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Review Article

Immunogenicity of Hydrolysate Formulas in Children (Part 1). Review of 202 Reactions

Abstract

Cow's milk (CM) protein hydrolyzed formulas (HFs) appeared in the 40's with the aim of decreasing or eliminating the allergenicity of CM proteins, and in addition of red¬ucing the risk of sensitization. In recent years the so-called "hypoallergenic" (HA) formulas have been devel¬oped. The use of such HFs is based on the premise that predi¬gested pro¬teins, when fed as amino acids and peptides, provide nutrients in a not antigenic form. Thus, pro¬tein HFs have been classified as HA. These formulas are pro¬ces¬sed by heat and enzymatic hydrolysis, and the conforma¬tional and se¬quential structu¬res are more or less changed. The formulas contain pep¬tides of lower molecular wei¬ght (MW) than the nati¬ve protein source, which are thought to be less immuno¬genic. HFs appear to be nu¬triti¬onally adequate and infants gen¬erally gain weight until they refuse the formu¬la be¬cause of its bad taste. However, caution should be taken when such formulas are given for prolonged peri¬ods since no data is available on nutritio¬nal assessment of in¬fants ex¬clusively fed HFs for several months. In this paper we report and discuss > 202 re¬actions to different HFs, including cases of anaphylactic shock and of apparent life-threatening events. The cross-reactivity betw¬een dif¬ferent HFs and CM proteins, and the potential immunoge¬n¬icity of such for¬mulas are dis¬cussed. We con¬clude that none of the HFs are non-aller¬genic, both for al¬lergic children and for high-risk (HR) babies. Moreover we suggest that double-blind placebo-con¬trolled food challenges (DBPCFC) stud¬ies in larger cohorts of babies evaluat¬ed with well-defined and -val¬i-dated diag¬nos¬tic methods may establish a more reliable prevalence of HF allergy.

Introduction

CM allergy (CMA) is a disease of infancy with onset in the first 3 months of life with a 58% incidence and lowers to 39% in the 4th-6th month [1] and to 24,9% (mean) in food-allergic children aged 1-18 [2]. Since the turn of the century, CM formulas have become progressively more common as breast milk (BM) substitutes when BM is unavailable, and CMA has thus gradually become a more common disorder [3]. The evaluation of infants for possible CMA is thus one of the more common problems encountered by pediatricians and CMA management in infants and children confronts pediatricians and allergists with one of the most demanding challenges. Unfortunately, both over- and under-diagnosis are frequently seen.

The ideal CM substitute should be hypoallergenic, easily available, inexpensive, and palatable in order to obtain a good compliance, and with an adequate nutritional value appropriate to the infant's age. As yet, the CM available substitutes are SPF (soy-protein formulas), home-made, meat-based formulas (HMMBF) [2], HFs and elemental diets. HFs have been developed with the aim of decreasing or eliminating the allergenicity of CM proteins, of reducing the antigenic load, and the risk of sensitization. Thus, protein HFs, classified as HA, have been used for feeding babies with CMA [4,5] and/or for the prevention of atopic disease in babies of atopic parents [6,7]. In this paper we report and discuss 202 reactions to different HFs.

HFs available for infant nutrition

According to the source of proteins there are four types of HFs, reduced to two types based on the degree of hydrolysis: highly and partially hydrolyzed (Table 1) [8].

Briefly, these formulas are processed using heat denaturation and enzymatic hydrolysis to reduce the MW of the peptides. The reduction of antigenicity is associated with a reduction of the palatability. The composition of HFs is dependent on several factors including the degree of digestion, post-hydrolysis processing, elimination of the enzymes used for the hydrolysis and protein source. Extensively HFs are considered the more HA among the HFs, whereas partially HFs are considered less HA and even dangerous in children with CMA [9,10]. However all HFs contain variable profile peptides with

Table 1: Data in favor of the relationship between Food allergy and AD.

* Children with AD show positive skin tests to foods and food specific IgE
* Children with AD may experience immediate Cutaneous food allergic reactions such contact urticaria or generalized urticaria or Non cutaneous such as Vomiting, Diarrhea, Rhinitis, etc.
* Children with AD may show improvement of AD after appropriate elimination diet.

very high MW, even greater than 6 kD (kilodaltons) [8], which are an index of the extent of their immunogenicity. These formulas are integrated with vegetable lipids, and Alfa-Rè, Alimentum and Pregestimil in addition contain medium chain triglycerides (Table 2). All HFs, excepted HA, are lactose free, and contain a small amount of carnitine. They are rather unpalatable (except HA) and compliance is therefore poor.

Studies in healthy children

Care should be taken when HFs are given for prolonged periods since no data is available on the nutritional assessment of infants fed exclusively HFs for several months. Rigo et al. [11] showed that a partly whey HF (PWHF) induced in full-term newborns fed this product for 6 days a significant increase in plasma concentration of several essential amino acids (AA), especially threonine and branched-chain amino acids. The total essential AA concentration and the ratio of essential to total AA concentration were higher in the babies fed the PWHF than in the babies fed BM or a whey hydrolysed formula (WHF). In a subsequent paper [12] the authors noted that at age 33 days the plasma threonine concentration remained twice as high and the plasma tyrosine, phenylalanine and proline levels were significantly lower in the PWHF group than in the BM-fed infants. Growth and most of the biological indices of protein metabolism were alike in the two groups. Finally the authors [13], while confirming the AA level alterations, observed a drastic reduction in fat Ca and P absorption with the use of a whey-casein HF. In preterm infants, compared with the standard preterm formulas, HFs led to a significant increase in plasma threonine, a decrease in tyrosine and phenylalanine concentrations, and a reduction in plasma histidine, valine, leucine, cystine, methionine and/or tryptophan [13].

Vandenplas et al. [14] studied from birth to 3 months of age 45 healthy infants, 20 receiving a WHF and 25 a PWHF. They found that except for the iron-binding capacity (IBC), zinc, and urea, which were higher in the PWHF-fed babies, at the end of the study the nutritional status was adequate in both groups. Apparently there was a 16% dropout rate only in the PWHF-fed infants. The increased IBC in the PWHF children (351 ± 58.4 mg/dl versus 301 ± 47.4, p = 0.006), was accompanied by similar Hb, Hct, and iron levels values which suggests an impaired iron absorption. Zinc levels were also significantly different in the two groups (79 ± 12.9 mg/dl in the PWHF babies versus 67.6 ± 8.9, p = 0.002 in the controls). The most intriguing issue is the statistically significant difference in plasma urea levels (20.5 ± 6.53 mg/dl versus 15.9 ± 3.45, p = 0.009) and its increased urinary output with an even higher statistical difference (p = 0.001). The authors discuss the data of an earlier study [15] in four adults with malabsorption who experienced a high retention of absorbed N when fed an elemental diet. However these patients underwent N balance studies in a crossover fashion, and were fed in addition solid food, and a diet partly containing a casein hydrolysate formula (CHF): following such diets plasma and urinary urea N levels declined. Therefore this study fails to support the thesis set forth by Vandenplas et al. In addition, the mean daily volume intake was smaller with the PWHF compared with the WHF (590 versus 680 ml/day, p = 0.002), while the protein equivalents/dl were almost similar [14]. These findings clearly show that the net retention of N appears to be lower in the PWHF-fed babies.

Table 2: Contributory factors to the unreliability of diagnostic diets in atopic dermatitis.

* Presence of the specific allergen in different foods
* Contamination with the offending food
* Failure to eliminate specific components of the offending food
* Unlabeled inclusion of the offending food as ingredient
* Cross reaction among closely related foods
* Transfer of the offending food through breast milk
* Multiple sensitivities
* Child and parents' compliance
* Environmental stimuli
* Infections

In 205 healthy term infants enrolled shortly after birth, and followed-up for 8 months, 72 of whom receiving a PWHF, 68 BM, and 65 a WHF [16], the growth rates were measured at one-month interval until the 4th month. The laboratory studies included the measurement of IgG and IgE anti-CM antibodies in 30.7% infants, and several common gastrointestinal (GI) symptoms were evaluated. The dropout rates for reasons related to the feeding regimen were 24% in the PWHF, and 21% in the WHF groups, higher than in the study by Vandenplas et al. [14]. To demonstrate that feeding an HF in the very first days of life does not influence the development of atopy, 128 infants were fed in a double-blind way a CM formula or a PWHF as a supplement to BM during the first 5 days. When the amount of early post natal CM supplementation was correlated with subsequent IgE levels, it was found that the total volume of supplements and the frequency with which they were received by the neonates before the start of breast feeding resulted in a statistically significant increase in the total IgE levels (p = 0.02). The dropout rates were 4% at day 150, and 26% at day 365 [17]. However, feeding a CHF in the neonatal period seems to influence the absorption of macromolecules. In 130 healthy term neonates randomly assigned at birth one of three feeding regimens for the first 3 days of life, and then exclusively breastfed, only those CHF-fed had at 2 months a significantly higher a-lactalbumin serum content in comparison to controls, in addition two babies of the CHF-fed group had CMA symptoms at 7-8 weeks of life [18].

So the results at our disposal do not allow a conclusion: on the one hand the clinical impact of variations of AA serum content in healthy infants is presently largely unknown, on the other in the children so far evaluated there are no visible differences in both growth and symptoms.

Clinical properties of HFs: Studies in atopic children

Use of HFs in treating CMA in HR children: Considering the pertinent studies, it is evident that less or more severe reactions were observed employing formulas with either whey or casein proteins more or less extensively hydrolyzed. We discuss first the "programmed" studies which demonstrated the allergenicity of HFs in vitro and in vivo in children with IgE-mediated CMA, using skin prick tests (SPT), RAST, and/or challenge studies:

a. Six out of 13 infants aged 3-32 months with high serum total IgE and CMA as diagnosed by an appropriate challenge had specific IgE antibodies directed against Pregestimil, Nutramigen, and Alfa-Rè [19];

b. The positivity of SPTs to Nutramigen employing casein/whey formulas with an increasing grade of hydrolysis was recorded in a group of 15 children (median age 8 years) with immediate-type CMA: no child had positive SPTs to the "regular", extensively CHF, however 5-7/15 (33.3-46.6%) reacted to the intermediately hydrolyzed preparations [20];

c. Dean et al. [21] have studied the allergenicity of several infant formulas, using RAST and RAST inhibition on sera of 16 patients (mean age 7,5 years) with known CMA: the RAST was > 3rd class to several HFs in 10/16 (62.5%), 7 to the only high-degree WHF, 1 to Nutramigen, 2 to Pregestimil, 3 to the soy/collagen HF, and 2 to a SPF. The RAST inhibition results were in agreement with the RAST ones;

d. Rugo et al. [22] used SPTs, RAST, RAST inhibition and titrated provocation tests in 8 children (aged 5 months-9,5 years) with known CMA; the challenges were in single blind fashion. Five out of 8 children (62.5%) reacted to low-degree WHF with symptoms similar to those induced on challenge by whole CM, 2/8 to extensively WHF (EWHF), 4/8 to a not marketed whey ultra-filtrated formula; 1/8 manifested only a perioral urticaria on challenge with the soy/collagen HF, and no child to CHFs;

e. After Sampson et al. [23] documented the safety of a new extensively CHF in 25 children with CMA who underwent a DBPCFC, Amonette et al. [24] demonstrated in a 7-year-old girl with CMA acute IgE-mediated reactions following a DBPCFC with the same formula and in addition, with another extensively CHF and a PWHF, whereas the girl had no reaction to a SPF.

I total, we report 202 reactions to HFs [4,5,24-54]. Reactions to extensively CHFs are shown in (Table 3) [4,5,24-44,50-54], and to partially and extensively WHFs in (Table 4) [23,24,26,27,29,39,40,45-50,52]. In particular, we first reported [50] 5 exclusively BM fed infants aged 3-8 months (median 5 months) with IgE-mediated CMA, who experienced anaphylactic reactions when first fed a small amount of a CHF (Table 3). All infants had AD (atopic dermatitis) during BM feeding, positive SPTs and RAST to CM proteins and to the HF; total IgE levels ranged from 45 to 2,990 U/ml. Subsequently the infants were successfully fed a SPF.

A new ultra-filtrated WHF was investigated in 66 children with CMA (mean age 1.9 years), who tolerated it on open food challenge (OFC), except four, only one of whom was positive at the first rechallenge and negative at the final one. However of the 35 subjects with IgE-mediated CMA 11% (3/28) had positive SPTs and 6% (2/35) IgEs to the WHF. The authors conclude that this formula is safe for children with CMA, and suggest to perform a rigidly controlled OFC in children with immediate reactions to CM before starting whatever HF [55].

Table 3: Contributory factors to the unreliability of diagnostic diets in atopic dermatitis.

Contamination with the offending food due to to errors during processing and preparations :
* Inadequate cleaning of equipment (Yunginger et al, J Food Prot 1983;
* Inadvertent use of certain ingredients or
* Use of the same utensils for different foods (Yunginger et al, JAMA 1988; 260: 1450)

Table 4: Contributory factors to the unreliability of diagnostic diets in atopic dermatitis.

Failure to eliminate specific components of the offending food :
* Lactose can be contaminated with CM proteins
* Lecithin can be contaminated with egg or soy proteins
* Starch can be contaminated with wheat, corn, tapioca proteins
* Many gluten-free products contain other wheat proteins

HFs can induce, in addition to immediate reactions, also intestinal lesions which both SPT and DBPCFC fail to detect, while they can easily documented with light microscopy [39]. Two babies aged 15 days-10 weeks were fed an EWHF being affected with diarrhoea Æ remission for 10 Æ days recurrence Æ resolution when put on a BM diet. The intestinal morphology improved, however following an OFC with the EWHF showed congestion and inflammatory infiltration of the corion of a lymphoplasmocytic type, and increased numbers of intraepithelial lymphocytes. Such lesions are sometimes discrete, and useful for diagnostic purposes only when they are compared with a previously regular biopsy (which is rarely performed in normal conditions). Therefore we are confronted with elusive forms, because their diagnosis is difficult with the presently available tests, in addition to making necessary the intestinal biopsy, and the differential diagnosis with all possible causes of diarrhoea in little infants.

About the case of urticaria to Alimentum reported by Oldæus et al. [25], the HF was administered in a SPF, however the patient had a positive RAST only to the HF, thus excluding the potential responsibility of the SPF.

Thus, considering the cases referred to in the literature, the use of HFs has provoked 202 reactions, many of which IgE-mediated, in 132 children, aged 20 days-15 years) to CHFs (1 case of shock, 5 of anaphylaxis, 7 of generalized urticaria, 1 apparent life-threatening event) (+ 2 localized), and in 70 children aged one month-15 years to WHF (either extensively or partially) (1 case of shock, 10 of anaphylaxis, 13 systemic reactions, 2 apparent life-threatening events), at variance with other studies [56,57]. In addition there is the unspecified number of significant allergic reactions to GS resulting in the removal of the HA designation from the label [46], and those ascribed to a CHF [5]. On the other side Buts et al. [58] affirm that a PWHF tolerated by >50% of babies who are highly allergic to CM can be called HA even if it can potentially trigger severe anaphylactic reactions in <50% of them.

Strategies in preventing atopy in HR babies with the use of HFs

Several studies in babies with a high hereditary risk, that is a severe single or dual parental heredity, tried to prevent atopy with the use of HFs. In several studies a highly CHF has been employed, in others a PWHF (Table 5) [6,7,59-73].

In a prospective, randomized study the avoidance of CM, egg, fish and peanuts, during the first 6 months of lactation with the

Table 5: Contributory factors to the unreliability of diagnostic diets in atopic dermatitis.
Unlabeled inclusion of the offending food as ingredient :
* Errors in processing and preparations
* Restaurant foods
* Unknown source of food ingredients: Lecithin (egg or soy-bean) Starch (wheat, corn, tapioca, potatoes, etc.)

supplementation of a CHF, significantly reduced the prevalence of AD and food allergy at the age of 1 and 2 years, however there was no difference at age 4 and 7 years [59-61]. The compliance to the avoidance regimen was poor, there being evidence of breaks in the diet, in addition the nursing mothers who avoided CM during the period of lactation were allowed to drink an extensively CHF [59].

Chandra et al. [62] showed that in babies drinking a PWHF the prevalence of atopic disease significantly decreased compared to that found in SPF- or CM-fed infants. However atopic disease was not wholly prevented, since the prevalence of allergic disorders in the study groups was 18% at 12 and 26% at 18 months. In addition, 4/5 infants who developed atopic symptoms while on PWHF had a positive SPT to CM proteins, against 2/25 SPF-fed (80% versus 8%) [62] (Fisher 0.0026), thus suggesting that sensitization to CM proteins in infants receiving this HF is by 1000% more frequent than in the SPF-fed babies.

In the studies by Vandenplas [6,64,65] performed in HR babies fed either a PWHF or a CM formula, the prevalence of CMA in the HF-fed group at 12 and 36 months of age varied from 6 to 25%.

In an Italian study [71] 279 babies received a dietary and environmental prevention programme, and 80 formed the non-intervention group. However, the babies were neither randomized nor observed blinded concerning evaluation, the SPTs were not always done, or specific IgE measured, neither details of the clinical methods employed, nor of the severity score used to distinguish different forms of AD were given. In addition we note the high prevalence of allergic disorders in the controls (> 42%).

Halken et al. [7] studied prospectively from birth to 18 months of age 105 HR infants: the study group was recommended BM and/or a HF (Nutramigen or Profylac), while the control group consisted of 54 HR babies born in 1985. The authors suggested environmental controls and dietary manipulations restricted to solid foods avoidance during the first 6 months of life, which were strictly followed by 85% of cases, therefore the high prevalence of atopic diseases in the two groups (32 and 74%, respectively) is unexpected. In a subsequent prospective and randomized study of the same group [55] on 141 HR infants fed the same HFs to evaluate their protective effect, the CMA incidence was 3.6%, while that of CM-related symptoms was 26-33% (15% in the 20 BM-fed babies); the figures of controls are not specified. None of the mothers had dietary restrictions, thus the compliance with the fundamental advice of strict and exclusive breast feeding until the 6th month was scarce: 46% for 1-2, and only 14% for 6 months, whereas the mothers in our preventive intervention programme were 100% [3].

Mallet and Henocq [68] have studied the effects of a high-degree CHF in 39 infants, in addition 53 were BM- (> 2 months) and HF-fed, and 65 infants fed an adapted CM formula, 33 of whom received also BM as above were the controls. Only 31% infants of the HF group (12/39) vs. 45% of BM-HF-fed (24/53) had a surely positive family history. Among the HF-fed, at two years 10/78 (13%) had asthma, and 9/78 AD (11.5%) vs. 12/61 (19,6%) and 26/61 (42,6%), at 4 years there were 8/70 (11.4%) cases of asthma and 5/70 (7%) of AD, vs 6/54 (11,1%) and 14/64 (25,9%), respectively. Therefore the results are difficult to interpret, apart the high prevalence of atopy in both the HF-fed and the control groups.

In conclusion, only two studies [23,55] show that Alimentum and Profylac fulfil the American Academy of Pediatrics (AAP) recommendations [74] for designing a formula as HA. This is stressed by the inclusion of children with different ages, clinical diseases, and a different diagnostic work-up, in the absence of well-defined and -validated methods [6,14,63,66], as instead suggested by ESPACI and AAP [57,74]. Also the absence of specialists may limit standardization of diagnosis [75]. The high prevalence of allergic disorders especially in the control children seems to be a common characteristic [6,7,62-65], and the 36-74% prevalence reported in several studies [6,7,62,63,65,66,68] are unexpected, as well as the CMA prevalence found in the PWHF-fed babies (21-35%) [7,60,62,65,66,68,73] (Table 5). However most of these studies included only infants with a very HR of allergic disease, therefore such high prevalences may be justifiable.

In addition, we have investigated the immunogenicity in the IgE system of a PWHF, 400 ml daily of which were given to 39 mothers of HR babies during the lactation period, while 39 control mothers of HR babies consumed 400 ml daily of CM. Although there was no significant difference in both the incidence and prevalence of CM-induced AD and of CMA in the babies at 0.5 and 1 year of age, according to the mother's diet, the number of babies with IgE antibodies to CM and with total IgE levels more than 2SD for normal values for age were significantly higher in the group of babies whose mothers received the HA formula ($p = 0.02$). We may speculate that when a mother drinks this product, a large amount of immunogenic peptides are easily absorbed through the intestinal mucosa, thus rapidly reaching the breast and then presented to the T and B cells of her baby. This data suggests that such PWHF seems to be more immunogenic in the IgE system than CM [76].

Nine babies breast-fed by mothers who strictly limited the assumption of CM experienced anaphylaxis when fed a PWHF [77]. The sensitization seems to have occurred in the very first days of life as a consequence of some feeds in the Maternity Hospital. [78] with the PWHF, which was given again at the 6th month for CMA prophylaxis. Although we cannot exclude that the infants were the victims of pirate bottles, nor we analyzed BM samples for the presence of CM proteins, the following points are in favour of this assumption: 1) The mothers totally avoided CM and dairy products during lactation, therefore a sensitization through BM can be ruled out; 2) The babies were healthy during breast feeding and did not show any symptom or sign suggestive of CMA; 3) high levels of IgE antibodies and strongly positive SPTs to the HF were present in the babies [77].

Chemical and immunological properties of HFs: a critical evaluation

The more or less severe reactions as yet reported were unexpected by the industry, that on the contrary tried to prepare HFs in the hope of reducing significantly the prevalence of CMA, by maintaining an adequate nutritional value [79]. It is therefore necessary to try to explain the allergenic activity of such HFs:

As regards the main technologies employed in processing, commercially available HFs devised for CMA or CM-intolerant infants are processed using two main technologies: heat treatment or enzymatic cleavage or both to reduce the MW of peptides to obtain alternatives of minor allergenic potential. Several different techniques are employed, together with enzymes, such as trypsin, chymotrypsin, pepsin, carboxypeptidase, etc [22]. Briefly, during infant formula processing, heat-induced denaturation, mainly affecting whey proteins, changes the protein structure of the allergenic molecule, and most conformational B epitopes are eliminated, thus facilitating the hydrolysis, but not of sequential T epitopes [9]. An enzymatic hydrolysis is necessary to degrade casein proteins, which reduces the antigenicity and allergenicity, mostly eliminating the sequential epitopes, then the ultrafiltration is necessary to remove peptides of high MW [22]. These different technical procedures are essential for obtaining an acceptable palatability, and a combination of these methods is also in use [80]. However the results cannot be considered as definitive: the degree of hydrolysis can be of 26% in WHFs, of 15% in whey HA formulas (range 1,3-32,5), and of 52% in the only CHF tested [81].

Another intriguing issue is the evaluation of higher or lesser MW of HFs. Since the HFs contain peptides of lower MW than the native protein, it is suggested that a low MW most likely decreases also their inherent sensitizing capacity, which is why the AAP Committee of Nutrition recommended HFs with a MW <1200 D supposedly not allergenic [82]. However in peptides as small as 1500 to 2000 D in size, or smaller, even when their ability to act as an allergen is eliminated, the immunogenicity of the absorbed fragments may theoretically be as strong as or even stronger than the native molecule [80]. Thus, the MW alone cannot suffice to guarantee antigenicity for any given HF, and especially for commercially available products [83], because the MW distribution declared by a manufacturer is approximate, and cannot assess a 100% non allergenicity of a given HF [84]. Hence the indiscriminate HA labelling, which literally means "less allergenic" than normal CM formula, and not "non- allergenic", is unconvincing because is not quantifiable, hence open to each evaluation [57]. Small but detectable amounts of residual peptides, even with MW >6.000 D can be demonstrated in HFs (Table 1), however in CHFs there is 15-20% of peptides with MW >3,850 D and 35-42% with MW between 340 and 3,850 D [86]. After protein enrichment by trichloroacetic acid (TCA) precipitation, the presence of high-MW polypeptides was shown in HFs, such as protein bands visible in SDS-PAGE with a characteristic pattern [87]. Partial hydrolyzed formulas show the higher amount of polypeptides with a diffused area ranging from 6.500 D and 71 kD, while extensively hydrolyzed products have a lower residue [87]. The study upon 11 WHF, 7 of which HA, one based on hydrolyzed casein, and one ultrafiltrated [81] shows the presence of a mean 55,5% content of peptides with a MW of 3 kD, 27% of 3-5 kD, 13,5% of 5-10 kD and 6,9% >10 kD, including two HA HFs with a 26,3% and 40,6% content, thus all tested products retain some residual antigenicity of one or more of the individual CM proteins [81]. Previously peptides with MW > 5.000 D were found in GS [88], however by tricine-SDS-PAGE and subsequent silver staining were identified residual protein fractions between <14 kD and 20 kD in Alimentum, Profylac and in the CHF Nutramigen and none in Alfa-Rè [56], Yet studies done initially in the animal model (see below) suggested that HFs even with peptides <3.400 D were not immunogenic and peptides with MW between 3.400 and 6.500 D would induce only weak reactions. The allergenic and immunogenic epitopes which can be "seen" in CM proteins by the human are not necessarily the same as those seen by a rabbit, or guinea pig, or lamb;

The AA sequence of an epitope can resist the physicochemical manipulations, in spite of extensive breakdown [89], thus HFs may contain an amount of residual intact proteins able to stimulate immune reactions in predisposed children [90]. New epitopes may arise due to structural changes in the ternary structure, or may be created during heat treatment, or be unmasked during the ready-to-feed preparation of CM formula, or following digestion of food peptides during the intestinal passage. The immunogenicity of new epitopes may be as high as that of native protein, or even higher [9]. Thereby the residual antigenicity and thus the potential allergenicity is dependent on the food-processing technologies applied [89]. On clinical grounds, the cells secreting IgM against trypsin- and pepsin-digested ßLG (ß-lactoglobulin) suggest that some antigenic epitopes may resist such treatments [91], and it is known from the Küstner case that epitopes not present in the original food protein can be formed or made accessible by digestion, unless the segments of peptides that resist enzyme hydrolysis are affected and inactivated by adequate denaturation, or some kind of filtration is used to remove the residual large peptides [89]. Lipids and carbohydrates not concerned by the hydrolysis, can bind protein structures, building up the substratum to make new epitopes. A number of small peptides may become antigenic and even allergenic, and bind IgE antibodies, by aggregating or cross-linking within each other or with other molecules, probably through covalent or very strong hydrophobic binding, thus binding a cell membrane that can be presented to T cells [83]. HFs contain protein fractions which resulted in a specific IgE binding after incubation with serum samples from patients with CMA [10]. As previously reported, HFs are not only allergenic in already sensitized babies, but may be immunogenic in the IgE system due to residual allergenic epitopes which bind IgE antibodies to CM [10]. Haptens can combine with albumin or other protein carriers [83]: so a peptide not recognized by the immune system will probably never be available [9].

Elimination of peptides in HFs may be arduous: antigenic structures, which are processed and cleaved into peptides of low MW, consist of 11 to 13 AAs. Schematically, when an antigen is captured by B cells via their surface immunoglobulin (Ig) molecules and internalized, and enzymatically degraded inside the cell, the resulting peptide fragments with low MW are presented on the B cell surface in association with HLA class II molecules [92]. The HLA-peptide complex at the surface of the APC (Antigen presenting cells) is recognized

by the T-cell receptor, an event which result in the activation of both T and B cells [93]. Subsequently, specific processes lead to IgE synthesis [94]: the processation with the unfolding of the native polypeptide chain and demasking epitopes hidden by the three-dimensional folding of the molecule, trigger a specific IgE reaction in individuals with atopic backgrounds [95]. In normal subjects, the AA sequences equipped with the functional properties necessary to be complexed with HLA molecules can bind to an HLA molecule only after the unfolding of proteins [96]. Thus it seems impossible that the food-processing procedures in vitro abolish all the epitopes present in the native protein source [89]. So we are totally sceptic about the idea of destroying or neutralizing the epitopes of so a peculiar structure: a breakdown of 90-95% of the peptide reactivity leaves sufficient antigen to fully stimulate an antigen-specific immune response, even if the allergen hydrolysis can reduce the immunogenicity dramatically. There does not exist a sophisticated technique insuring the selective destruction of such specific epitopes. Such processes are operative already in the neonate/little infant [97]: in an infant ingesting CM the IgE reaction to the non-self-proteins always takes place, as shown by dendritic cells active in the neonate mice [98]. The ability of antigen fragments to interact with specific B lymphocytes is related to the retention of epitopes that can bind surface Ig receptors: the AA sequence for a peptide from bovine serum albumin bound to HLA class was described [99]. It is easily understood that a "final product" consisting of two or three AA residues (and one epitope) would be more sufficient to trigger allergic reactions in HR children than the clearly recognized by T cells seven or eight AA sequence with ≥ two epitopes [100].

Casein has been found in whey HFs and vice versa: residual casein epitopes in all the HA formulas Alfa-Rè, LHA and Pregomin were detected [101]. Trace amounts of casein are present in commercial whey preparations, accordingly casein IgE epitopes could be demonstrated in a number of such products [81]. In another study [23] Alimentum and Nutramigen had detectable casein and whey proteins, however GS had more than 700 times the amount of detectable whey proteins, more than 100 times the amount of casein proteins, and more than 700 times the detectable whole CM proteins than the other two HFs. GS was found to have 2.625 mg of casein/g protein [88]. A recent study [56] has shown by inhibition-ELISA that Nutramigen has a 64,7% of whey conten and Alimentum about 50%, whereas Profylac haa a 36,6% of casein content and Alfarè of 17,9% [56]. The apparent contradiction of casein in WHFs and vice versa can be easily understood, since employing the TCA precipitation and SDS-PAGE to obtain the separation of whey proteins from casein [87], aliquots of casein may remain in the whey fraction due to casein-derived low-MW peptides [95]. Heating casein at a T = 1210C for 15 minutes is not enough, and boiling it for 30 minutes is necessary to reduce its antigenicity and immunogenicity significantly [89]. Most IgE antibodies to CM proteins bind short chains of AAs that have mostly sequential epitopes, T epitopes [92], in addition to the high allergen city of the casein k fraction [90].

Additional effects are seen in nursing newborns. To appreciate how the elimination of all the epitopes of CM proteins is challenging [102], we point out that nursing babies can be sensitized to such proteins even through BM. Once consumed by the nursing mother, after a first denaturation with cooking, the CM peptides undergo in the maternal gut an enzymatic hydrolysis and further denaturation and assimilation, pass through biologic membranes, and go into the blood and to the breast (enteromammary axis). Reaching thus the infant's gut, they are again hydrolyzed, absorbed, and enter into the APC where they experience their hundredth transformation, yet CM peptides are still immunogenic [80] despite the presence of secretory IgA in BM [103]. Such data explain also why babies who apparently never received CM native proteins, but were surely fed a HF supplement in their very first days of life, at the age of few months when still exclusively BM-fed experienced anaphylaxis after a few ml of the same formula [7] as the babies fed whole CM in the Maternity [78]. However, we face the crude reality that 50-60% if not 100% of HR children receive a CM formula in the nursery without our knowledge [78]. In our hospital, and surely others too, also babies who are meant to be BM-fed receive a pirate bottle during the night if they cry [2,78].

Minimal levels of ßLG in BM (Table 6) [25,84,103-109] can be allergenic at the point of triggering anaphylaxis [33], although IgA antibodies to CM proteins can play an important role in the exclusion and elimination of ßLG [103]. According to data shown in (Table 6) BM has less mean ßLG levels than ready-to-use Nutramigen: 7-12 times after drinking 400 ml of CM, and 84 in basal conditions. It follows that the assumption of HFs declared to be HA containing little amounts of residual ßLG, however in concentrations much higher than those found in BM, can trigger severe anaphylactic reactions in babies with IgE-mediated CMA, elicited by IgE cross-reactive with epitopes present despite the enzymatic hydrolysis or even multiplied by it [85]. It is of note that certain extensively HFs contaminated by proteins left intact by enzymatic hydrolysis, contain significant amounts of ßLG detected with the methods of ELISA and RAST-inhibition: in dry powder a CHF contains 0,0056 ± 0,0005 mg/g, a WHF 200: in the ready-to-use formulas the levels varied between 0,84 ± 0,07 and 31.200 ± 3.744 mg/l (Table 6). It has been shown that products of digestion of ßLG retain allergenic activity [91]: Haddad et al demonstrated that 10 CM-allergic children with or without IgE antibodies to undigested ßLG all had IgE antibodies to the peptic or peptic and tryptic digests of ßLG [110]. Indeed ßLG is stable to digestion for 60 min, thus as other intact proteins is capable of crossing the gut mucosal membrane and of entering the circulatory system, with all likelihood to be absorbed, hence eliciting allergenic responses [111].

Several studies have shown in animal models that CM protein HFs failed to elicit an IgG antibody response to CM protein or induce passive cutaneous anaphylaxis [79,102,112-114]. Subsequently, Boner et al. [115] evaluated the allergenicity of a PWHF in guinea pigs fed ad libitum CM, pasteurized CM (PCM), or PWHF. On day 37 were

Table 6: Contributory factors to the unreliability of diagnostic diets in atopic dermatitis.

Cross reactivity among closely related foods :
* Cow, sheep and goat milk(Juntunen et al, Kiel Milkwirt Forschungsbr 1983, 35: 439)
* Chicken, turkey, duck and goose eggs (Langeland, Allergy 1983; 39: 399)
* Protein hydrolysates and CM proteins(Lorenz et al, Nestlè Nutrition 1988; 17: 215)
(Businco et al, Ann Allergy 1989; 62: 333)

IV challenged: one fatal reaction was provoked by the sequence PWHF-PWHF or PCM-PWHF, and nonfatal reactions by the same sequences, and the sequence PCM-PWHF [115]. Again in guinea pigs even employing the more rigorous challenge schedule, the extensively hydrolyzed formulas failed to cause evident symptoms of anaphylaxis [116]. Cordle et al. [117] using the rabbit hyperimmunization model, have measured with the ELISA immunoassay the immunologic active bovine whey protein (IAW) and active bovine casein (IAC) in HFs demonstrating that either formula containing intact, or partially, or extensively hydrolyzed proteins, elicited immune responses (mg/g protein): the partially hydrolyzed 1060-1210 IAC and 140,000-210,000 IAW, the highly hydrolyzed 7.68-12.2 IAC and 19.1-25.6 IAW, while the intact CM-formulas 400,000-800,000 IAC and 180,000-600,000 IAW).

Consequently, the protein molecules, even if hydrolyzed in peptides of very low MW, retain all their patrimony of immunoreactive epitopes, capable either of stimulating the immunocompetent cells of the baby, or inducing the synthesis of IgE antibodies. Hence all the epitopes of a CM protein, native or manipulated, are potentially immunogenic, but they can defined as such only with reference to the sensitized individual. [85].

Laboratory diagnosis of children with HF allergy

Laboratory diagnosis can be done employing in vitro and in vivo tests, useful also for clinical testing in at risk infants before the use of HFs, and clinical testing of formulas with standardized procedures [2,57,74].

The most reliable methods are those using the RAST, the ELISA, and their inhibition variants, since their results are not crucially dependent on epitope density. In Table 6 are shown the results obtained with the ELISA and RAST inhibition tests. The very great differences demonstrated between PWHFs and CHFs have been commented earlier. Analyzing the dry weight concentrations, the PWHFs appear to be 585 times less allergenic, and the EWHFs 21x106 less allergenic than CM. Evaluating the ready-to-use levels, the figures are 128 and 4.760.000, respectively.

As an alternative, the FAST inhibition test can be utilized (118). Using sera from patients with CM anaphylaxis and high IgE activity to CM, casein, ßLG, and a-lactalbumin, the authors evaluated Nutramigen, Alimentum, GS, and Alfa-Rè. All the HFs bound IgE specific to CM and its allergens, although in general the HFs bound significantly less IgE than the CM formulas, with the notable exception of GS (Tables 7, 8, 9).

SPTs, RAST and DBPCFC have been employed as detailed before [18-20, 23]. Among 26 children with different forms of CMA, there was a group with more severe reactions to CM, which also had positive SPTs to CHFs (Alimentum, Nutramigen), while children with less severe form of CMA had not positive SPTs to these products, but all had positive SPTs to the PWHF [119]. However, both SPTs and RAST failed to correlate with clinical reactivity to Nutramigen [56], while 2 out of 11 CMA children (18,2%) with SPTs positive to the formula experienced a clinical reaction following an OFC [37]. Thus it appears that babies with SPTs positive to HFs cannot ingest HFs with impunity.

Table 7: Main properties of SPFs.

Not reacting with CM
Lower allergenicity (IgE Abs) than CM
Similar antigenicity (IgG Abs) to CM
Nutritional adequacy similar to CM formulas Better palatability than highly HFs Less expensive than highly HFs

CM = cow milk, HS = hydrolysate formulas Adapted from 93

Table 8: Prerequisites of an ideal CM substitute.

Adequate nutritional value
Hypoallergenicity
Pleasant taste
Not too expensive
Easily available
Adaptability to individual needs

Table 9: Possible consequences of inappropriate diets.

Failure to thrive
Osteoporosis and hypocalcemia
Anemia
Hypoprotidemia
Zinc deficiency
Malnutrition of mother, fetus and infant

The RAST inhibition may be useful to predict the reactivity or cross-reactivity of IgE anti-CM antibodies with infant formulas. Accordingly, 1-4% of infants with CMA would recognize epitopes of CM proteins in about one of the HFs. Six out of 13 infants with CMA (46%) indeed reacted to four HFs, Alimentum, Alfa-Rè, GS, and Nutramigen [120].

Clinical testing of formulas in children with HF allergy

Clinical testing of HFs with standardized procedures has been recommended by the ESPACI [57], and the Subcommittee of Nutrition and Allergic Disease of the AAP [74]. The AAP Subcommittee has recommended that DBPCFC studies be conducted with standardized procedures, also to exclude the possibility of a type II error in the data. In the case of HFs, infants and children with documented CMA should be studied in conformity. A sufficient number of such patients should be enrolled in a prospective study organized in such a way that it can be projected with 95% confidence that 90% of children with CMA will not react to the HF studied. This model was followed by two studies [23,55].

Considering the 90% as a cut-off point, and following the procedures, a group fed HFs should be formed by 28 children with CMA free of clinical symptoms. If 1 or 2 infants react, the sample should be increased up to 46 or 61 children, respectively. If the 90% level is judged a too low cut-off, to use a 95% probability level, the sample should consist of 120 children with no reaction, but if only one infant reacts to the HF, the group must be enlarged to encompass approximately 400 infants (plus 400 controls equally representative). In our opinion it would be inconceivable to recruit so many infants,

who in addition should comply with that way of approaching the above recommendations. Facit: before embarking on a new study or reporting an anaphylactic reaction one should consult an expert in statistics. Halken et al. [55], have recommended to perform a strictly supervised OFC in children with immediate reactions to CM before starting whatever HF. In two infants the anaphylactic symptoms appeared as soon as one drop of CM or Alfa-Ré was administered, and 5 ml to the other three [50].

It might be appropriate that HFs, before they are employed in vivo in infants with IgE-mediated allergies, be studied with adequate methods such as RAST-inhibition, CRIE, immunoblotting, ELISA, etc, and studies be organized according to previous specifications [57,74,75]. The laboratory animal hyperimmunization model proposed to evaluate the immunogenicity of protein HFs (117) could reduce the costs, the risks and the time inherent in the DBPCFC studies.

Management of children with allergy to HFs

For the treatment of atopic children, the suitable CM substitutes are BM if still available, SPFs [3], HMMBF [2], and L-amino acid formula (AAF) [37,40,41,75]. As regards the AAF, the above references seem to be sufficient for a correct use. HMMBFs were first employed in 1973 [121] and are HA in the strict sense, since reactions to HMMBF were never reported, are very well accepted by newborns, infants [122], children aged 8-9 years [123] and even parents, also insuring a regular growth [122].

Concluding Remarks

We stress that many concerns regarding the HFs depend by the striking lack of scientific data provided by the Companies regarding a lot of technical aspects, not useful even to the Companies. However, the real composition of these HFs remains largely unknown. Moreover, varying the technologies according to the different Companies, at least this technical information should be given to the medical specialists or in any case to the doctor prescribing the HF. Not even topics such as the possible contamination of the product during fabrication or packing procedures, or the lot-to-lot variability of HFs have been discussed, and we think that a word of caution is needed.

Although the proteins of HFs have been processed by physico-chemical procedures and therefore contain peptides of lower MW than the native protein source, the peptides still have allergenic potency and can be recognized by cell-bound IgE of a child with CMA. We point out that the changes in the intestinal mucosa [39], which unless carefully sought after are likely to be missed. One child who in spite of a biparental history of atopy was not subjected to prophylactic measures was dead 8 hours after the first of two feedings of PHWF (12 hours after the last SPF was given) [124].

On clinical grounds, reactions to casein were first observed by Glaser in 1944 [125]. We have documented >202 allergic reactions induced by HFs, from 17 cases of shock-anaphylaxis (ome every 3.3 years), 3 apparent life-threatening events, and 18 systemic reactions (17.8%) to worsening of eczema lesions and GI symptoms (Tables 3, 4). In comparison, SPFs have provoked one case of anaphylaxis/22.3 years [3], with a difference of 676%. Sampson has proposed a 10-18% prevalence for HF allergy (in high atopic children) [37,56], but in Milan 86% of neonates are recommended HFs (14% partially and 70% extensively), also in the absence of a history of family atopy [126]: how much can increase the prevalence of HF allergy and of adverse reactions? On scientific grounds, we conclude that neither SPTs nor RAST are reliable, whereas only DBPCFC (OFC in infants) should be considered diagnostic [57,74,75]. Thus, further large trials should be performed using well-defined samples of HR infants.

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