% and 55% in the whey hydrolysate group. In the comparison group, only 6% of allergic and 20% of the tolerant infants were exposed. In the CM group, the infants with CMA had been given smaller volumes of CM than the tolerant infants (*Figure 3B,C*). Of the 17 allergic infants in the comparison group, only 1 had been exposed to CM (daily median 20 ml) at 2-8 weeks of age.

9.2.3 Parental atopy and environmental factors (I)

The percentage of allergic infants with a history of obvious parental atopy was almost double that of tolerant infants: 36% vs. 19% ($p<0.001$). Of the allergic and tolerant infants, 33% and 18% in the CM group ($p=0.01$), 38% and 19% in the human milk group ($p=0.008$), 35% and 20% in the whey hydrolysate group ($p=0.06$) and 47% and 19% in the comparison group ($p=0.004$) had 1 or both parents atopic. The incidence of CMA was 1.3% in the infants born between March and May, 1.6% in those born between June and August, 2.4% in those born between September and November and 1.8% in those born between December and February (Saarinen KM, unpublished results). The incidence was higher in the autumn than in the spring group (2.4% vs. 1.3%, $p=0.03$, χ^2 test). The percentages of allergic and tolerant infants with siblings (46% vs. 54%) and with smoking mothers (8% vs. 12%) were similar.

9.2.4 Multivariate analysis of risk factors for CMA (I)

Among the 5317 infants given supplementary feeds at hospital, exposure to CM while in hospital and obvious parental atopy increased the risk of CMA (*Table 5*).

Table 5. *Effects of parental atopy and environmental factors on the risk of developing CMA among the infants who required supplementary feeds at the hospital.*

Variable	Reference category	OR (95% CI)	p value
Supplementary feeding while in hospital			
CM (43/1758)*	No CM† (58/3559)*	1.54 (1.04-2.30)	0.03
Obvious parental atopy			
Yes (35/1014)*	No (66/4303)*	2.32 (1.53-3.52)	<0.001

Factors tested but found not to be significant were parental smoking and the presence of siblings and furred pets. *Number of infants with CMA/ total number of infants in each category. †Pasteurised human milk or hydrolysed whey formula used as a supplement.

9.2.5 Immunoglobulins, TGF-b1, IL-6 and IFN-g in colostrum (II)

The mean concentrations of IgA, IgM and IgA CM antibodies and TGF-β1 were similar in maternal colostrum of infants with CMA and of controls (*Figure 4*). IL-6 was measurable in less than 50% and IFN–γ in less than 30% of samples and the percentages of samples with low contents of both cytokines were similar in the 2 groups. The mean levels of measurable IL-6 and IFN–γ were similar in the 2 groups (*Figure 4*).

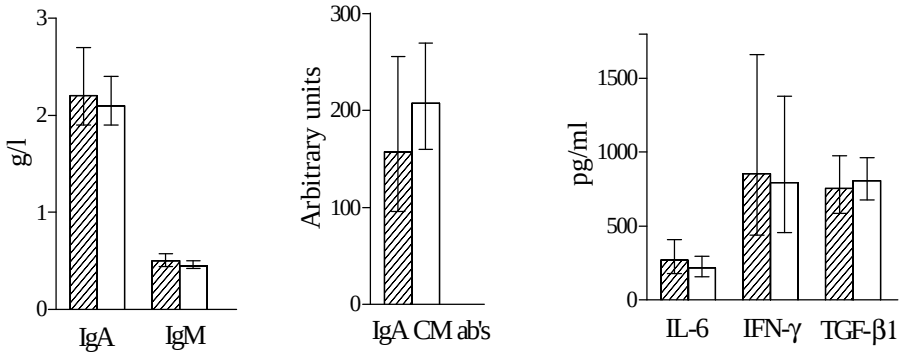

Figure 4. *Mean (95% CI) concentrations of IgA, IgM, IgA CM antibodies, IL-6, IFN-g and TGF-b1 in colostrum samples of mothers of infants with CMA (hatched bars) and of control mothers (open bars).*

9.3 Factors affecting the immune responses to CM in infants with CMA

9.3.1 CM formula and breast-feeding (III, IV)

Exposure to CM at the maternity hospital tended to elicit the development of IgE CM antibodies, whereas exposure to CM during the first 8 weeks at home correlated with the development of IgE-negative CMA (*Table 6*). Of those exposed, the IgE-positive infants had been given smaller amounts of CM during a shorter period (*Figure 5*).

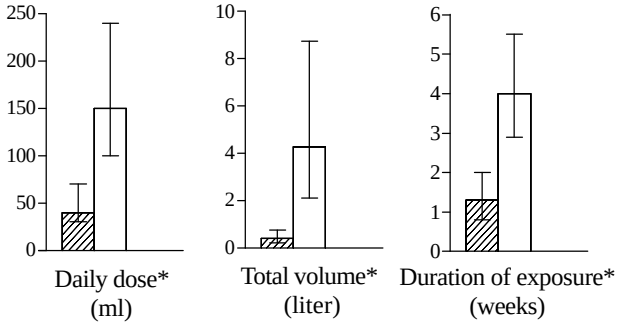

Figure 5. *Means (95% CI) for volume of CM and duration of exposure per infant in the IgE-positive (hatched bars) and in the IgE-negative (open bars) groups during the first 8 weeks at home (*p<0.001, t test).*

Among the 239 challenged infants, SPT was more frequently positive in those exposed to CM at hospital: 46/84 (55%) vs. 55/155 (36%), (p=0.004); the difference was similarly significant in the infants with a negative challenge. On the other hand, SPT was less frequently positive in the infants exposed to CM at home during the first 8 weeks than in those exclusively breast-fed: 38/120 (31%) vs. 63/118 (53%), (p=0.001).

Fifty infants with CMA developed their first adverse symptoms during exclusive breast-feeding; 18 of them had been given CM at maternity hospital (*Table 6*). Thus 32 infants were sensitised during exclusive breast-feeding. The IgE-positive infants were breast-fed longer: 8.4 (7.8-9.1) vs. 4.5 (3.5-5.7) months, (p<0.001).

Table 6. *Infant-feeding patterns in the IgE-positive and -negative groups of infants with CMA.*

	Number (%)		
Feeding pattern	IgE-positive n = 75	IgE-negative n = 43	χ^2 test p value
Exposure to CM at maternity hospital	31 (41)	12 (28)	0.15
Exposure to CM at home <8 weeks of age	30 (40)	26 (61)	0.03
Cumulative exposure to CM at 0-8 weeks of age	46 (61)	28 (65)	0.68
Symptoms during exclusive breast-feeding	37 (49)	13 (30)	0.04
Sensitised during exclusive breast-feeding	23 (31)	9 (21)	0.25

9.3.2 TGF-b1 in colostrum (II)

The mean concentration of TGF–β1 in the maternal colostrum of infants later developing IgE-positive CMA was lower than in samples from mothers of infants developing IgE-negative CMA (p=0.012, *t* test) (*Figure 6*). The level of the controls did not differ from either one. The mean concentrations of IgA, IgM and IgA CM antibodies and IL-6 and IFN–γ were similar in all 3 groups (data not shown). Of the 315 mothers whose colostrum samples were analysed, 255 (81%) had no atopy and 60 (19%) had obvious atopy. Maternal atopy had no influence on any parameters measured in colostrum (data not shown).

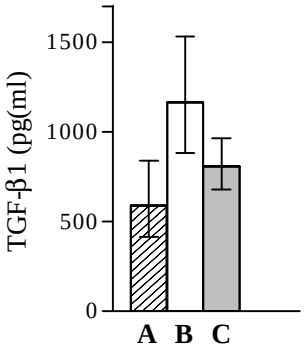

Figure 6. *Mean (95% CI) concentration of TGF-b1 in maternal colostrum of infants with IgE-positive (**A**, n=65) and IgE-negative CMA (**B**, n=37) and in controls (**C**, n=126), (p=0.015, ANOVA).*

The concentration of TGF–β1 correlated with the level of IgA BLG antibodies and with IgG α-casein and CM antibodies measured from the sera of the infants with CMA, whereas the diameters of SPTs to CM and the SIs to α-casein and to BLG correlated negatively with TGF–β1 (*Table 7*).

Table 7. *Correlations between the concentration of TGF-b1 in colostrum and the serum levels of antibodies to CM and its fractions, the diameters of the SPT to CM, and SIs to CM proteins in infants with CMA measured at the time of the CM challenge.*

Measurement	n	Correlation coefficient	p value
IgA antibodies to β-lactoglobulin	100	0.204	0.04
IgG antibodies to β-lactoglobulin	99	0.184	0.07
IgA antibodies to α-casein	100	0.138	0.17
IgG antibodies to α-casein	99	0.237	0.02
IgA antibodies to whole CM formula	100	0.165	0.10
IgG antibodies to whole CM formula	99	0.240	0.02
Total serum IgE	100	-0.168	0.09
IgE antibodies to CM	96	-0.138	0.18
SPT to CM	102	-0.228	0.02
SI to α-casein	54	-0.282	0.04
SI to β-casein	54	-0.241	0.08
SI to β-lactoglobulin	54	-0.347	0.01

9.3.3 Multivariate analysis of risk factors for IgE-positive CMA (II, III)

The risk of developing IgE-positive CMA was increased by exposure to CM at the maternity hospital, and by no exposure or exposure to only small amounts of CM at home during the first 8 weeks of life (*Table 8*). The risk was also increased by long breast-feeding.

The prevalence of a family history of atopy was 40% in the IgE-positive and 44% in the IgE-negative group. In the IgE-negative group, more mothers smoked: 7 (16%) vs. 2 (3%) ($p<0.01$). The prevalences of siblings and furred pets were similar in the 2 groups.

Table 8. *Effects of dietary and other environmental and genetic factors on the risk of an IgE-positive reaction to CM at the time of diagnosis in infants with CMA.*

Variable	Reference category	OR (95%CI)	p value
Exposure to CM while in hospital	none	3.5 (1.2-10.1)	0.02
Total amount of CM formula at home during the first 8 weeks			
- No exposure	>1.5 l	5.1 (1.6-16.4)	0.006
- 0.01 l to 1.5 l	>1.5 l	5.7 (1.5-21.6)	0.01
Breast-feeding for 7.5 months or longer	<7.5 months	3.9 (1.6-9.8)	0.004

Factors tested but found not to be significant were age at first exposure to CM at home, a family history of atopy, maternal smoking, the presence of siblings and furred pets and the content of TGF-β1 in colostrum.

10. DISCUSSION

10.1 Development of CMA

10.1.1 Cumulative incidence

When strict criteria for diagnosis were applied, i.e. a positive response to challenge by the study protocol, the cumulative incidence of CMA in the whole cohort was 1.9%, a percentage in good accord with earlier studies on unselected infants using similar criteria for diagnosis (Jakobsson and Lindberg 1979, Høst et al. 1988). In addition, according to the data obtained from the Social Insurance Institution, CMA was diagnosed elsewhere in 81 infants, giving a cumulative incidence of 3.2%. This is also comparable with earlier reports (Høst 1994) and is close to the assumed percentage of 3.5 used in calculations of sample size (Savilahti et al. 1991). This latter study comprised only 200 infants. Although the incidence of atopy in general is rapidly increasing (Holgate 2000), no increase in the incidence of CMA has been found from the 1950's to the 1990's (Høst 1994).

10.1.2 Feeding of CM formula

This prospective study comprised an unselected group of 6209 healthy, full-term newborn infants, 5385 of whom (87%) were given supplementary feeds while in hospital. Among these infants, exposure to CM for an average of 2 days after birth increased the risk of subsequent CMA. The ill effects of early exposure to CM may be due to the immaturity of the infants' gastrointestinal tract, for its permeability is highest during the first few days of life (Kuitunen et al. 1994). Furthermore, the immune system of newborn infants is functionally immature, Th2 skewed and the capacity to produce INF-γ (Th2 suppressor) and IL-4 (TGF-β enhancement) is undeveloped, especially in those with atopic heredity (Koning et al. 1996, Prescott et al. 1999). Thus, early contact with an antigen may accentuate the prevailing Th2 response and lead to sensitisation in susceptible individuals.

Very early exposure to CM, i.e. at the maternity hospital, seemed to be the only factor that increased the risk of CMA. Similar percentages of infants in the randomised groups were given CM at home during the first 8 weeks: 54% of the allergic and 55% of the tolerant infants were exposed. The permeability of the gut decreases rapidly after birth, absorption of α-lactalbumin being only 19% at 1 month and 5% at 2 months of that measured 3-4 days post partum in healthy infants (Kuitunen et al. 1994). In a rat model, early feeding of CM delayed the

normal decrease in gut permeability (Arvola et al. 1993). We suggest that, for the development of oral tolerance in full-term infants, the first few days after birth constitute the most critical period.

In addition, development of an immune response is influenced by the dose of the antigen (Hanson et al. 1996, MacDonald 1998). Our results suggest that larger amounts of CM are more likely to induce tolerance than smaller amounts: in the CM group, the allergic infants had been exposed to smaller amounts of formula at hospital and also at home during the first 8 weeks than the tolerant ones. Oral tolerance may develop during the neonatal period (Hanson et al. 1995) or later during the first year of life (Vaarala et al. 1995) and is promoted by administration of large amounts of antigen (Fritsché et al. 1997).

10.1.3 Feeding of whey hydrolysate formula and pasteurised human milk

Infants given either whey hydrolysate formula or pasteurised human milk at the maternity hospital developed CMA less often. The percentage of infants exposed to CM formula at home during the first 8 weeks was similar in the 3 randomised groups. Furthermore, the prevalence of atopic heredity in these groups was comparable. Thus, the only significant difference between the groups was the type of early feeding at hospital. Feeding of infants at high risk of atopic diseases with extensively hydrolysed formula for several months after birth has been found to reduce the risk of food allergies (Chandra et al. 1989a, Halken et al. 1992, Zeiger and Heller 1995). These formulas contain hardly any antigenic material (Mäkinen-Kiljunen and Sorva 1993), and serum IgE concentrations were lower in the infants given extensively hydrolysed formula for the first 3 days of life than in those fed human milk or CM (Juvonen et al. 1996). The allergenicity of several other extensively hydrolysed whey- and casein-based formulas is as low as that of Pepti-Junior® used in this study (Businco et al. 1993) thus, similar result would probably have been achieved by using any of these. Although human milk contains measurable concentrations of BLG (Sorva et al. 1994), pasteurised human milk, as used in the present study, may be less immunogenic, because pasteurisation denatures many antigenic proteins (Lee 1992, Fiocchi et al. 1998) but does not completely destroy its secretory IgA (Ford et al. 1977). The extensively hydrolysed formula proved to be at least as efficient in preventing CMA as pasteurised human milk, which at present is the preferred supplement in Finnish maternity hospitals.

10.1.4 Breast-feeding

Surprisingly, the incidence of CMA in the infants exclusively breast-fed at hospital was similar to that among the infants given CM at hospital. In a recent

study, breast-fed infants who were exposed to either CM or placebo during the first 3 days of life were at similar risk of developing allergies by the age of 2 years (de Jong et al. 1998). In contrast, in the study of Høst et al., none of the 210 infants solely breast-fed while in the hospital developed CMA (Høst et al. 1988). In our study, all but 1 of the 17 allergic infants who were exclusively breast-fed at hospital continued on exclusive breast-feeding for at least the first 8 weeks of life. Exclusive breast-feeding for at least 1 month after birth has been shown to prevent food allergy at 1 to 3 years of age (Saarinen and Kajosaari 1995). In contrast to the randomised groups, this self-selected comparison group was formed on the basis of the mother's ability to breast-feed her infant fully while in hospital. These mothers were non-smokers and more often multiparous, factors associated with a reduced risk of atopic diseases (Zeiger 1990, Strachan 1996) and an increased rate of successful breast-feeding (Feinstein et al. 1986). Moreover, a positive parental history of atopy was not more frequent in the comparison group than in the randomised groups. Nor were there any differences in the composition of maternal colostrum between the 4 groups or as compared with the samples of control mothers (data not shown). In fact, there is no good explanation for the surprisingly high incidence of CMA in the group of infants exclusively breast-fed at hospital.

The small amounts of CM proteins present in breast milk (Axelsson et al. 1986) may elicit a reaginic reaction to CM (Jarrett 1984, Isolauri et al. 1999). Furthermore, infants with a positive atopic heredity may react to the minute amounts of food antigens in breast milk (Hattevig et al. 1990), and the incidence of food allergies (Zeiger and Heller 1995) and development of specific IgE antibodies (Hattevig et al. 1990) were reduced when both the nursing mother and her infant avoided CM. In the present study, 50 infants showed their first symptoms during exclusive breast-feeding, but 18 of these had been exposed to CM at hospital. Thus, at least 32 infants (0.5% of the whole study population) were probably sensitised to CM proteins present in breast milk. However, other routes of sensitisation, such as inhalant exposure to food antigens, are also possible (Witteman et al. 1995, Vaswani et al. 1999). A similar incidence (0.4%) of sensitisation to CM during exclusive breast-feeding was reported in another study (Jakobsson and Lindberg 1979). In the present study, some mothers eliminated CM from their diet after the infant's symptoms had developed, but none avoided CM initially. We infer that CM antigens in breast milk are capable of both sensitising the infant and triggering an adverse immune reaction to CM.

10.1.5 Family history of atopy and environmental factors

A positive history of obvious parental atopy, which is known to be a risk factor for CMA (Høst 1994), was the most significant risk factor in this study also increasing the risk of CMA by 2.3 times. In earlier Finnish studies, the

prevalence of atopy in infancy was twice (Savilahti et al. 1987) to 3 times (Pöysä et al. 1989) as frequent in infants with a positive atopic heredity than in those without such heredity. In infants with atopic heredity, functional maturation of T cells is delayed and IFN-γ production reduced, both being features associated with the development of allergies (Björkstén 1999).

The incidence of CMA was highest in the infants born in autumn, i.e. between September and November. Similar results have been published earlier (Aalberse et al. 1992, Nilsson et al. 1997). However, the reason for this seasonal effect still remains unexplained.

10.1.6 Immune factors in colostrum

We found no differences between the levels of total IgA or CM-specific IgA antibodies in colostrum samples from the 118 mothers of infants developing CMA and from 207 mothers of tolerant infants. This disagrees with the earlier findings (Machtinger and Moss 1986, Savilahti et al. 1991). In these 2 studies the number of samples from mothers of allergic infants was small, only one-tenth of that in the present study or even fewer. Neither did we find we any differences between the mothers of allergic and of tolerant infants in the concentrations of total IgM, TGF-β1, IFN-γ or IL-6. We measured IL-6 because it is an important switch factor for IgA, and IFN-γ because it directs T cell differentiation towards type 1, promoting cell-mediated reactions (Mosmann and Sad 1996). (TGF-β1: see section 10.2.2.) The mother's own allergy had no effect on the levels of immunoglobulins, CM antibodies or cytokines, as was also found recently by Rudloff et al. (1999).

10.2 Development of immune responses in infants with CMA

10.2.1 CM formula and breast-feeding

The present series of 118 infants with CMA showed that different feeding patterns during the first few months of life modulate the subsequent hypersensitivity reaction to CM: breast-feeding exclusively or combined with infrequent exposure to small amounts of CM during the first 8 weeks of life was associated with development of IgE CM antibodies, whereas infants showing reactions not associated with specific IgE antibodies had been exposed to larger volumes of CM more frequently. The latter infants had received, on average, almost 4 times as much CM daily, and the cumulative volume was 10 times as great. In animal studies, feeding small amounts of antigen stimulated IgE production (Jarrett 1984), whereas the specific IgE response was suppressed by a larger antigen dose, the degree of suppression depending on the dose (Fritsché et

al. 1997, Pecquet et al. 1999). Previously, in a group of 23 infants with CMA, the highest concentrations of specific IgE antibodies were found in those who had been exposed to the smallest volumes of CM formula before diagnosis, and infrequent exposure to CM was correlated with development of IgE CM antibodies (Firer et al. 1981). Similarly, we found that the infants who were IgE-positive had received formula less frequently during the first 8 weeks of life than those who were IgE-negative.

The neonatal period is the critical period for induction of a specific immune response to dietary antigens (Hanson et al. 1995). Two-thirds of the infants in both groups were given CM during the first 8 weeks of life, but those in the IgE-positive group tended to have been exposed earlier. Exposure to CM at hospital, when followed by exclusive breast-feeding or infrequent exposure to small amounts of CM formula, significantly increased the risk of developing IgE CM antibodies. It has been speculated that feeding low doses of antigen induces the development of oral tolerance by active suppression characterised by secretion of TGF-β by Th3 cells and of IL-4 and IL-10 by Th2 cells (Weiner 1997). We hypothesise, that in susceptible neonates, TGF-β secretion is defective and, if it is not sufficiently compensated by TGF-β in colostrum (see section 10.2.2), exposure to small amounts of CM leads to an enhanced immune response of IgE type.

Both those infants who were sensitised during exclusive breast-feeding and those who showed symptoms during exclusive breast-feeding but had been exposed to CM at hospital were more frequently IgE-positive. This further confirms that small amounts of CM favour the development of an IgE response. The IgE-positive infants were breast-fed for almost twice as long as the IgE-negative infants, which accords with earlier studies showing a correlation between the length of breast-feeding and the development of specific (Kaplan and Solli 1979) and total IgE (Juto and Björkstén 1980).

SPT positivity to CM correlated with hospital exposure to CM, even among the infants with a negative challenge. In healthy infants, a humoral immune response to CM proteins was strongest when CM had been introduced early during the neonatal period (Tainio et al. 1988). A transient low-level IgE response is regarded as a normal sign of initial antigen contact when followed by antigen-specific immunological tolerance (Hattevig et al. 1984, Koning et al. 1996).

10.2.2 TGF-b1 in colostrum

This is the first study to show that a cytokine present in colostrum, TGF-β1, may play an important role in determining the intensity and type of CM specific immune response in infants prone to CMA. The mean TGF-β1 concentration in maternal colostrum of the infants later developing IgE-positive CMA was

significantly lower than in that of the infants developing IgE-negative CMA. This association was reinforced by the negative correlation between the concentration of TGF-β1 in colostrum and the strength of the SPT in infants with CMA. Furthermore, in the infants with CMA, the concentration of colostrum TGF-β1 was correlated negatively with the reactivity of the PBMCs to CM protein fractions. Our findings are in accord with the results of another Finnish group published shortly after ours (Kalliomäki et al. 1999). In that study the concentrations of both TGF-β1 and TGF-β2 were lower in the maternal colostrum of infants showing symptoms of atopic dermatitis during exclusive breast-feeding than in that of those who had symptoms only after weaning. The authors suggest that TGF-β in colostrum may prevent the development of atopic disease during exclusive breast-feeding.

The concentrations of TGF-β1 that we found were of the same magnitude as those found by Saito and colleagues (1993). They reported that, of the total amount of TGF-β in the colostrum samples, about half was in the active form (Saito et al. 1993). Very little is known of the function of TGF-β in breast milk. In mice, feeding human milk or TGF-β had similar effects on antibody production against sheep red cells: depending on the route of delivery of the red cells, the response was either suppressed or fortified (Ishizaka et al. 1994). TGF-β is a major factor in the development of hyporesponsiveness to oral proteins (Weiner 1997) and, in mucosal membranes, may regulate inflammation and antagonise IFN-γ (Strober et al. 1997). Homozygous TGF-β1 null mice die of devastating autoimmune disease soon after weaning, presumably having been rescued until that age by transfer of maternal TGF-β1 across the placenta and in the mothers' milk (Letterio et al. 1994). That study suggested that TGF-β1 is absorbed from the milk and distributed to several organs. Thus, TGF-β1 could have both local effects on the intestine and generalised effects on the immune system. TGF-β1, even in very low concentrations, strongly inhibits proliferation of T lymphocytes (Kehrl et al. 1986, Ahuja et al. 1993). This type of systemic effect is suggested by our findings of reduced proliferation of PBMCs to CM proteins in infants with CMA in whose mothers' colostrum there was a high concentration of TGF-β1.

However, a low concentration of TGF-β1 may not be the sole factor predisposing to CMA. We found no difference in the TGF-β1 content of maternal colostrum between infants later developing CMA and tolerant infants, as also recently reported (Kalliomäki et al. 1999). According to our results, a low level of TGF-β1 in colostrum favours the development of an IgE-mediated reaction to CM; whereas, at a higher level, other types of abnormal immune reaction prevail, or at least IgE has a less important role. This accords with the role of TGF-β in participating in the class switch of immunoglobulins. It inhibits IgE switching (Stavnezer 1995) and directs switching to IgA (Ehrhardt et al. 1992). Both we and the other group (Kalliomäki et al. 1999) found an interesting positive correlation between the TGF-β concentration in colostrum and the

infants' IgA responses to CM proteins. Both studies suggest that TGF-β in colostrum enhances the subsequent development of an IgA response to food antigens.

10.3 Diagnosis of CMA

10.3.1 Clinical and immunological features at challenge

Most infants show adverse symptoms to CM during the first year of life (Bock and Sampson 1994), in the present study the mean age of challenged infants was 7 months. As in earlier studies (Dannaeus and Johansson 1979, Hill et al. 1986, Baehler et al. 1996), most of the infants in the IgE-positive group showed symptoms within 2 h of challenge, the most frequent rapidly developing symptoms being urticaria and exanthema. Similar percentages of infants in the IgE-positive and -negative groups reacted to the challenge between 2 to 24 h, but their symptoms differed: of the IgE-positive infants, 68% had urticaria or exanthema, whereas, of the IgE-negative infants, 71% exhibited erythema and pruritus as early symptoms of atopic dermatitis. Most of the delayed reactions were seen among the IgE-negative infants and were associated with eruption of atopic dermatitis and gastrointestinal symptoms, as described in earlier studies (Dannaeus and Johansson 1979, Hill et al. 1986, Baehler et al. 1996). However, an increasing number of studies have shown that specific IgE antibodies are implicated in the late-phase reactions of atopic dermatitis (Eigenmann et al. 1998). In the present study, 5 infants in the IgE-positive group reacted only after 24 h, all with eruption of atopic dermatitis, corresponding to the findings of Hill et al. (1986). Five infants with multiple symptoms at challenge developed allergic conjunctivitis, which is a rare but recognised symptom of CMA (Sampson 1997). Anaphylactic reactions to foods are rare (Sampson et al. 1992) but may occur on exposure to CM (Goldman et al. 1963). Therefore, in 6 infants we based our diagnosis on a positive SPT to CM combined with a history of serious symptoms. In spite of this, 1 infant unexpectedly had an anaphylactic reaction at challenge.

10.3.2 Predictability of challenge outcome from the history and immunological tests

None of the 4 tests, alone or in a combination, were able to predict CMA satisfactorily. Parallel use of an SPT, CM-specific IgE, a patch test and ECP at low cut-off levels detected 85% of the infants with CMA with a poor specificity of 0.23; at high cut-off levels the respective figures were 54% and 0.94.

Of the individual tests, serum CM-specific IgE at low levels (≥0.35 kU/l) gave the best value for sensitivity, detecting 72% of the challenge-positive

infants, but 51% of the responses were false-positive (poor specificity). The SPT at a cut-off level ≥3 mm produced the best value for overall agreement (0.69; overall agreement is the total proportion of patients correctly classified by a given test) with a sensitivity of 0.61 and a specificity of 0.76. This accords with an earlier study, in which the predictive indices of the SPT to different foods at a cut-off level of 3 mm were comparable to indices derived at higher cut-off levels (Eigenmann and Sampson 1998). When we used all 4 tests in parallel (a positive reaction to at least 1 test being considered to be a positive response), 73% of the patients were correctly classified with a combination of SPT ≥3 mm, CM-specific IgE ≥0.7 kU/l, a positive reaction to CM protein fractions in the patch test and ECP ≥20 µg/l, with a sensitivity of 0.76 and specificity of 0.67. The parallel use of several tests improved the detection rate, probably because several immunological mechanisms are involved in CMA (Sampson 1997). The 4 tests used in parallel detected 94% of the infants with immediate reaction, but as also shown earlier (Tainio and Savilahti 1990, Vanto et al. 1999), the tests usually failed to detect those challenge-positive infants who had delayed reaction with gastrointestinal symptoms.

The rate of false-positive reactions (1-specificity) to an individual test varied from 2% to 51%, depending on the test and cut-off level used. Low levels of circulating CM-specific IgE antibodies detected in 51% of the infants with a negative challenge may reflect the natural maturation of the immune system. This agrees with the theory that the neonatal immune system is Th2 skewed (Prescott et al. 1999) and with that transient low-level IgE responses to food antigens are commonly seen during the first year of life and do not always signify a clinical disease (Hattevig et al. 1984). Moreover, some of the infants in the present study may have lost their clinical reactivity before the challenge, which was performed only when the infant required supplementary milk, i.e. independently of the time of appearance of symptoms. The time taken to overcome food hypersensitivity is often only a few months (Bock 1987), whereas immunological reactivity often lasts beyond the development of clinical tolerance (Hill et al. 1994).

The sensitivity of the skin-patch test ranged from 0.26 to 0.43 and the specificity from 0.72 to 0.92, depending on the antigens used, with whole CM those being of the same magnitude as previously reported (Majamaa et al. 1999b). We found a positive correlation between patch test results and measurements of specific IgE, as also found earlier (Darsow et al. 1997, Vanto et al. 1999). A positive reaction to a patch test tended to correlate with the immediate-type reaction at challenge whereas, in other studies, positive patch-test responses have been associated with delayed-type reactions to CM (Räsänen et al. 1992, Isolauri and Turjanmaa 1996) or no associations have been found (Vanto et al. 1999). When CM protein fractions were used as antigens, good specificity (0.92) was achieved with a low sensitivity of 0.26, as reported earlier (Räsänen et al. 1992). When the patch test was used in parallel with other tests,

the detection rate of CMA increased, as previously suggested (Räsänen et al. 1992, Isolauri and Turjanmaa 1996).

Serum ECP, measured on challenge day 4, distinguished poorly between the challenge-positive and -negative infants. Although the increase in serum ECP during the challenge was significant in infants with a positive but not in those with a negative challenge, the test did not discriminate between the challenge-positive and -negative infants at low cut-off levels. However, the small number of high values (≥24.7 µg/l) were mostly confined to the infants with a positive challenge. Others have measured the post-challenge ECP earlier than we did in the present study (Niggemann et al. 1994, Suomalainen et al. 1994). In the study of Niggemann et al. (1994) on infants with food-sensitive atopic dermatitis, serum ECP reached a maximum 24 h after a positive food challenge and by 48 h after a clinical reaction had returned to the baseline value. We measured the post-challenge ECP 96 h after the start of the challenge. Therefore, the difference in pre- and post-challenge ECP values might have been greater if the second samples had been taken earlier. The late second visit in our study was selected to ensure that we found all the patients with a delayed reaction. In fact, in the present study the slowest symptoms appeared 90 h after the beginning of the challenge.

The history of symptoms suggestive of CMA reported by the parents did not predict the challenge outcome. The occurrence of urticaria produced the best value for overall agreement (0.62) with a sensitivity of 0.46 and a specificity of 0.77. A positive challenge was more likely in infants with early appearance of adverse symptoms. Among the infants with a positive SPT, a dermal CM challenge produced a sensitivity of 0.46 and a specificity of 0.85. Earlier, in older infants with a positive SPT and suspicion of CMA not confirmed by a challenge test, a higher reaction rate of 69% to dermal CM challenge was reported (Salo et al. 1986).

10.4 Methodological aspects

The study population was collected during a 16-month period in 3 maternity hospitals in the Helsinki region. Infant-feeding habits differ between the provinces of Finland (Hasunen et al. 1996). The present data on infant-feeding practices reflect the situation in the province of Uusimaa in 1994-1995. The study was explained to the mothers immediately after delivery, when many of them were exhausted, especially those who had had a Caesarean section. In these circumstances, a rapid decision to participate may have felt impossible, which probably accounts for the quite low participation rate of 41%. However, more than 1700 infants were enrolled in each randomised group, which exceeded the projected sample size of 1640. Any seasonal effects, and possible inter-hospital

variation were eliminated by using a different milk supplement in each hospital at the same time and changing the supplement monthly in each hospital.

All the results of this study are based on classification of infants as having or not having CMA. Interpretation of the challenge outcome, especially that of delayed reactions, is difficult (Hill and Hosking 1996) and even specialists may have different opinions (Vanto et al. 1999). In this study, all the patients were investigated by a single observer (KMS) and emphasis was placed on discovering the infants with delayed symptoms. Even so, in 8 infants the diagnosis was uncertain: they were challenged according to the study protocol and diagnosed as not having CMA but, according to the register of the Social Insurance Institution, they were later diagnosed elsewhere as having CMA. This reflects the difficulty of diagnosing CMA or disagreement with the parents about the diagnosis (Kaila and Isolauri 1997). We were not able to investigate why the parents of the 73 infants diagnosed as having CMA elsewhere did not contact us. We can only assume that some families had moved from the area, or the others may have forgotten their participation in the study when they were struggling with the infant's adverse symptoms. Moreover, some initial symptoms may not have been recognised by the parents as suggestive of CMA, which may have led to contact with medical personnel elsewhere.

Although the optimal test would be a double-blind placebo-controlled food challenge (Bock et al. 1988), we performed 237/247 CM challenges openly, since in this age group open and double-blind challenges have produced similar reaction rates (Isolauri and Turjanmaa 1996). In 10 randomly selected infants we used the double-blind placebo-controlled method. Of these challenges, 50% were positive, a reaction rate equal to that of the open challenges performed by us.

The presence of skin-fixed and circulating CM-specific IgE antibodies was assessed at the time of diagnosis (mean age: 7 months). However, classification of the CM-provoked reactions as IgE-positive or -negative according to the tests used may not have been entirely valid, since the sensitivity of these tests is lower in infants under 1 year of age (Høst and Halken 1990, Bock and Sampson 1994). Moreover, some patients with negative skin and blood tests for specific IgE have been found to have a local intestinal IgE response to CM (Caffarelli et al. 1998).

The data on early infant feeding were prospectively collected in 2 questionnaires. The personnel in the hospitals rapidly became used to recording supplementary feeds as part of their daily routine. There were no differences in recording or in administration between the 3 study supplements. All the parents of infants with CMA and 76% of those of tolerant infants returned the data on infant feeding recorded at home during the first 8 weeks. The symptoms suggestive of CMA appeared (mean: 3 months) near to the time of returning the questionnaire, which probably served as an extra reminder to the parents of allergic infants. However, similar proportions of the parents of tolerant infants in the 4 groups returned the records. Nor do we see any reason why the parents of

exclusively breast-fed, partially breast-fed and fully bottle-fed infants should return the records differently. Thus, we regard the hospital and home records as comparable within and between the groups.

Data on a family history of atopy were recorded at hospitals soon after birth and were available for 100% of the study participants. We collected these data again at 6 months of age (questionnaire returned by 77%), by which age, most of the CMA-prone infants had already developed the first symptoms suggestive of CMA. This knowledge may have increased parental recall of past symptoms of atopy and so have caused bias (Odelram et al. 1995). We therefore elected to use only the data collected in the hospital, i.e. before the infants showed atopic symptoms.

10.5 Recommendations and future aspects

The results of the present study show that, to prevent CMA, feeding healthy newborn infants with CM should be avoided during the first few days of life. We suggest that, if supplementary feeds are needed, extensively hydrolysed whey- or casein-based formula or pasteurised human milk should be used in the maternity hospital. However, after the first few days of life, adapted CM-based formula can be used without increasing the risk of CMA as compared with exclusive breast-feeding. The same is recommended for infants with a positive family history of atopy, who are at even higher risk of developing CMA. Breast-feeding of all newborn infants should be promoted because of the multiple benefits related to it but, as our results show, exclusive breast-feeding cannot be argued to prevent CMA.

The effects of early feeding practices on the development of other atopic diseases in this cohort are also being evaluated. In future studies, the content of immunoactive and growth-promoting agents in breast milk, such as TGF-β1, should be evaluated in serial samples to further determine the role of these factors in the development of allergies. The development of oral tolerance and the critical period for this in infants needs to be studied in greater detail. In addition, the amount of food allergen seems to be critical: the development of CMA, especially when IgE-mediated, was related to exposure to small amounts of CM. Further human studies, with measurements of the degree of exposure, are needed to confirm these findings. The immunological mechanisms responsible for the development of delayed-type CMA need to be verified in future studies; the characteristics and cytokine profile of T cells are of especial interest.

11. CONCLUSIONS

This prospective study on CMA explored the incidence, aetiological factors and clinical and immunological findings in relation to infant feeding practices. The study comprised a cohort of 6209 unselected, full-term infants who were given 1 of 3 supplementary feeds or were exclusively breast-fed at the maternity hospital and were followed prospectively for the development of CMA. The main conclusions of the study are:

1. Healthy newborn infants who are exposed to CM formula while in hospital after birth are at higher risk of developing CMA than those given extensively hydrolysed whey formula or pasteurised human milk. Feeding of larger amounts of CM is more likely to induce tolerance than feeding of smaller amounts.

2. A positive parental history of obvious atopy is a significant risk factor for CMA: in the present cohort the risk was 2.3 times higher.

3. Exclusive breast-feeding at the maternity hospital and at home during the first 8 weeks of life does not reduce the risk of CMA. Symptoms of CMA may develop even during exclusive breast-feeding.

4. Short exposure to CM at the maternity hospital and prolonged breast-feeding exclusively or combined with infrequent intake of small amounts of CM stimulates specific IgE antibody production in infants who are prone to develop CMA, whereas frequent feeding with larger volumes of CM induces development of the non-IgE-mediated delayed-type hypersensitivity to CM.

5. The cytokines in human milk may be of functional importance for the immune responses of infants. In infants prone to develop CMA, TGF-β1 in colostrum inhibits cell- and IgE-mediated reactions and enhances production of IgG and IgA antibodies to the proteins in CM.

6. In infants under 1 year of age, the CM challenge outcome cannot be predicted by a history of adverse symptoms or by the SPT, the patch test, serum ECP or CM-specific IgE antibodies. A combination of these 4 tests used in parallel detected 76% of the patients with a specificity of 0.67. Therefore, it is essential that a diagnosis of CMA is confirmed by a clinical CM elimination-challenge test.

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