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Safety of a new extensively hydrolysed formula in children with cow's milk protein allergy: a double blind crossover study

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Abstract

Background: Formulae for infants with cow's milk protein allergy (CMA) should be based on extensively hydrolysed protein. 'Extensively' however is not strictly defined. Differences in molecular weight and peptide chain length may affect its clinical outcome. We studied the safety of a new extensively hydrolysed casein based formula (Frisolac Allergycare®: FAC) for children with IgE mediated CMA.

Methods: Thirty children, aged 1.5 – 14.8 years old (median 4.9 years) with persistent CMA were enrolled in this double-blind reference product (Nutramigen®: NUT) controlled crossover study. All had positive skin prick tests (SPT) and IgE mediated allergy, showing immediate reactions after ingestion of small amounts of milk. Twenty-five children also had positive radio allergen sorbent tests (RAST) to cow's milk. Formulae provided consisted of 80% elementary formula in combination with 20% reference or test product. Crossover periods lasted for two weeks. From both products molecular weight (MALDI-TOF method and HPLC) and peptide chain length distribution (adapted Edman degradation) were determined.

Results: Maximum molecular weights of NUT and FAC are 2.1 and 2.56 kDa, respectively. The contribution of free amino acids and small peptides <0.5 kDa is 46% for FAC and 53% for NUT. About 50% of the protein fraction of both products consists of peptides longer than four amino acids. Three children did not complete the study. The other children all tolerated FAC very well; no adverse reactions were reported.

Conclusions: The new extensively hydrolysed casein-based formula (FAC) can safely be used in children with IgE mediated cow's milk allergy.

Background

Cow's milk protein allergy (CMA) is an increasing problem in infancy, and a result from an abnormal immunologic reaction to cow's milk protein [1]. About 3% of all new-borns will suffer from CMA within the first year of life. Although breast milk is the best to provide, up to

1.5% of breast-fed infants will develop CMA [2]. Treatment of CMA in infants and young children means total avoidance of cow's milk and use of 'hypoallergenic' formulae. It has been stressed by both the European Society for Paediatric Allergy and Clinical Immunology (ESPACI) and the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) that only extensively hydrolysed formulae should be used in IgE mediated CMA owing to their proven safety and hypoallergenicity [3–6]. Partially hydrolysed formulae should be avoided in infants having CMA due to the unacceptable frequency of adverse, at times even severe, reactions associated with their ingestion [4,7–9]. However, these hydrolysates may be useful in prevention of CMA in high-risk infants as has been shown in four recently published studies [10–13]. The terms 'partially' and 'extensively' are not well defined. Although molecular weight is an important classifier, studies have shown that products with hydrolysates of comparable molecular weight may have different preventive or treatment effects [12] or is of less predictive value than suggested [11]. Other characteristics, such as peptide chain length distribution may be necessary to judge the effectiveness of the protein hydrolysate. However, this has to be studied and for the time being the only way to determine the safety of a hydrolysate-based product is to test it in those with CMA as also indicated by the American Academy of Pediatrics [7] and the European Community [14]. The aim of this double blind, cross-over study was to determine whether a new extensively hydrolysed casein based formula (Frisolac Allergycare®; FAC), with about 22% free amino acids and a maximum molecular weight of 2.56 kDa can be administered safely to children with IgE mediated CMA. As reference product Nutramigen® (NUT) was used.

Methods

Peptide characteristics of the used products were studied by three methods. Determination of absolute molecular weight was done by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method (MALDI-TOF), a rapid and sensitive quantitative method with high resolution for peptides as described by Kaufmann [15] and Soerpyapranata [16]. Molecular size distribution was measured with high-performance gel-permeation chromatography (HP-GPC, Superdex 75 HR10/30 column) with a phosphate-sulphate buffer (pH 6.65), and spectrophotometric detection at 20 nm. For calibration, the following proteins and peptides were used: bovine serum albumin (68 kDa), bovine α-lactalbumin (14.40 kDa), cytochrome C (12.32 kDa), insulin A oxidized (2.53 kDa), bradikinin (1.06 kDa), Arg-Lys-Asp-Val-Tyr (0.68 kDa), Pro-Phe-Gly-Lys (0.447 kDa), Thr-Tyr-Ser (0.369 kDa), Tyr-Arg (0.337 kDa), and TRP-Gly (0.261 kDa). This HP-GPC method is often used as an alternative for molecular weight measurement. An accurate distribution of peptide chain length was obtained by using an adapted Edman degradation [17].

Thirty children under treatment (23 boys and 7 girls) were included. Characteristics of participants are described in Table 1. All children showed immediate IgE mediated hypersensitivity reactions after challenge with or ingestion of small amounts of cow's milk at the time of inclusion. The reactions to cow's milk were exacerbation of the pre-existing eczema (morbilliform-like rash), oral allergy syndrome, wheeze and sneeze, vomiting, rhinoconjunctivitis and even anaphylactic reaction.

Skin prick tests (SPT) were performed using extracts of cow's milk. Results were evaluated after 15 minutes. A positive test was classified as a weal of 3 mm or larger. Histamine (10 mg/ml) and a diluent solution were used as positive and negative controls respectively. All children had positive SPT to cow's milk and 25 of 30 also had positive radio allergen sorbent test (RAST) to cow's milk. All children used extensively hydrolysed formulae from different manufacturers. None of them had signs of allergy.

The study had a cross-over, double-blind reference product controlled design. To guarantee the double-blind character of the study, the tested formulae were mixed with an elementary formula, home-made and prescribed by the Wilhelmina Children's Hospital in severe CMA, in a ratio of 80% elementary formula and 20% test formula. Organoleptic tests (taste, smell and outer appearance) by independent inspectors showed that in this ratio the test formula, FAC (Friesland Nutrition, Leeuwarden, The Netherlands) was indistinguishable from the reference product NUT (Mead Johnson, USA) which is been widely used for CMA treatment. The formulae were mixed under aseptic conditions and coded.

After informed consent was obtained, the children were randomly assigned to group A or B. Group A started with the test formula in the first period (weeks 1 and 2) and was fed with the reference product the second period (weeks 3 and 4). Group B started with the reference formula and changed to the test formula the second period. In case children had had an anaphylactic reaction in the past to cow's milk, an open challenge with the test formula was performed at the day care centre of the Wilhelmina's Children's Hospital. When no adverse reaction occurred during challenge, the test formula was considered to be safe to ingest by the child during this study.

A weekly questionnaire containing questions about skin, gastro-intestinal tract, respiratory tract, behaviour, fever, and anaphylaxis was answered. Questions were scored 1 point when answered positive, 0.5 points when answered ±, and 0 points when answered negative. Questionnaire

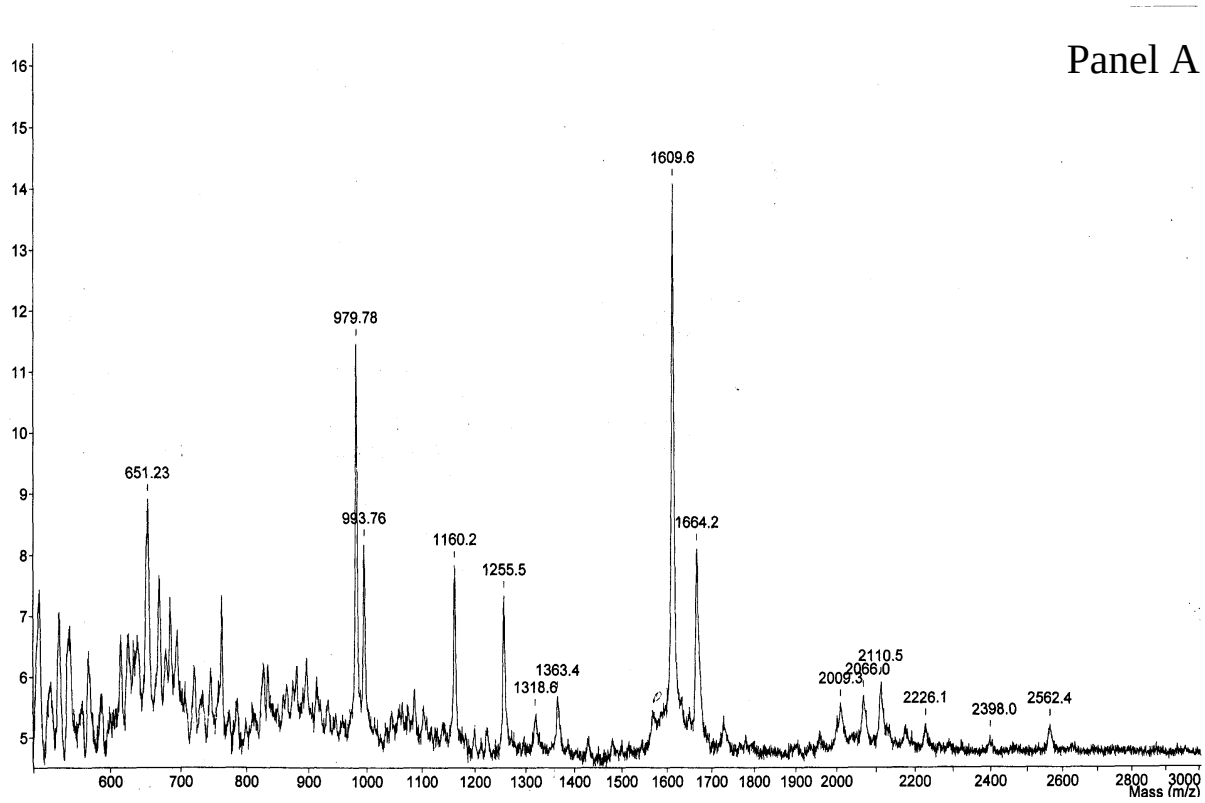

Figure 1
Molecular weight profile of the hydrolysate used in Frisolac Allergycare® (FAC). The molecular weight profile is determined with the MALDI-TOF method as described by Schols [19] and Kaufmann [15] and Soeryapranata [16].

scores were analysed according to the Wilcoxon signed-rank test, testing the hypothesis that no difference exists between the test and the placebo formula. $P < 0.05$ was considered statistically significant.

This study was approved by the Medical Ethical Committee of the University Hospital Utrecht, The Netherlands.

Results

The absolute maximum molecular weights (MALDI-TOF) as determined for the protein fraction of the complete products were 2.108 kDa and 2.562 kDa for NUT and FAC, respectively. Remarkable was the difference between the molecular weight of the hydrolysate used in FAC and the final product FAC. The maximum molecular weight increased from 1.613 kDa to 2.562 kDa. For NUT this was not studied since we did not have the hydrolysate used in this product.

Molecular weight profile and peptide length profile of the hydrolysates are shown in Figures 1, 2 and 3. Figures 1 and 2 show no relevant differences in absolute molecular weight between FAC and NUT. In peptide length profile (Figure 3), the test hydrolysate showed to have more di- and tripeptides and a somewhat lower free amino acid content compared with NUT.

Twenty-seven of the 30 children completed the study. Of the three children who failed to complete the study, two children refused to drink the formula after two weeks and one child had an exacerbation of dermatitis in the NUT period.

Group A comprised 12 children and group B 15 children. Distribution of age, gender, RAST, atopic disease and questionnaire score at start of the study between the two groups was comparable (Table 2). None of the 27 chil-

Table 1: Patients' characteristics

Number	30
Age	19 – 176 months
Median age	51 months
Gender	23 boys, 7 girls
Atopic disease	
Eczema	23
Asthma/wheezing	17
Gastro-intestinal symptoms	3
≥ 2 signs	10
Positive RAST	25
Positive SPT	30
Symptoms-after challenge with cow's milk	
Oral allergy	7
Wheeze	5
Urticaria	8
Exacerbation eczema (rash)	10
Angio-edema	3
Vomiting	2
Rhino-conjunctivitis	2
Anaphylaxis	1
≥ 2 symptoms	8

dren showed an adverse reaction or exacerbation of the allergic symptoms to the test formula.

The mean score after using the reference formula NUT was 1.28 (S.D.: 1.87) and after using the test formula FAC 0.89 (S.D.: 1.28). Analysis according to the Wilcoxon signed-rank test indicated that the two formulae did not differ significantly ($p = 0.135$).

Discussion

In this study, FAC is as effective as NUT which indicates that Frisolac Allergycare is suitable for infants and children with CMA. Both products have maximum molecular peptide weights lower than 3 kDa (Figure 1 and 2), a molecular size that is thought to have a low probability on at least two antibody binding sites which is a prerequisite for inducing an allergic response [19]. Based on the peptide length distribution (Figure 3), FAC contains less free amino acids, which is in favour of taste.

A first indication of safe use of the FAC hydrolysate in a study with children with proven CMA was based on preceding animal studies (not published). No development of antibodies specific for the hydrolysate was seen in mice and rats, whereas no active systemic anaphylaxis could be initiated in guinea pigs.

Although both products FAC and NUT had molecular weights less than 3 kDa based on our methods, Rosendal and Barkholt [18] showed that even extensively hydrolyzed formulae like NUT may contain peptide fractions of molecular weight above 5 kDa as determined by SDS-PAGE. They suggest that this may explain the rare occasions of allergic reactions on these formulae. However, no epitopes for anti-whey, -casein or -soybean protein antibodies were detected. They also found small amounts of β-lactoglobulin in NUT, based on sandwich ELISA although the principal protein source used is casein. The presence of a whey protein in such a product may be explained by co-precipitation during isolation of the casein fraction, or contamination during processing, it also may point to an overestimation by ELISA. Therefore, the accurate way to study a hydrolyzed formula is in a clinical study with cow's milk protein allergic patients.

A higher maximum molecular weight in the final product FAC compared with the used hydrolysate may be explained by aggregation of smaller peptides to larger particles, or contamination despite careful processing. It is known that due to heat treatment, enzymatic hydrolysis, and changes in protein structure aggregation sites such as SH-groups may become available for the formation of S-S bridges [19]. Rosendal and Barkholt [18] however, did not find substantial amounts of aggregated or polymerized material in 12 products with different degrees of hydrolysis.

A point of dicussion is that all children did well on the reference products before inclusion. In this way, children that may not tolerate the small amounts of larger peptides that may be present in the reference product were excluded.

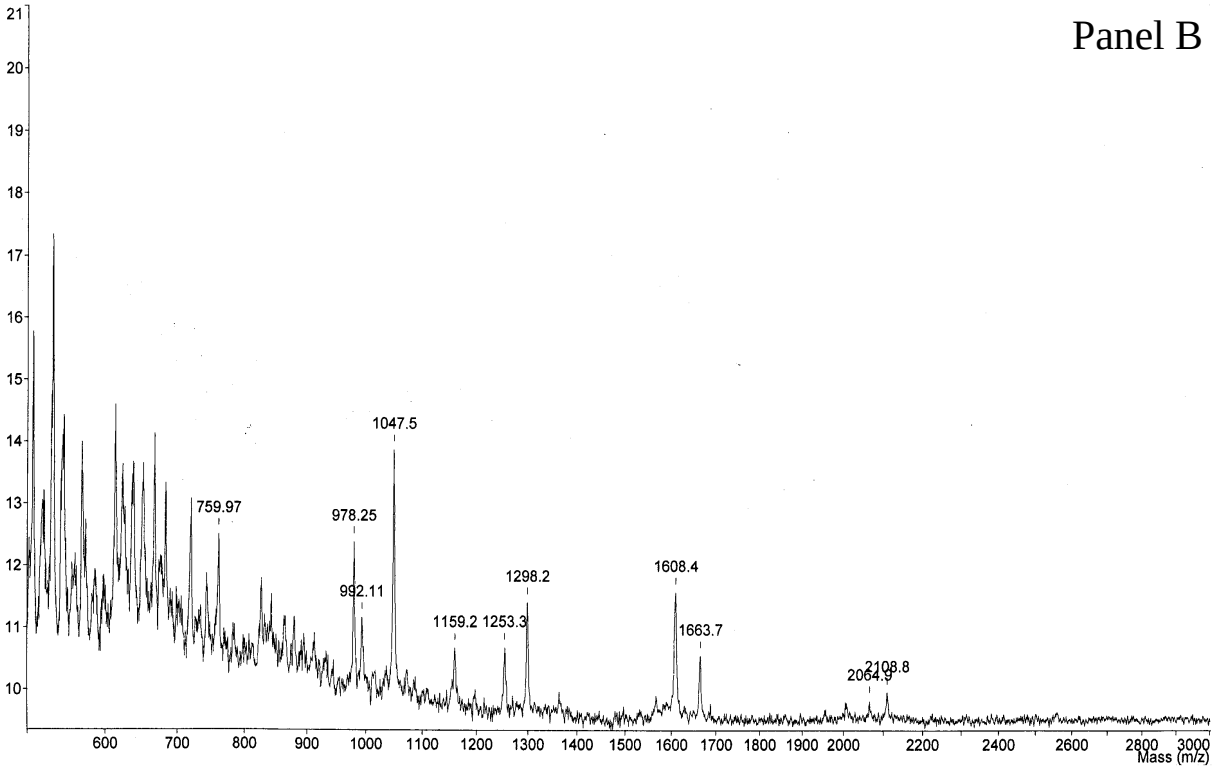

Figure 2
Molecular weight profile of Nutramigen® (NUT). The molecular weight profile is determined with the MALDI-TOF method as described by Schols [19] and Kaufmann [15] and Soeryapranata [16].

Table 2: Group characteristics at the start of the study

	Group A	Group B
Number	12	15
Mean age (months)	64.4	64.0
Gender (boys/girls)	10/2	11/4
RAST (positive/negative)	10/2	12/3
Atopic disease (number):		
Dermatitis	5	5
Asthma	2	6
Dermatitis and asthma	4	3
Dermatitis and GI-symptoms	1	1
Mean questionnaire score at start of the study	1.98	1.78

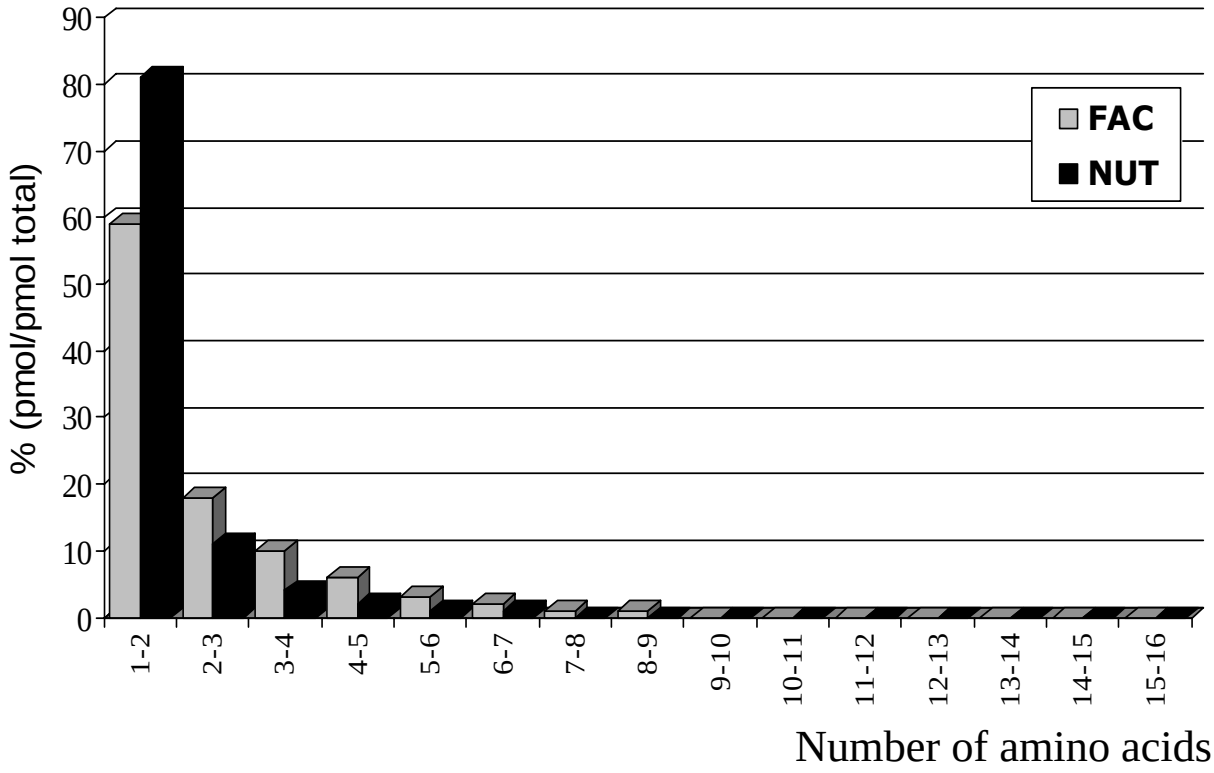

Figure 3
Peptide length profile of the hydrolysate used in Frisiolac Allergycare® (FAC) and Nutramigen® (NUT). The peptide length profile is determined with an adapted Edman degradation method as described by Siemensma et al. [17].

Another point of discussion may be the inclusion of older infants in this study. However, to our opinion these children with a persistent CMA can be considered to be a more vulnerable group.

Conclusion

Because children with persistent and proven IgE mediated CMA did not show adverse reactions after consumption of a new extensively hydrolyzed formula (Frisolac Allergycare®), this formula can be regarded as safe for this target population.

Competing interests

Friesland Nutrition, manufacturer of Frisolac Allergycare®, funded this study. Although two of the authors are employees of Friesland Nutrition (IK and AS), they could not influence the final outcome of the study since the practical work was done by independent researchers of the Wilhelmina Childeren's hospital (STL and EK). The outcome of this study will also not influence financial interest of Friesland Nutrition since this product is a special formula with only a very small contribution to overall financial interest. The only reason for this study was to get information on the safety and efficacy of the formula for infants and children with cow's milk protein allergy.

Authors' contributions

STL carried out the practical part of the study and participated in the evaluation of data and preparation of the manuscript. IK contributed to the study protocol, working group and data evaluation. AS contributed to the study protocol and preparation of the manuscript. EW super-

vised the study, contributed to the practical work, evaluation of data and preparation of the manuscript.

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