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Section 2: Methodology

The outline of the consensus guideline was the result of the considered opinion of the whole panel. Narrative parts, that is, sections 1-8, 9-13, 15-17, and 19 included the relevant CMA literature as searched using the algorithms reported in Appendix 1. For these sections, the relative weight of the suggestions retained for the purpose of DRACMA reflects the expert opinion of the panel. They may contain general indications, but no evidence-based recommendations. The consensus on these indications was expressed by the panelists using a checklist itemizing the clinical questions considered relevant after analysis of the literature. The panel decided to use a GRADE methodology for defining some treatments and diagnostic questions.

The DRACMA worked with the GRADE members on this panel the clinical questions and their scope after various fine-tuning stages. The GRADE panelists independently searched the relevant literature for sections 9, 14, 18. Their analysis was independent of the other panel lists. For question formulation, guideline panel members explicitly rated the importance of all outcomes on a scale from 1-9, where the upper end of the scale (7–9) identifies outcomes of critical importance for decision making, ratings of 4-6 represent outcomes that are important but not critical and ratings of 1-3 are items of limited importance. Evidence summaries were prepared following the GRADE Working Group's approach (1–6) based on systematic reviews done by an independent team of the GRADE Working Group members (JLB and HJS supported by 5 research associates).

The GRADE approach suggests that before grading the quality of evidence and strength of each recommendation, guideline developers should first identify a recent well-done systematic review of the appropriate evidence answering the relevant clinical question, or conduct one when none is available. This should be followed by preparing a transparent evidence summary, such as creation of GRADE evidence profiles, on which the guideline panel will base their judgments (7). We prepared 3 systematic reviews addressing the clinical questions covered by the guideline (about the diagnosis, use of formula and immunotherapy of the CMA). We searched MEDLINE, EMBASE, and the Cochrane Library (including Cochrane Central Register of Controlled Trials, DARE, NHS EED) for relevant studies. We included studies published up to September 2009. We developed GRADE evidence profiles (summary of findings tables) for the clinical questions based on the systematic reviews. The summaries of evidence were reviewed by the panel members and corrections and comments were incorporated.

We assessed the quality of the evidence according to the methodology described by the GRADE system (1–3, 8). In this system quality of supporting evidence is assessed based on explicit methodological criteria and classified as either "high," "moderate," "low," or "very low."

The DRACMA guideline panel reviewed the evidence summaries and the draft guidelines,

and made recommendations. We reached consensus on all recommendations. Formulating the recommendations included explicit consideration of the quality of evidence, benefits, harms, burden, cost, and values and preferences described as the "Underlying values and preferences" or in the "Remarks" sections of each recommendation as outlined earlier (9). Statements about the underlying values and preferences and the remarks are integral parts of the recommendations and serve to facilitate accurate interpretation of the recommendations. They cannot be omitted when citing or translating DRACMA guidelines. In this document, the expression "values and preferences" refers to the relative weight one attributes to particular benefits, harms, burdens, and costs to determine their balance. We used the decision framework described previously to determine the strength of recommendations (1, 10).

Little information about costs of diagnosis and treatment of IgE-mediated cow's milk allergy was available to the panel and it is very likely that it varies considerably across geographical areas and jurisdictions. Cost, therefore, plays a limited role in these recommendations. However, whenever we considered cost and resource expenditure, we used health system perspective (11). For individual patients, cost may not be an issue if the service or treatment strategy is provided at reduced price or free of charge. Clinicians and patients should consider their local resource implications when interpreting these recommendations.

After the GRADE approach we classified recommendations in these guidelines as either "strong" or "conditional" (also known as weak)/weak. The strength of recommendations depends on a balance between all desirable and all undesirable effects of an intervention (ie, net clinical benefit), quality of available evidence, values and preferences, and cost (resource utilization) (1). In general, the higher the quality of the supporting evidence, the more likely it is for the recommendation to be strong. Strong recommendations based on low or very low quality evidence are rare, but possible (12).

For strong recommendations we used words "we recommend" and for conditional recommendations, "we suggest." We offer the suggested interpretation of "strong" and "weak" recommendations in Table 2-1. Understanding the interpretation of these 2 grades (strong or conditional) of the strength of recommendations is essential for clinical decision making.

Table 2-1. Interpretation of "Strong" and "Weak" Recommendations

Implications	Strong Recommendation	Weak Recommendation
For patients	Most individuals in this situation would want the recommended course of action and only a small proportion would not. Formal decision aids are not likely to be needed to help individuals make decisions consistent with their values and preferences	The majority of individuals in this situation would want the suggested course of action, but many would not.
For clinicians	Most individuals should receive the intervention. Adherence to this recommendation according to the guideline could be used as a quality criterion or performance indicator.	Recognize that different choices will be appropriate for individual patients, and that you must help each patient arrive at a management decision consistent with his or her values and preferences. Decision aids may be useful helping individuals making decisions consistent with their values and preferences.
For policy makers	The recommendation can be adapted as policy in most situations.	Policy making will require substantial debates and involvement of various stakeholders.

How to Use These Recommendations

The DRACMA guidelines are not intended to impose a standard of care for individual countries and jurisdictions. They should, as any guideline, provide a basis for rational decisions for clinicians and their patients about the management of cow's milk allergy. Clinicians, patients, third-party payers, institutional review committees, other stakeholders, or the courts should never view these recommendations as dictates. Strong recommendations based on high quality evidence will apply to most patients for whom these recommendations are made, but they may not apply to all patients in all circumstances. No recommendation can take into account all of the often-compelling unique features of individual clinical circumstances. Therefore, nobody charged with evaluating clinicians' actions should attempt to apply the recommendations from the DRACMA guidelines as rote or in a blanket fashion.

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Section 3: Epidemiology of CMA

Overview

There are no surveys of population and geographical trends in food allergy in adults or children (though the situation is different in pediatric asthma and rhinitis) and this unmet need is particularly felt for CMA. The *perception* of milk allergy is far more frequent than *confirmed* CMA. Patient reports of CMA range between 1 and 17.5%, 1 and 13.5%, and 1 to 4% in preschoolers, at children 5 to 16 years of age and adults respectively. Cow's milk-specific IgE sensitization point prevalence progressively decreased from about 4% at 2 years to less than 1% at 10 years of age in the German Multi-Centre Allergy Study. The most reliable data in epidemiology are those from birth cohorts that are free from selection bias. There are 5 such challenge-confirmed studies. The CMA prevalence during infancy ranged from 1.9% in a Finnish study, 2.16% in the Isle of Wight, 2.22% in a study from Denmark, 2.24% in the Netherlands, and up to 4.9% in Norway.

Patients with CMA develop gastrointestinal symptoms in 32 to 60% of cases, skin symptoms in 5 to 90%, and anaphylaxis in 0.8 to 9% of cases. This frequency of anaphylaxis is the main concern pointed out in many CMA studies. In a review, nearly one third of children with atopic dermatitis (AD) received a diagnosis of CMA after an elimination diet and an oral food challenge, and about 40 to 50% of children less than a year of age with CMA also had AD. Finally, with actual population and geographical trends remaining unknown, allergists are primarily in need of more detailed epidemiological surveys on a global scale. One large such epidemiological study supported by the European Commission is ongoing and aims to furnish the first prevalence data regarding the suspicion of CMA, sensitization to cow's milk, and oral food challenge-confirmed diagnosis in 10 European birth cohorts.

Introduction

Around 11–26 million of the European population are estimated to suffer from food allergy (1). If this prevalence was consistent around the world and projected to the 6,659,040,000 people of the world's population (2), it translates into 220–520 million people and represents a major global health burden. Although there are surveys on the natural history and prevalence trends for symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood (3), we do not have a study assessing the prevalence of food allergy and its time-trends. The problem is complicated by the fact that perceived food allergy (ie, the self-reported feeling that a particular food negatively influences health status) is not actual food allergy. Allergy prevalence is much greater in the public's belief than it has ever been reported by double-blind studies. Back in the 1980s, the perceived incidence of allergy to food or food additives in mothers with young children was reported between 17 (4) and 27.5% (5). Thirty percent of women reported that they or some

member of their family were allergic to some food product (6). In the after decade, a British study using a food allergy questionnaire reported a 19.9% incidence of food allergy (7). From the mid-1990s onwards, self reports began to be compared with challenge-confirmed diagnoses; reported incidence data of between 12.4 and 25% could be confirmed by oral food challenge in only 1.5 to 3.5% of cases, illustrating how reports of adverse reactions overestimate true food allergy (8, 9). This was further confirmed when prevalence figures of 2.3 to 3.6% were confirmed by challenge procedures in unselected patient populations (10, 11). In the 1990s, it was also confirmed that only a minority of subjects who report food-related illness also test positive by skin prick test using the same food (12).

Thus, 2 separate "food allergy epidemiologies" can be distinguished:

a. Self-reported food allergy; although this does not represent actual food allergy epidemiology, it is useful as a proxy measure of the potential demand for allergy medical services, and may guide public health allergy service users between general and specialist medicine (13), and more generally for public health planning.
b. Actual food allergy (ie, confirmed by a positive oral food challenge) represents the real extent of this clinical problem.

In general, food allergy is more frequent in the pediatric, rather than the adult, population. According to a recent Japanese multicenter trial, the prevalence of CMA is 0.21% in newborns and 0.35% amid extremely premature babies (< 1000 g) (14). Food allergies are a cause of particular concern for children. Incidence is estimated to be greater in toddlers (5-8%) than it is in adults (1-2%) (15–17). Earlier prospective challenge-based studies have shown that in a population of 480 newborns followed up in the setting of a U.S. general pediatric practice through their third birthday, a parental report of 28% food allergy translates into a challenge-confirmed CMA rate of 8% (18, 19), with 2.27 to 2.5% occurring in the first 2 years of life.

Perceived Cow's Milk Allergy

Similar considerations can be applied to cow's milk allergy perception. Self-report is common. In a large European survey of above 44,000 telephone contacts, 5 million European respondents claimed to be milk-allergic, with adult women as the group making most of these claims. There were also wide national differences ranging from 13.8% of reports from Greece to 52.3% from Finland. In this survey milk was the most often reported offending food in children (38.5% of reports) and the second food most often implicated by adults (26%) (20). In a group of 600 children less than 4 years, CMA was reported by the parents of 18 children (3%) (21). Milk reactions were reported by the parents of 2% of children without wheeze and by 16% of wheezers (22).

In the literature, the bulk of studies based only on self-reports of CMA is staggering, compared with reports that include an objective measure to assess the condition (23). Currently, at least a score of studies have evaluated the self-perception of CMA over the last 20 years in preschoolers (24–33), school-age children (5-16 years), (20, 34–38), and young adults (20, 39–45). From these studies, reviewed in the only meta-analysis in the field,35 the prevalence of self-reports varies between 1 to 17.5% in preschoolers, 1 and 13.5% in 5 to 16-year-olds, and between 1 and 4% in adults.

The children from these studies neither underwent sensitization testing nor oral food challenge. In a population of 6-year-olds, 1 out of 7 cases was based on self-reports whereas less than one out of 2 children with a positive cow's milk specific skin prick test was confirmed allergic by DBPCFC, thereby confirming that most parent-reported symptoms of CMA are unreliable (46). Not only parents, but also health care professionals, allergists, and nonallergists alike, cite cow's milk-induced reactions as the most common food allergy affecting children (47). Thus, the incidence of self-reports of CMA remains of interest for public health authorities, health maintenance organizations and the processed food industry as a metric for policy planning, planning diagnostic services; (48) tabling labeling legislation and even meeting the demand for milk-free products. However, as such, this proxy cannot represent the full extent of the clinical issues at stake.

Sensitization to Cow's Milk Proteins

The number of studies on CM sensitization in unselected populations is limited. The meta-analysis carried out by Rona and colleagues (23) identified 7 studies reporting a sensitization rate of 0.5 to 2% of preschoolers, of 0.5% at 5 to 16 years of age, and in less than 0.5% of adults (23, 25–33). In a later cohort of 543 children from the Isle of Wight followed-up from birth and tested at 1, 2, and 3 years of age, a positive milk sensitization test was found in 2 infants at 12 months (0.37%), in 5 at 2 years (0.92%), and in 3 at 3 years

(0.55%) (49). In the German Multicenter Allergy Study, 1314 children initially recruited were followed from birth for 13 years. The longitudinal data were analyzed for 273 children testing positive for serum cow's milk specific IgE antibody and were obtained at age 2, 5, 7, and 10. The point prevalence of sensitization to cow's milk progressively decreased from about 4% at 2 years to less than 1% at 10 years (50).

Epidemiology of Challenge-Confirmed CMA

The epidemiology of oral food challenge-confirmed CMA of the last 10 years consists of the following 5 studies:

a. In a Danish study of 1,749 newborns followed for 12 months, 39 (or 2.22%) were confirmed allergic (51)
b. In a study from Finland 6,209 newborns followed for 15 months, 118 (1.9%) had positive DBPCFC (52)
c. In a Norwegian study of 193 premature and 416 full-term infants, 27 of 555 (or 4.9%) were diagnosed with an allergic reaction to cow's milk on the basis of an open challenge but not all children were tested; interestingly, all had symptoms before 6 months of age (53)
d. In an Isle of Wight cohort of 969 newborns followed for 12 months, 21 (2.16%) reported CMA but only 2 (0.21%) were actually with IgE-mediated CMA (54)
e. In a newborn cohort from the Netherlands 1,158 infants prospectively followed through 12 months of age reporting "cow's milk protein intolerance" (defined as two positive cow's milk elimination/challenge tests) reported 26 allergic children (or 2.24%) of 211 (or 18.2%) suspected cases (33).

In this series of challenge-based studies, the Danish study further suggested that reproducible clinical reactions to CMP in human milk were reported in ~0.5% of breast-fed infants (55). Data from cross-sectional studies (analyzed by Rona and coworkers (2)) demonstrated a rate of 0.6 to 2.5% prevalence in preschoolers, 0.3% at 5 to 16 years of age, and of less than 0.5% in adults (23, 56–58).

While most of our information on cow's milk allergy prevalence comes from northern European and Spanish studies, there are methodological and geographical differences in clinical evaluation, which must be considered in assessing the epidemiological features we discuss here. Some studies may consider only immediate reactions, while others include delayed reactions; not all studies include IgE sensitization assessments; some studies are based on open oral food challenges, some performed blinded oral food challenge tests. Methods used across studies in this literature of oral food challenges with (59) cow's milk are not standardized (see section on Diagnosis).

Thus, among the unmet needs of epidemiological research in this field are high-quality community studies based on patient data objectively confirmed by DBPCFC to close the current knowledge gap on the prevalence of CMA in the population. To address this, the European Commission launched the EuroPrevall Project (http://www.europrevall.org) in 2005 in concert with more than 60 partners including patient organizations, the food industry and research institutions from across Europe, Russia, Ghana, India, and China. This translational endeavor involves basic and clinical research components, and large epidemiological studies of both children and adults (60). The first results, will include data on suspicion of CMA, on sensitization to cow's milk and of oral food challenge-confirmed diagnosis from 10 birth cohorts (61).

Different Clinical Presentations of CMA

In a Danish birth cohort, 60% of children with CMA presented with gastrointestinal symptoms, 50 to 60% with skin issues, and respiratory symptoms present in 20 to 30% while 9% developed anaphylaxis (62, 63). In the Norwegian cohort noted above, young infants experienced pain (48%), gastrointestinal symptoms (32%), respiratory problems (27%), and atopic dermatitis (4.5%) (53). In the Finnish cohort, presentation symptoms included urticaria (45.76%), atopic dermatitis (89.83%), vomiting and/or diarrhea (51.69%), respiratory symptoms (30.50%), and anaphylaxis (2.54%). The same children reacted at oral food challenge with symptoms of urticaria (51.69%), atopic dermatitis (44.06%), vomiting and/or diarrhea (20.33%), respiratory symptoms (15.25%), and anaphylaxis (0.84%) (52). In the British study quoted above, infants reacted to oral food challenges with eczema (33%), diarrhea (33%), vomiting (23.8%), and urticaria in 2 children who immediately reacted to the challenge meal (one with wheeze and the other with excessive crying) (54). Dutch infants with CMA from the study noted above developed gastrointestinal (50%), skin (31%), and respiratory (19%) symptoms (33).

Several other studies have assessed the incidence of CMA in populations selected for referral by other care givers to a tertiary

institution for specialist assessment of their symptoms and therefore requires caution in generalizing the results of such studies. As a case in point, in a long-term study of 97 children with challenge-confirmed CMA, 21% had atopic dermatitis at the final follow-up evaluation (at 8 years) (62). In another follow-up study of 42 infants with IgE-mediated CMA, 57% of children had developed atopic dermatitis at the median age of 3.7 years (63).

Thus, CMA appears with GI symptoms in 32 to 60% of cases, cutaneous symptoms in 5 to 90%, anaphylaxis in 0.8 to 9% of cases. Respiratory complaints, including asthma, are not rare. Clearly, in most of the populations studied, there are overlapping presenting symptoms and multiple symptoms are often confirmed during challenge.

CMA in Different Clinical Conditions

Reversing the point of view, milk sensitization and CMA are reported with different frequencies in different clinical presentations. In 2184 young children aged 13-24 months with atopic dermatitis, the frequency of positive serum IgE responses against cow's milk protein was 3% (64). Among 59 breast-fed children with moderate-severe AD, 5 (8,5%) were SPT-positive with milk extracts (65). In a consecutive series with moderate atopic eczema referred to a University-affiliated dermatology department, SPT showed 16% of infants with IgE against CMP (66). In a group of infants and children (mean age 17.6 months) with AD and no other allergic manifestations, 20/54 children (37%) had a diagnosis of CMA (67). Among 90 children with IgE-mediated food allergy, 17% were allergic to cow's milk (68). Thus, as reviewed some years ago, nearly one third of AD children have a diagnosis of CMA according to elimination diet and challenge tests, and about 40-50% of children < 1 year of age with CMA have AD (67).

An exception to the uncertainty of information about epidemiology of CMA is anaphylaxis. In a prospective survey of hospital admissions for food-allergic reactions, conducted through the British Pediatric Surveillance Unit, covering the 13 million children in the United Kingdom and Ireland, 229 cases were reported by 176 physicians in 133 departments, yielding a rate of 0.89 hospital admissions per 100,000 children per year. With a 10% rate, milk was the third most frequent allergenic trigger, after peanut (21%) and tree nuts (16%) (69). In the UK, there are 13 million individuals less than 16 years of age, and over the past 10 years 8 children died of anaphylaxis (incidence of 0.006 deaths per 100 000 children 0-15 years per year). Milk caused the greatest number of fatal reactions (four of eight) (70), in line with reports of both the frequency and severity (71) of reactions to milk.

Secular Trends of CMA

In such a leopard-skin epidemiological context, it is hardly surprising that there is no continuum that can be identified across studies regarding time variations in CMA frequency (72). Is CMA prevalence on the rise? Utilizing surrogate indicators, we can only infer changes in CMA prevalence based on studies of general food allergy. Among those, a British study found that the admission rates per million population between 1990 and 2004 rose form 5 to 26 for anaphylaxis, from 5 to 26 for food allergy, and from 16 to 107 specifically for pediatric food allergy (73). Reinforcing this picture, eczema rose from 13% in 1991 to 16% in 2003(3).

Geographical Trends in CMA

Is milk the most important offender in food allergy in children? From self-reports, it appears that this may be the case. However, given the paucity of epidemiological studies, we do not have sufficient information to argue the relative importance of CMA in different parts of the world. The maximum information comes from Spain, Scandinavian countries, the UK, and Germany. Inadequate information from different areas in the world are available, including Italy, Australia and North America where many cross-sectional and referral studies come from. Table 3-1 shows the comparison of the 3 main food allergens in the child studies. The pan-European RedAll survey estimated milk as the most frequently reported offender in children (38.5% of reports) and the second in adults (26.2%) (20). In France, 29/182 school-aged children with reported food allergy are milk-allergic in 11.9% of cases (24). Accordingly, the Rona (23) metanalysis indicates milk as the major food offender in challenge-based studies, followed by egg and fish. However, cow's milk accounts for less than one third of any food that can be blamed for food allergy among the studies significantly combined ($P < 0.001$) (74). Similarly a review of studies of various designs (surveys, reviews, clinico-epidemiological studies) indicated egg as the most frequently found allergen in children (75). The pattern is repeated in Japan, where CM accounts for 22.6% of children with food allergy (76). The same may not be true in other parts of the world, where the

prevalence will largely reflect local factors such as exposure to foods, mode of preparation, and cultural attitudes. As an example, in Israel sesame is the third most frequently implicated offending food, probably because of its widespread consumption. Among young Australian adults, the major offender was peanut, followed by shrimp, wheat, egg, and milk (44). In Iranian children CM is the most common offender identified during diagnostic provocation challenge (77). Thus, it may be said that the most representative allergen is a hand-maiden to local customs.

Table 3-1. Comparison of the Three Main Food Allergens In Children Studies 75

Country	1st	2nd	3rd
USA	Egg	Cow's milk	Peanuts
Germany	Egg	Cow's milk	Wheat
Spain	Egg	Cow's milk	Fish
Switzerland	Egg	Cow's milk	Peanuts
Israel	Egg	Cow's milk	Sesame
Japan	Egg	Cow's milk	Wheat

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Section 4: Allergens of Cow's Milk

Overview

The main allergens of cow's milk are distributed among the whey and casein fractions.

The whey allergens include:

a. Alpha-lactalbumin (*Bos d 4*): its role in milk allergy is controversial and prevalence data across studies vary between 0 and 80% of patients reacting to this protein.

b. Beta-lactoglobulin (*Bos d 5*), the most abundant cow's milk whey protein; it occurs in the milk of many other species but is not present in human milk. Thirteen to 76% of patients are found to react to this protein.

c. Bovine serum albumin (*Bos d 6*): involved in other allergies such as beef; it accounts for between 0 and 88% of sensitization events, while clinical symptoms occur in up to 20% of patients.

d. Bovine immunoglobulins (*Bos d 7*): are seldom held responsible for clinical symptoms in CMA.

The casein allergens (collectively known as *Bos d 8*) consist of 4 different proteins ($alpha_{s1}$, $alpha_{s2}$, beta, and kappa casein) which share little sequential homology. Despite this, simultaneous sensitization to these caseins is frequently observed. Patients are more often sensitized to alpha (100%) and kappa caseins (91.7%).

Of clinical relevance, milk allergens of various mammalian species cross-react. The greatest homology is among cow's, sheep's and goat's milks protein as *Bos* (oxen), *Ovis* (sheep), and *Capra* (goat) are genera belonging to the Bovidae family of ruminants. Proteins in their milks have less structural similarity with those from the Suidae (pig), Equidae (horse and donkey), and Camelidae (camel and dromedary) families and also from those of humans. Its noteworthy that the milks of camels and dromedaries (and human milk) do not contain *Bos d 5*. All this is relevant for later considerations on formula (section 13).

There is no clear relationship between digestibility and protein allergenicity. Milk allergens are known to preserve their biologic activity even after boiling, pasteurization, ultra-high-temperature processing, or evaporation for the production of powdered infant formula. To obtain hypoallergenic formulas, extensive hydrolysis and further processing, such as heat treatment, ultrafiltration, and application of high pressure are necessary. Attempts have been made to classify formulas into partial and extensively hydrolyzed products according to their degree of protein fragmentation, but there is no agreement on the criteria on which to base this classification. Nevertheless, hydrolyzed formulas have until now proven to be a useful and widely used protein source for infants suffering from CMA (section 12).

Introduction

Milk can give rise to several food hypersensitivities, usually classified as milk allergy or milk intolerance (1). The mechanism of intolerance to cow's milk is not IgE antibody-mediated and has been blamed on the functionality of a specific enzyme deficiency, commonly lactose intolerance, attributable to beta-galactosidase (lactase) deficiency. DRACMA will not address lactase deficiency or other cow's milk-induced hypersensitivity not mediated by immune mechanisms, which have been described in detail elsewhere (2–5). Cow's milk allergy is an adverse clinical reaction associated with the binding of immunoglobulin (IgE) to antigens capable of eliciting an immune response (6). Where allergy is not mediated by IgE, other classes of immunoglobulin, immune complexes, or a cell-mediated reaction have been proposed to be involved. In IgE-mediated allergy, circulating antibodies recognize specific molecular regions on the antigen surface (*epitopes*), which are classified according to their specific amino acid sequence (*sequential epitopes*) or the folding and configuration of their protein chains (*conformational epitopes*). In this section, we describe the chemical characteristics of cow's milk allergens, how they are involved in cross-reactivity among mammalian species, their resistance to digestion and proteolysis and their response to technological processing.

Chemical Characterization of Cow's Milk Allergens

Cow's milk contains several proteins that could each in principle elicit an allergic reaction in a sensitized individual. Some of these proteins are considered major allergens, some minor ones, while others have rarely or never been associated with reports of clinical reactions. The casein and whey proteins of cow's milk are listed in Table 4-1. Each of these 2 fractions contains 5 major components (7–9). The casein fraction contains 80% of the total protein of cow's milk while alpha$_{s1}$ and beta-casein make up for 70% of this fraction. Whey proteins are less abundant, and beta-lactoglobulin (BLG) accounts for 50% of this fraction. Because BLG is not present in human milk, this protein was previously considered the most important cow's milk allergen, but it has since been shown that other proteins, such as the caseins, are also critically involved in the etiology of the disease.

By convention, allergens in the international nomenclature are designated by an abbreviation formed by the genus (capitalized; abbreviated to the first 3 letters) and species (reduced to one letter) names of the Linnaean taxonomical system in italics, followed by an Arabic numeral reflecting the chronological order in which the allergen was identified and characterized (eg, *Bos d*[omesticus] 4) (10).

Table 4-1. The Proteins of Cow's Milk

Fraction	Protein	Allergen10	g/L	% Total Protein	MW (kDa)	# AA	pI
Caseins		*Bos d 8*	~30	80			
	α_{s1}-casein		12–15	29	23.6	199	4.9–5.0
	α_{s2}-casein		3–4	8	25.2	207	5.2–5.4
	β-casein		9–11	27	24.0	209	5.1–5.4
	γ_1-casein				20.6	180	5.5
	γ_2-casein		1–2	6	11.8	104	6.4
	γ_3-casein				11.6	102	5.8
	κ-casein		3–4	10	19.0	169	5.4–5.6
Whey			~5.0	20			
proteins	Alpha-lactalbumin	*Bos d 4*	1–1.5	5	14.2	123	4.8
	Beta-lactoglobulin	*Bos d 5*	3–4	10	18.3	162	5.3
	Immunoglobulin	*Bos d 7*	0.6–1.0	3	160.0	–	–
	BSA*	*Bos d 6*	0.1–0.4	1	67.0	583	4.9–5.1
	Lactoferrin	–	0.09	Traces	800.0	703	8.7

*Bovine serum albumin.

Alpha-Lactalbumin (*Bos d 4*)

Alpha-lactalbumin (A-LA) is a whey protein belonging to the lysozyme superfamily. It is a regulatory subunit of lactose synthase and is, able to modify the substrate specificity of galactosyl-transferase in the mammary gland, making glucose a good acceptor substrate for this enzyme and allowing lactose synthase to synthesize lactose (11, 12). A-LA is produced by the mammary gland and has been found in all milks analyzed so far. Table 4-2 shows its main chemical characteristics.

A-LA contains 8 cysteine groups, all forming internal disulphide bonds, and 4 tryptophan residues. It contains high-affinity calcium binding sites stabilizing its highly ordered secondary structure. The role of A-LA in milk allergy is controversial and prevalence data across studies vary between 0 and 80% of patients reacting to this protein (reviewed in (13)). This heterogeneity is probably linked to whether skin prick test, specific IgE determinations, immunoblotting, or other method of sensitization assessment was used.

Table 4-2. Characteristics of Alpha-Lactalbumin (*Bos d 4*)

Parameter	Description
Allergen nomenclature	*Bos d 4*
Entry name	LALBA_BOVIN
Synonyms	Lactose Synthase B protein
Sequence databases	Genbank: M18780 PIR: A27360, LABO Swiss-Prot: P00711
Number of aminoacids	123 residues
Molecular weight	14.2 kDa
Isoelectric point	4.8
Involvement in allergic sensitization to cow's milk	0–80% CM allergic subjects 75% CM allergic children by SPT

Beta-Lactoglobulin (*Bos d 5*)

Beta-lactoglobulin (BLG) is the most abundant cow's milk whey protein; it occurs in the milk of many other mammalian species but is not present in human milk. *Bos d 5* belongs to the lipocalin allergen family and is synthesized by the mammalian gland. Its function is unknown, although it may be involved in retinol transport, with which it readily binds (14). Table 4-3 shows its main physical and chemical characteristics. It contains 2 internal disulphide bonds and one free-SH group. Under physiological conditions, BLG exists as an equilibrium mixture of monomer and dimer forms but, at its isoelectric point, the dimers can further associate to octamers. There are 2 main isoforms of this protein in cow's milk, the genetic variants A and B, which differ only by 2 point mutations at amino acids 64 and 118. Because it is lacking from human milk, BLG has long been believed to be the most important cow's milk allergen. The literature indicates that the prevalence of allergic subjects reacting to this protein is between 13 and 76% (15).

Table 4-3. Characteristics of Beta-Lactoglobulin (*Bos d 5*)

Parameter	Description
Allergen nomenclature	*Bos d 5*
Entry name	LACB_BOVIN
Synonyms	–
Sequence databases	Genbank: X14712 PIR: S10179, LGBO Swiss-Prot: P02754
Number of aminoacids	162 residues
Molecular weight	18.3 kDa
Isoelectric point	5.13–5.23 (variants)
Involvement in allergic sensitization to cow's milk	13–76% CM allergic subjects 73.7% CM allergic children by SPT

Bovine Serum Albumin (*Bos d 6*)

Bovine serum albumin (BSA) is the main protein of whey. It can bind water, fatty acids, hormones, bilirubin, drugs, and Ca^{2+}, K^{+}, and Na^{+}. Its main function is the regulation of the colloidal osmotic pressure in blood (15). The tertiary structure of BSA is stable, and its 3-dimensional conformation is well documented. The protein is organized into 3 homologous domains (I to III) and consists of 9 loops connected by 17 covalent disulphide bridges. Most of the disulphide bonds are well protected in the core of the protein and are not readily accessible to the solvent. Table 4-4 shows some of its characteristics.

Table 4-4. Characteristics of Bovine Serum Albumin (*Bos d 6*)

Parameter	Description
Allergen nomenclature	*Bos d 6*
Entry name	ALBU_BOVIN
Synonyms	BSA
Sequence databases	Genbank: M73993 PIR: A38885, ABBOS Swiss-Prot: P02769
Number of aminoacids	583 residues
Molecular weight	67.0 kDa
Isoelectric point	4.9–5.1
Involvement in allergic sensitization to cow's milk	0–88% CM allergic subjects 62.5% CM allergic children by immunoblotting

Bos d 6 is involved not only in milk allergy but also in allergic reactions to beef (15). It induced immediate allergic symptoms (lip edema, urticaria, cough, and rhinitis) in children allergic to beef who received the protein in a double-blind placebo-controlled food challenge (DBPCFC) (16). The prevalence of patients with cow's milk who react to this protein ranges from 0 to 88%, while clinical symptoms may be found in as many as 20% of patients (17).

Immunoglobulins (*Bos d 7*)

Bovine immunoglobulins are present in blood, tissues, fluids, and secretions such as milk. Some characteristics of the bovine IgG are shown in Table 4-5. Bovine IgG seldom cause clinical symptoms in CMA (18).

Table 4-5. Characteristics of Cow's Milk Immunoglobulin G

Parameter	Description
Allergen nomenclature	*Bos d 7*
Entry name	–
Synonyms	IgG
Sequence databases	–
Number of aminoacids	–
Molecular weight	160.0 kDa
Isoelectric point	–
Involvement in allergic sensitization to cow's milk	Frequency unknown

Caseins (*Bos d 8*)

Most of the casein aggregates as colloidal particles (the casein micelle) and its biologic function is to transport calcium phosphate to the mammalian newborn. More than 90% of the calcium content of skim milk is attached to or included in casein micelles. Caseins consist of 4 different proteins (alpha$_{s1}$, alpha$_{s2}$, beta, and kappa casein) with little sequential homology. Another group, the gamma caseins, are present in very low quantities in milk and are by-products of beta casein proteolysis. A distinguishing feature of all caseins is their low solubility at pH 4.6; another common characteristic is that caseins are conjugated proteins, most with phosphate groups esterified to the amino acid serine. Caseins contain no disulphide bonds, while the high number of proline residues causes pronounced bending of the protein chain, which inhibits the formation of close-packed, ordered secondary structures. Characteristics of *Bos d 8* are reported in Table 4-6.

Table 4-6. Allergenic Characteristics of Caseins

Parameter	α_{s1}-casein	α_{s2}-casein	β-casein	κ-casein
Allergen nomenclature	*Bos d 8*	*Bos d 8*	*Bos d 8*	*Bos d 8*
Entry name	CAS1_BOVIN	CAS2_BOVIN	CASB_BOVIN	CASK_BOVIN
Synonyms	None	None	None	None
Sequence databases	G X00564/ M33123 P S22575/ KABOSB S P02662	G M16644 P JQ2008/ KABOS2 S P02663	G M16645/ X06359 P I45873/ KBBOA2 S P02666	G X14908/ M36641 P S02076/ KKBOB S P02668
No. aminoacids	199	207	209	169
Molecular weight	23.6 kDa	25.2 kDa	24.0 kDa	19.0 kDa
Isoelectric point	4.9–5.0	5.2–5.4	5.1–5.4	5.4–5.6
Involvement in allergic sensitization to cow's milk-1. whole casein	65–100%	65–100%	65–100%	65–100%
Involvement in allergic sensitization to cow's milk-2. single casein	54% 100%	54% 100%	39% 66.7%	NT 91.7%

Despite the poor sequence homology between proteins of the casein fraction, poly-sensitization to many caseins is frequently observed; this may be because of cross-sensitization through shared or closely related epitopes (8). Patients are almost always sensitized to alpha (100%) and kappa caseins (91.7%) (19).

Cross-Reactivity Between Milk Proteins from Different Animal Species

Cross-reactivity occurs when 2 proteins share part of their amino acid sequence (at least, the sequence containing the epitopic domain) or when the 3-dimensional conformation makes 2 molecules similar in binding capacity to specific antibodies. In general, cross-reactivity between mammalian proteins reflects the phylogenetic relationships between animal species and evolutionary conserved proteins that are often cross-reactive (20). Table 4-7 shows the sequence similarity (expressed in percentages) between milk proteins from different mammalian species (22).

Table 4-7. Sequence Homology Between Mammalian Milk Proteins (in Percentage, Relative To Cow's Milk Proteins)

Protein	Goat	Ewe	Buffalo	Sow	Mare	Donkey	Dromedary	Human
ALA	95.1	97.2	99.3	74.6	72.4	71.5	69.7	73.9
BLG	94.4	93.9	96.7	63.9	59.4	56.9	Absent	Absent
Serum alb.	–	92.4	–	79.9	74.5	74.1	–	76.6
α s_1 CAS	87.9	88.3	–	47.2	–	–	42.9	32.4
α s_2 CAS	88.3	89.2	–	62.8	–	–	58.3	–
β CAS	91.1	92.0	97.8	67.0	60.5	–	69.2	56.5
κ CAS	84.9	84.9	92.6	54.3	57.4	–	58.4	53.2

The greatest homology is between cow's, sheep's and goat's milk proteins as *Bos* (oxen), *Ovis* (sheep), and *Capra* (goat) that are genera belonging to the Bovidae family of ruminants. The proteins in their milks consequently have less structural similarity with those from the Suidae (pig), Equidae (horse and donkey), and Camelidae (camel and dromedary) families and also with those in human milk. It is noteworthy that the milks of camels and dromedaries (as well as human milk) do not contain BLG.

However, phylogeny does not explain everything. In 1996, a clinical trial in France showed that 51/55 children with cow's milk allergy tolerated goat's milk for periods ranging from 8 days to 1 year (22), but subsequent research showed that other subjects allergic to cow's milk did not tolerate goat's and sheep's milks (23). This is consistent with the pattern of IgE cross-reactivity shown by several independent studies in vitro, for instance the cross-reactivity between milk proteins from different mammalian species (including goat's milk) (24). Furthermore, selective allergy to goat's and sheep's milk but not to cow's milk has also been reported in 28 older children with severe allergic reactions, including anaphylaxis. In one study, IgE antibodies recognized caseins from goat's milk but cow's milk caseins were not or scarcely recognized (25). This is not an isolated finding (26, 27), however, and a case report of an adult with goat's milk allergy without CMA found specific IgE to caprine ALA (28). Finally, allergy to sheep's milk can also evolve into allergy to cow's milk (29). Mare's and donkey's milks have proved sometimes useful to some patients (30–32), but

uncertainties remain about chemical composition and hygienic control. The same considerations apply to Camellidae (camel and dromedaries) milks, which could represent an alternative to cow's milk for allergic subjects because of their low sequence homology with cow's milk and the absence of BLG, if problems related to availability and technological processing to avoid new sensitization (33).

Figure 4-1 shows the electrophoretic patterns of milk from several mammalian species. The pronounced similarity is evident for milk from cows, goats, and sheep, while the protein profiles of mare's, donkey's, and camel's milks present some specificities. The low cross-immunoreactivity of horse/donkey milk and the absence of BLG in camel's and human milk is easily visible in immunoblots using antibodies against bovine BLG.

Structural Modifications and Cow's Milk Protein Allergenicity

The 3-dimensional structure of most antigenic proteins is unknown, even where the amino acid sequence has been precisely identified, because the conformation is not immutable but is influenced by the surrounding environment. This problem is even more significant for milk proteins since their organization is complex and the presence of micelles in caseins makes their investigation difficult. We discuss here the structural modifications brought about by gastrointestinal digestion or technological treatments and their role in allergenic potential where this is known or can be inferred.

Digestibility and Cow's Milk Protein Allergenicity

Food proteins are digested by gastrointestinal enzymes; it is generally believed that proteins resistant to proteolysis are the more powerful allergens. However, it has been shown that there is no clear relationship between in vitro digestibility and protein allergenicity (34). Caseins are thought to be easily digestible, but they coagulate in an acidic medium (at gastric pH). Acidification increases the solubility of minerals, so that the calcium and phosphorus contained in the micelles gradually become soluble in the aqueous phase. As a result, casein micelles disintegrate and casein precipitates. Whey proteins are more soluble in saline solution than caseins and theoretically they should be more easily digested by proteases that work in aqueous medium. However, the correlation between water solubility and digestibility is not linear. Caseins are digested faster than whey proteins by the commonest food-grade enzymes (eg, pepsin, trypsin, and thermolysin) (35).

Although BSA is very soluble in water and rich in amino acids broken-down by gastrointestinal enzymes, it is also relatively resistant to digestion. Sequential epitopes were unaffected for at least 60 minutes when BSA was digested with pepsin (36). Its 9 loops are maintained by disulphide bonds, which are not easily reduced under physiological conditions, and slow the fragmentation of BSA into short peptides that have decreased antigenic activity.

Heating and Cow's Milk Protein Allergenicity

Cow's milk is only marketed after it has been subjected to technological process, usually pasteurization, which reduces potential pathogen load (70-80°C for 15-20 seconds). Ultra-high-temperature (UHT) processing with flash heating (above 100°C for a few seconds), evaporation for the production of powdered infant formula (dry blending or wet mixing-spray drying process) have a minor or no effect on the antigenic/allergenic potential of cow's milk proteins. Boiling milk for 10 minutes reduces the SPT response in patients who react to BSA and beta-lactoglobulin, whereas wheal diameter remains the same in those sensitized to caseins (37). Comparative studies have shown no difference in antigenicity between raw and heated milks (38), however, and in some cases the aggregation of new protein polymers capable of binding specific IgE have been demonstrated. After boiling BSA at 100°C for 10 minutes, dimeric, trimeric, and higher polymeric forms increased, and all maintained their IgE-binding properties (39).

The persistence of allergenicity in heat-treated milk is clinically confirmed by the fact that in

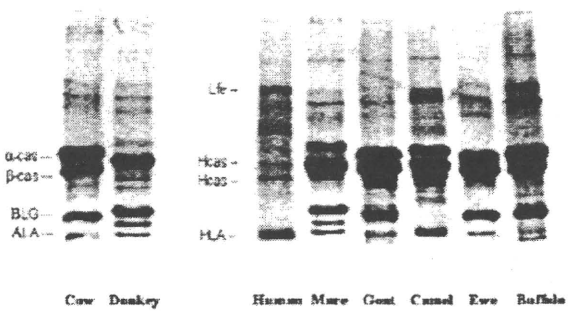

Fig. 4-1 SDS-PAGE of mammalian milk samples. Hcas = human casein; HLA = human lactalbumin; Lfe = human lactoferrin; α-cas = bovine alpha casein; β-cas = bovine beta casein; BLG = bovine β-lactoglobulin; ALA = bovine α-lactalbumin.

some children CMA develops after the ingestion of heat-treated milk. Furthermore, heating processes can only modify conformational epitopes, which might lose their binding capacity to specific IgE antibody, while sequential epitopes maintain their allergenic potential even after heating (40). Milk proteins contain both types of epitopes and, even though a slight reduction of antigenicity can be observed with whey proteins, insignificant alterations in binding properties are reported with caseins. To complicate the picture, vigorous heating (such as that used for certain sterilization processes [121°C for 20 minutes]) but also the less drastic pasteurization process, have also been shown to enhance some allergenic characteristics (41). Furthermore, milk proteins can be oxidized during industrial treatment, resulting in the formation of modified/oxidized amino acid residues, particularly in BLG, which may be responsible for the development of new immunologically reactive structures (42).

Technological Treatments and Cow's Milk Protein Allergenicity

Hypoallergenic formulas can be prepared by hydrolysis and further processing, such as heat treatment, ultrafiltration, and application of high pressure. Attempts have been made to classify formulas into partial and extensively hydrolyzed products according to the degree of protein fragmentation, but there is no agreement on the criteria on which to base this classification (see section "CM hydrolyzed formula"). Nevertheless, hydrolyzed formulas have until now proved a useful and widely used protein source for infants suffering from CMA. Because undigested protein can still be present as residue at the end of proteolysis (43), further processing is necessary in combination with e enzymatic treatment. Another attempt to eliminate antigenicity involves the use of proteolysis combined with high pressure. Different authors have shown increased fragmentation of BLG if proteolysis occurs after or during the application of high pressure (44). The partial ineffectiveness of proteolysis under ordinary atmospheric conditions may be because of the inability of enzymes to reach epitopes that are less exposed. Heat treatment is also often combined with proteolysis to unfold the protein and modify the 3-dimensional structure of conformational epitopes. However, thermal denaturation can also cause the formation of aggregates with greater resistance to hydrolytic attack, as is the case with BLG (45).

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Section 5: Immunological Mechanisms of Cow's Milk Allergy

Overview

CMA designates objectively reproducible symptoms or signs initiated by exposure to cow's milk protein at doses tolerated by normal persons. CMA can be either antibody-mediated or cell-mediated; occasionally both mechanisms may be involved. CMA may be mediated by any of the 4 basic types of immunologic reactions, as outlined by Gell and Coombs: 1) Type I or IgE-mediated hypersensitivity, 2) Type II (cytotoxic reactions), 3) Type III (Arthus-type reactions), and 4) Type IV (delayed T cell reactions). Type I reactions are the best characterized and represent the classic immediate allergic reactions. The 3 other types, collectively described as non-IgE-mediated allergy, are less well understood.

The suppression of adverse immune responses to nonharmful ingested food antigens is termed oral tolerance. Ingested milk proteins are normally degraded by gastric acid

and luminal digestive enzymes. The exact mechanisms involved in tolerance development remain unclear. The primary immunologic mechanisms include deletion, anergy, suppression, "ignorance," and apoptosis of T-cells. The balance between tolerance (suppression) and sensitization (priming) depends on several factors, including: 1) genetic background, 2) nature and dose of the antigen, 3) frequency of administration, 4) age at first antigen exposure, 5) immunologic status of the host, and 6) antigen transmission via breast milk.

The acquisition of tolerance to milk is seen as a T_H1 (T helper cells type 1)-skewed immune response. After intestinal mucosal exposure to cow's milk antigens, antigen-presenting cells (APCs) interact with subepithelial T and B lymphocytes. Recognition of antigens by the T cell receptors (TCR) involves major histocompatibility complex (MHC) molecules. Activated T and B cells of lymphoid follicles migrate via the lymphatic system, and then via the circulation to several target organs, including the gastrointestinal tract, respiratory system or skin. If tolerance is not achieved, T and B cells will be activated and give rise to an inflammatory reaction in the target organ, resulting in the clinical manifestations of CMA.

The innate immune system has the ability to modulate adaptive immune responses to food proteins. In this process, dendritic cells (DC) and Toll-like receptors (TLR) play a central role. Intestinal microbiota have been shown to exert diverse effects on TLRs and regulatory T cell responses. TLR can recognize specific pathogen-associated molecular patterns (PAMP). The mechanisms by which TLRs influence *Treg* responses are incompletely understood. *Treg* promote tolerance to milk antigens via the production of tolerogenic cytokines, including interleukin (IL)-10 and transforming growth factor beta (TGF-β).

CMA is believed to result from either the failure to develop normal tolerogenic processes, or their later breakdown. In the case of IgE-mediated CMA, activation of milk-specific T helper cells type-2 (T_H2) leads to the production of milk-specific IgE. Non-IgE-mediated reactions may be because of T_H1-mediated inflammation. Decreased *Treg* activity has been identified as a factor in both allergy mechanisms. The development of tolerance in children with a history of CMA was associated with the up-regulation of *Treg* responses.

The events after intestinal allergen exposure are complex as digestion and cooking may modify the allergenicity of bovine proteins. Intact allergenic epitopes on food proteins will interact with the mucosal immune system. Dietary proteins that escape proteolysis can be taken up by intestinal epithelial cells. Early exposure to relatively large doses of soluble protein is thought to promote tolerance. Factors that modulate the risk of sensitization include: 1) nature and dose of the antigen, 2) efficiency of protein digestion, 3) immaturity of the host, 4) rate of absorption of milk proteins, 5) antigen processing in the gut, and 6) the immunosuppressive milieu of Peyer's patches. The type of gut microbiota may also modulate the risk of sensitization in young infants.

Introduction

Acquired immunologic tolerance of environmental agents is an active mechanism of adaptive immunity that is mediated by polarized cells of the T helper type I lymphocyte subset but when, in an atopic individual, the predisposition to secrete IgE antibody to cow's milk antigen goes into overdrive, homeostasis breaks down and mast cells can become sensitized anywhere in the body, thereby expressing an often baffling array of symptoms in one or more organs which the clinician identifies as CMA (1). A basic understanding the underlying cellular and mediator mechanisms of CMA is therefore necessary to be proactive about diagnostic and treatment options.

Gut Barrier

The mucosal immune system must adapt and be able to discriminate between pathogens and harmless antigens and respond accordingly, that is, to protect the neonate from enteric pathogens while establishing a state of tolerance to dietary proteins and commensal bacteria. This important task is undertaken by cells of the gut-associated lymphoid tissue, the largest immunologic organ in the body (2). Many studies have reported increased macromolecular transport across the gut barrier in children with atopy (3, 4) which is thought to be because of mucosal damage induced by local hypersensitivity reaction to

foods (5) Dual sugar intestinal permeability studies (lactulose/mannitol) showed that in breast-fed infants with atopy, gut barrier function improved when breast-feeding was stopped and hypoallergenic formula started (6).

Oral Tolerance

The mucosa allows nutrients to be transferred from the intestinal lumen to the systemic circulation, while protecting against pathogens by inducing immune responses. Any down-regulation of immune responses to nonharmful ingested antigens is termed oral tolerance (7). Normally, mature lymph node lymphocytes become hyporesponsive after oral administration of these antigens (8).

Ingested milk proteins are degraded and their conformational epitopes are destroyed by gastric acid and luminal digestive enzymes, which often results in the destruction of immunogenic epitopes. In animal models, disrupting the process of digestion can inhibit milk tolerance and lead to hypersensitivity. Untreated bovine serum albumin (BSA) is immunogenic when administered to mice by means of ileal injection, but administering a peptic digest of the protein in the same manner results in immune tolerance (9).

Regulatory events after mucosal exposure to antigen have not been well characterized and remain controversial. In general, the acquisition of tolerance to milk is seen as a T_H1-skewed response, which on the one hand may prevent harmful mucosal immune reactions but on the other may contribute to adverse responses in a susceptible individual. The process starts with the contact of milk allergens with the intestinal mucosa. Here they interact with mucosal T and B cells either directly or through antigen-presenting cells (APCs): macrophages, dendritic cells, or microfold cells (M cells). T cell recognition of antigen by T cell receptors (TCR) involves the major histocompatibility complex (MHC) molecules (class I and II) of APCs. Activated T and B cells of lymphoid follicles migrate first via the lymphatic system and then via the circulation to any of several target organs including the gastrointestinal tract, the respiratory system, the skin, and the central nervous system, a process referred to as "homing." If tolerance is not achieved, T and B cells will activate at a homing site upon contact with their specific food antigen and release their cytokines, vasoactive peptides and antibodies, giving rise to an inflammatory reaction in the affected organ and resulting in the clinical manifestations of food hypersensitivity (10).

In this context, dendritic cells play a central role in taking up milk proteins and migrating to the draining mesenteric lymph nodes, where they induce regulatory CD4 T-cell differentiation. The primary mechanisms by which tolerance may be mediated include deletion, anergy, suppression, "ignorance," and apoptosis of T-cells (11).

The balance between tolerance (suppression) and sensitization (priming) depends on several factors, such as: 1) genetic background, 2) nature and dose of antigen, 3) frequency of administration, 4) age at first antigen exposure, 5) immunologic status of the host, 6) antigen transmission via breast milk, and others.

Overall, there is evidence in rodents that multiple low-dose feeds are likely to induce regulatory cytokines (eg, TGF-β, IL-10, IL-4) in part secreted by $CD4^+$ $CD25^+$ T-regulatory cells. Despite the powerful suppressive effects of oral autoantigen exposure observed in experimental models of autoimmune diseases (including bystander suppression), their translation into clinical trials of autoimmune diseases has not yet yielded the expected beneficial results. The same can be said for CMA (12).

In normal individuals with tolerance, systemic and secretory food-specific IgA antibodies are generally absent, indicating that mucosal IgA production is regulated similarly to that of systemic immunity (13). However, mucosal IgA response to foreign antigens remains active (14). In population surveys, more allergic sensitization was seen in subjects with an IgA level at the lower end of the normal range (15–17). The significance of IgM, IgG, and IgG subclass antibodies (eg, the role of IgG4) in food allergy is less well understood and remains controversial. It has long been known that milk-specific IgM and IgG antibodies are produced after single or repeated feedings of relatively large doses of milk proteins in both healthy and allergic persons (18).

Thus, unresponsiveness of the immune system to milk antigens ("oral tolerance") is believed to involve the deletion or switching off (anergy) of reactive antigen-specific T cells and the production of regulatory T cells (*Treg*) that suppress inflammatory responses to benign antigens (19, 20).

Innate Immunity and Tolerance Development

The innate immune system has the ability to modulate adaptive immune responses to food proteins. In this process, dendritic cells (DC) play a central role (21). In addition, TLR directly interact with innate immune cells. TLR recognize food antigens, and specific bacterial surface

markers, so-called PAMP (21). However, the exact mechanisms by which TLR influence *Treg* responses are incompletely understood. Regulatory T-cells are involved in the control of immune responses to food antigens via the production of tolerogenic cytokines, including IL-10 and TGF-β (22, 23). Intestinal microbiota may have a diverse effect on TLR and immune responses. Several types of intestinal Bifidobacteria have been shown to promote tolerogenic immune responses. The type of gastrointestinal microbiota of the newborn infant is crucial in this context. The probiotic effects of complex oligosaccharides in human milk promote the establishment of a bididogenic microbiota which, in turn, induces a milieu of tolerogenic immune responses to foods. Several probiotic bacterial strains have been shown to have similar properties. For example, *Lactobacillus paracasei* inhibits T_H1 and T_H2 cytokine production, and induces $CD4^{(-)}$ T cells to produce TGF- and IL-10, that is, induces a tolerogenic response (24). It appears possible that the recent decrease in exposure to early childhood infections and harmless environmental microorganisms in the westernized environment has contributed to an increase in T-cell dysregulatory disorders and autoimmunity (25, 26).

Dysfunctional Tolerance

CMA is believed to result from the failure to develop normal tolerogenic processes or their later breakdown. In the case of IgE-mediated CMA, a deficiency in regulation and a polarization of milk-specific effector T cells toward type-2 T helper cells (T_H2) both lead to B-cell signaling to produce milk protein-specific IgE (27, 28). Non-IgE-mediated reactions may be because of T_H1-mediated inflammation (29). Dysfunctional *Treg* cell activity has been identified as a factor in both allergy mechanisms (30). Additionally, the induction of tolerance in children who have outgrown their CMA has been shown to be associated with the development of *Treg* cells (31, 32). Much research is currently focused on manipulating the activity of dendritic cells (specialized antigen-presenting cells important in programming immune responses) to induce *Treg* cells and/or to redress T_H1/T_H2 imbalances to promote tolerance to allergenic foods.

Allergen Exposure and Sensitization

The events after allergen exposure in the gut are complex. Digestion (33) and cooking preparation (34, 35) slightly modifies the allergenicity of bovine proteins. Proteins that are not digested and processed in the lumen of the gut will come in contact with the epithelium and mucosal immune system in various ways. In the gut, dendritic cells can sample antigens by extending processes through the epithelium and into the lumen. M cells that overlie Peyer's patches can take up particulate antigens and deliver them to subepithelial dendritic cells. Soluble antigens possibly cross the epithelium through transcellular or paracellular routes to encounter T cells or macrophages in the lamina propria. Dietary proteins that escape proteolysis in the gut can be taken up by intestinal epithelial cells. The epithelial cells can act as nonprofessional APCs and can present antigen to primed T cells. Thus, food allergens (and microorganisms and nonviable particulate antigens) reach $CD4^+$ and $CD8^+$ T cells in the Peyer's patch, resulting in active immune responses (36). Early gastrointestinal encounters with relatively large doses of soluble protein almost always induce tolerance (37). Data from rodent models suggest that the effect of milk allergen exposure on the host depends on many factors, including:

a. Nature and dose of the antigen
b. Efficiency of digestion
c. Immaturity of the host
d. Rate of absorption of milk proteins
e. Antigen processing in the gut
f. The immunosuppressive milieu of the Peyer patch (38).

All of these factors can favor the induction of peripheral tolerance to dietary proteins rather than systemic hypersensitivity. In this context, the presence of commensal flora in the gut can lower the production of serum milk-specific IgE during the primary immune response; also, IgE production persists longer in germ-free mice. Conversely, the absence of gut microbiota significantly increases the milk-specific immune response in mice (39). This raises the possibility of prevention and treatment of milk allergy through the manipulation of the gastrointestinal flora.

Milk Allergy

An effect of dysfunctional tolerance, "milk allergy" designates objectively reproducible symptoms or signs initiated by exposure to cow's milk at a dose tolerated by normal persons (40). The term CMA is appropriate

when specific immunologic mechanisms have been demonstrated (see "definitions" in introductory section). Milk allergy can be either antibody-mediated or cell-mediated, or occasionally both may be involved. If IgE is involved in the reaction, the term "atopic food allergy" is appropriate. If immunologic mechanisms other than IgE are predominantly involved, the term "non-IgE-mediated food allergy" should be used. All other reactions should be regarded to as nonallergic food hypersensitivity (41).

Enhanced immune-mediated reactivity may come about though any, or a combination of, the 4 basic types of immunologic reactions outlined by Gell and Coombs:

a. Type I or IgE-mediated hypersensitivity leads to immediate symptoms, such as urticaria, angioedema and/or other anaphylactic reaction.
b. In type II (cytotoxic) reactions, the antigen binds to the cell surface and the presence of antibodies (IgG, IgM, or IgA) disrupts the membrane, leading to cell death.
c. In type III (Arthus-type) reactions, antigen-antibody-complement immune complexes (IgG, IgM, IgA, and IgE antibodies) get trapped in small blood vessels or renal glomeruli.
d. Type IV (delayed) reactions are mediated by sensitized T lymphocytes.

Type I reactions are the best understood, and they are often referred to as the most common and classic allergic reactions. The 3 other types, collectively described as non-IgE-mediated allergy, are more difficult to investigate and hence less well understood. In an individual, several types of immune responses may be activated, although IgE-mediated reactions are more usually measured.

IGE-Mediated CMA (IMMEDIATE HYPERSENSITIVITY)

IgE-mediated allergy is the best understood allergy mechanism and, in comparison to non-IgE-mediated reactions, is relatively easily diagnosed. Since the onset of symptoms is rapid, occurring within minutes to an hour after allergen exposure, IgE-mediated allergy is often referred to as "immediate hypersensitivity." (42) It occurs in 2 stages. The first, "sensitization," occurs when the immune system is aberrantly programmed to produce IgE antibodies to milk proteins. These antibodies attach themselves to the surface of mast cells and basophils, arming them with an allergen-specific trigger. Subsequent exposure to milk proteins leads to "activation" when the cell-associated IgE binds the allergenic epitopes on the milk proteins and triggers the rapid release of powerful inflammatory mediators.

IgE-mediated, acute onset CM allergies can affect several target organs: the skin (urticaria, angioedema), respiratory tract (rhinitis/rhinorrhea, asthma/wheeze, laryngoedema/stridor), gastrointestinal tract (oral allergy syndrome, nausea, vomiting, pain, flatulence, and diarrhea), and/or the cardiovascular system (anaphylactic shock) (43, 44). Life-threatening anaphylactic reactions to cow's milk may occur, but are fortunately rare (45). Since reactions to cow's milk proteins can occur on contact with the lips or mouth, strategies to reduce allergenicity by improving protein digestibility in the gut are unlikely to be effective for all allergic individuals. Simple diagnostic procedures, such as skin-prick tests (SPT) and specific serum IgE determinations (immuno-CAP), can be used to identify individuals with IgE-mediated CMA, although either of these tests can produce false-positive results (46). Food elimination and challenge testing are sometimes required to confirm milk allergy, and double-blind, placebo-controlled, food challenge (DBPCFC) testing remains the gold standard for diagnosis. IgE-mediated CMA may occur in neonates on first postnatal exposure to the food (47). IgE-mediated reactions account for about half of the CMA cases in young children (48), but are rare in adults (49, 50). In contrast to adults, atopic CMA in childhood (often a part of the "allergic march") resolves in more than 85% of cases (51, 52).

Non-Ige-Mediated CMA (DELAYED HYPERSENSITIVITY)

A significant proportion of infants and the majority of adults with CMA do not have circulating milk protein-specific IgE and show negative results in skin prick tests and serum IgE determinations (immune-CAP) (53, 54). These non-IgE-mediated reactions tend to be delayed, with the onset of symptoms occurring from 1 hour to several days after ingestion of milk. Hence, they are often referred to as "delayed hypersensitivity." As with IgE-mediated reactions, a range of symptoms can occur, but are most commonly gastrointestinal or cutaneous (55). The gastrointestinal symptoms, such as nausea, bloating, intestinal discomfort, and diarrhea, mimic many symptoms of lactose intolerance and may lead to diagnostic mislabeling. Anaphylaxis is not a feature of non-IgE mediated

mechanisms. IgE- and non-IgE-mediated reactions are not mutually exclusive and reactions to milk can involve a mixture of immunologic mechanisms.

The precise immunologic mechanisms of non-IgE-mediated CMA remain unclear. A number of mechanisms have been suggested, including T_H1-mediated reactions (Fig. 5-1) (56–63), the formation of immune complexes leading to the activation of complement (64, 65), or T-cell/mast cell/neuron interactions inducing functional changes in smooth muscle action and intestinal motility (1, 66, 67). A necessarily incomplete picture of such mechanisms indicates that T cells act through secretion of cytokines such as IL-3, IL-4, IL-5, IL-13, and GM-CSF, activating eosinophils, mastocytes, basophils, and macrophages. Macrophages, activated by CM protein allergens by cytokines, are able to secrete in turn vasoactive mediators (PAF, leukotriens) and cytokines (IL-1, IL-6, IL-8, GM-CSF, TNF-α) that are able to increase the cellular phlogosis. This involves epithelial cells, which release cytokines (IL-1, IL-6, IL-8, IL-11, GM-CSF), chemokines (RANTES, MCP-3, MCP-4, eotaxin) and other mediators (leukotrienes, PGs, 15-HETE, endothelin-1). This mechanism results in chronic cellular inflammation (at GI, cutaneous, and respiratory levels) and ultimately in CMA symptoms. When the inflammatory process is localized at GI level, immune phlogosis can contribute to maintaining epithelial hyper-permeability and potentially to increased exposure to antigenic CM proteins. This involves TNF-α and IFN-γ, antagonists of TGF-β and IL-10 in mediating oral tolerance (68). It has been shown that the pattern of TNF-α secretion is different in children with CMA manifested by digestive or cutaneous symptoms, and the use of TNF-α secretion in response to cow's milk antigens has been proposed as a predictive test of relapse in CMA children undergoing oral provocation.(69). In addition, CMP sensitization of T_H1 and T_H2 lymphocytes has been shown at the systemic level in conditions out of the CMA spectrum as neonatal necrotizing enterocolitis (70).

From the discrepancy between reportedly higher rates of natural recovery during childhood from non-IgE-mediated CMA than in IgE-mediated CMA (71–73) and the predominance of non-IgE-mediated CMA in adult populations (49) it has been postulated that a non-IgE-mediated CMA population emerges later in life. One study reported an increasing incidence of non-IgE-mediated food allergies with increasing age (50). However, the emergence of a new CMA population in adults remains to be conclusively demonstrated. Good epidemiological data for non-IgE-mediated CMA in both adults and children remain scarce because laborious DBPCFC trials remain the only conclusive diagnostic tests to confirm this form of allergy. In many cases, gastrointestinal food allergy remains undiagnosed or is classified as irritable bowel syndrome.

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