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**Preclinical Brown Norway rat models for the assessment of infant
formulas in the prevention and treatment of cow's milk allergy**

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Abstract**Background**

Infant formulas (IFs) based on hydrolysed cow's milk proteins are central in the management of cow's milk allergy (CMA) in infants and small children. New IF compositions with improved prevention and treatment properties are needed along with appropriate preclinical animal models to evaluate these properties before introduction into humans.

Objectives

Develop preclinical models for the assessment of the primary preventive and desensitising capacity of cow's milk IF in allergy prone high IgE-responder Brown Norway rats.

Method

Preventive capacity was assessed in cow's milk naïve rats given a 2 or 4-week regimen of whey-based extensively hydrolysed IF (eHF), partially hydrolysed IF (pHF), or intact beta-lactoglobulin (BLG) *ad libitum* in drinking bottles followed by intraperitoneal (IP) immunisation with BLG.

Desensitising capacity was assessed in orally BLG-sensitised rats after a 3 or 6-week regimen of eHF, pHF, or intact BLG administration in drinking bottles followed by IP challenges with BLG.

Primary preventive and desensitising capacity were analysed by serum BLG-specific IgG1 and IgE.

Results

The preventive regimens did not induce detectable BLG-specific IgG1 or IgE in cow's milk naïve rats. A preventive regimen consisting of pHF or BLG, but not eHF, induced complete tolerance to BLG, as demonstrated by the absence of BLG-specific IgE following IP immunisation. Desensitising regimens had a limited effect on BLG-specific IgG1 or IgE when comparing before and after treatment in sensitised rats. IP challenge with BLG increased BLG-specific IgE in all treatment regimens except for the BLG group, suggesting a limited desensitising capacity of IF based on hydrolysates and a need for the presence of intact allergen for desensitisation.

Conclusions

The presented models highlight that different mechanisms are at play in the induction of *de novo* tolerance to cow's milk proteins and in the desensitisation of CMA. Different IF products may be needed for primary prevention and treatment of CMA.

INTRODUCTION

Cow's milk allergy (CMA) is one of the most common IgE-mediated food allergies in infancy and early childhood. It affects proximately 2-3 % of infants [1,2], but the majority of afflicted infants outgrow the disease during childhood [3]. In general, the only way of managing food allergy is strict avoidance of the offending food. However, for infants with CMA or infants at the risk of getting CMA, alternatives exist as cow's milk based hypoallergenic infant formulas (IFs) are available when breastfeeding is not possible or sufficient.

Hypoallergenic IFs have a lower allergenic potential compared to standard IF as the protein fraction has been hydrolysed leading to destruction of allergenic epitopes. Based on the degree of hydrolysis and the peptide distribution, the hypoallergenic IF can be classified as either extensively hydrolysed formula (eHF) or partially hydrolysed formula (pHF) [4]. The eHFs are recommended for management of allergy in infants with a diagnosed CMA due to the low allergenicity of these formulas and thus reduced risk of elicitation of symptoms [4,5]. On the other hand, pHFs are recommended for infants at the risk of developing CMA, since it is believed that the larger peptides can be recognised by the immune system giving rise to tolerance development in the absence of intact allergen [6]. Clinical studies indicate that both eHF and pHF may have primary preventive capacity [5,7–10]. However, the evidence is not strong [11]. It is unclear which biochemical and biophysical properties are important for a tolerogenic effect of a hypoallergenic IF are unclear, although it has been suggested that the degree of hydrolysis (DH) is not the only contributing factor [12]. Furthermore, oral immunotherapy is emerging as a viable treatment option of food allergy [13,14]. Thus, it would be of clinical interest if IFs could be developed to include a desensitising capacity for the treatment of CMA.

In summary, improved IFs with documented primary preventive and desensitising capacity are needed for the management of CMA in infants and young children. Development of new IFs calls for better animal models in the assessment of different formula compositions in terms of DH, peptide distribution, biochemical, biophysical and immunological properties. Recent changes in the EU regulation of IFs require health claims to be clinically documented, which include completion of human trials. This requirement further highlights the need for appropriate preclinical models in the selection of formula compositions for testing in humans. Here we present models for assessment of primary preventive and desensitising capacity of IF in allergy prone high

89 IgE-responder Brown Norway (BN) rats [15] mimicking atopic predisposition in humans. We
90 compared the primary preventive and desensitising capacity of whey-based eHF and pHF to intact
91 beta-lactoglobulin (BLG), the main food allergen in whey. The presented models can be used to
92 assess the degree of tolerance induced by different formulas, as well as the capacity to induce
93 desensitisation. Interestingly, our results indicate that intact protein should be present in IFs to
94 obtain desensitising capacity.

MATERIALS AND METHODS

Cow's milk protein products

Arla Foods Ingredients (Videbæk, Denmark) kindly provided the cow's milk protein products used for the development of animal models. The products included: 1) Peptigen® IF-3080 (eHF) - an extensively hydrolysed whey protein product suitable for use in hypoallergenic IF with a DH of 27 (peptide distribution: <375 Da (1-3 AA): 16,6 %, 375-750 Da (4-6 AA): 38,7 %, 750-1250 Da (7-10 AA): 26 %, 1250-2500 Da (11-20 AA): 15,8 %, >2500 Da (>20 AA): 3 %). 2) Peptigen® IF-3087 (pHF) - a partially hydrolysed whey protein suitable for use in infant formulas with reduced allergen content with a DH of 18,3 (peptide distribution: <375 Da (1-3 AA): 15,4 %, 375-750 Da (4-6 AA): 26,6 %, 750-1250 Da (7-10 AA): 22,1 %, 1250-2500 Da (11-20 AA): 23,9 %, >2500 Da (>20 AA): 12 %). 3) Purified intact BLG (> 90% purity).

Animal experiments

BN rats were bred and raised in-house at the National Food Institute, Technical University of Denmark, and kept on a special diet free from cow's milk proteins for a minimum of 3 generations to avoid tolerance to cow's milk proteins. Rats were housed in macrolon cages at 22 ± 1 °C and 55 ± 5% relative humidity with a 12 h light-dark cycle. Studies were approved by the Danish Animal Experiments Inspectorate (authorisation number 2015-15-0201-00553-C1). The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use. Liquid consumption was monitored daily in cages each containing three animals, and calculated as the total consumption pr. animal each week.

Primary prevention model

Primary prevention of CMA was induced by *ad libitum* administration of cow's milk protein products in rat drinking bottles for 2 or 4 weeks starting at 3-5 weeks of age. The cow's milk protein product concentration was comparable to commercial IF (12.5 g/L) and dissolved in Milli-Q (Millipore Corporation, USA) purified water. The degree of induced tolerance was assessed one week later by 3 IP immunisations with 100 µg BLG/rat without adjuvant at one-week intervals. Blood was collected one week after the primary prevention regimen and after each IP injections for the assessment of BLG-specific IgG1 and IgE in serum (Figure 1A).

Desensitisation model

Rats were sensitised to cow's milk by daily oral gavage of 10 mg BLG with 2 µg cholera toxin (CT; Sigma) for 6 weeks starting at 3-5 weeks of age. One week later, desensitisation was induced by *ad libitum* administration of cow's milk protein products in rat drinking bottles for 3 to 6 weeks. The milk protein product concentration was comparable to commercial IF (12.5 g/L) and dissolved in Milli-Q purified water. Only rats with measurable BLG-specific IgE before treatment were included in the analysis of the desensitisation regimen effect. The degree of desensitisation was assessed one week later by 3 IP challenges with 50 µg BLG/rat at one-week intervals. Blood was collected after sensitisation, after desensitisation, and a week after the third IP challenge for assessment of BLG-specific IgG1 and IgE in serum (Figure 2A).

BLG-specific IgG1 and IgE assays

BLG-specific IgG1 antibodies were measured in serum using in-house developed indirect ELISAs. Maxisorp plates (96-well, NUNC, Roskilde, Denmark) were coated with 100 µL/well of 10 µg/mL intact or denatured BLG in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃; pH 9.6) over night at 4°C. Following, plates were incubated for 1 h at room temperature (RT) with 50 µL/well of 2-fold serial diluted rat serum samples in PBS-T (PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄; pH 7.2) with 0.01% (w/v) Tween 20 (P1379, Sigma)) starting at 1:8 dilution. Subsequently, the plates were incubated for 1 h with 50 µL/well of 20.000x diluted horseradish peroxidase (HRP)-labelled mouse-anti-rat-IgG1 (3060-05, Southern Biotech, Birmingham, AL, USA).

BLG-specific IgE antibodies were measured in serum using in-house developed IgE capture ELISAs. Maxisorp 96-well plates were coated with 100 µL/well of 0.5 µg/mL mouse-anti-rat-IgE (HDMAB-123, Patricell LTD, Nottingham, United Kingdom) in carbonate buffer over night at 4°C. Plates were blocked for 1 h at 37 °C with 200 µL/well PBS-T containing 5% horse serum (v/v; S0900-500, Biowest, France). Subsequently, plates were incubated for 1 h at RT with 50 µL/well of 2-fold serial diluted rat serum samples in PBS-T starting at 1:8 dilution. The plates were incubated for 1 h at RT with 50 µL/well of 0.05 µg/mL digoxigenin (DIG)-coupled BLG (coupling 10:1) in 5% horse serum (v/v) followed by incubation with 50 µL/well of 1.000x diluted anti-DIG (11633716001, Roche GmbH, Mannheim, Germany) in 5% horse serum for 1 h.

Both assays included washing of plates 5 times with (PBS-T) between each step. Plates were developed by incubating with 100 μ L/well of 3,3',5,5'-tetramethylbenzidine (TMB)-one (4380A, Kementec Diagnostics, Taastrup, Denmark) for 12 min and stopping the reaction with 100 μ L/well of 0.2 M H₂SO₄. The absorbance was measured at 450nm - 630nm. The results were expressed as Log₂ titre values with a cut-off value set higher than the mean absorbance for the negative control + three times the standard deviation. Each plate included positive and negative control samples for quality control of day-to-day and plate-to-plate variation.

Denaturation of BLG

BLG was denatured by reduction and alkylation as previously described [16].

Statistics

Differences between water control group and prevention regimen groups were tested using Kruskal-Wallis test with Dunn's correction for multiple testing at each sampling time point. Differences between paired data were tested using Wilcoxon matched-pairs signed-rank test. Differences in liquid consumption and desensitisation post-challenge Ig levels between water control group and regimen groups were tested using ANOVA with Bonferroni's correction for multiple testing. All statistical analyses were performed in GraphPad Prism 7.04 for MS Windows. P-values below 0.05 were considered statistically significant. Significance levels were indicated as *p<0.05, **p<0.01, and ***p<0.001 in figures.

RESULTS

Model for primary prevention of cow's milk allergy

We developed a model in BN rats for the assessment of primary prevention against milk allergy by cow's milk whey-based IFs. The model included a prevention regimen with *ad libitum* oral administration of IF in drinking bottles followed by IP immunisation with BLG to assess the degree of established tolerance (Figure 1A). This model was based on pilot investigations performed to optimise milk protein concentrations, number of IP immunisations, BLG IP concentration, the use of adjuvant, and rat age and sex (data not shown). It was decided that the protein concentration of the administered hydrolysates should be equivalent to commercial IFs (12.5 g/L). Since there were no significant differences between a 2 and 4 week primary prevention regimen, the results shown combined data from experiments using both regimens (Figure 1B-D).

The prevention regimen alone did not induce native BLG-specific IgG1 or IgE in rats receiving only water, eHF, pHF, or intact BLG in the drinking bottles (Figure 1B-C). IP immunisations with BLG induced increasing native BLG-specific IgG1 and IgE levels in rats not receiving an active preventive regimen (water alone), demonstrating that IP administration induced BLG sensitisation. Rats receiving a prevention regimen containing pHF or BLG, but not eHF, were found to have significantly reduced serum native BLG-specific IgG1. The induction of native BLG-specific IgE was largely undetectable after pHF or BLG prevention regimens, indicating that these regimens induce complete tolerance to milk proteins. Only rats having received a preventive regimen containing eHF had a significantly lower levels of IgG1 specific for denatured compared to native BLG (Figure 1D).

Liquid consumption was similar in all prevention groups (Figure 1E), indicating that the differences in preventive capacity between milk protein products cannot be ascribed to differences in consumed dosages.

Model for desensitisation in cow's milk allergy

We developed a model in BN rats to assess the desensitising effect of cow's milk whey-based infant formulas. The model used orally BLG-sensitised rats receiving a desensitisation regimen with *ad libitum* oral administration of IF in drinking bottles followed by IP challenges with BLG (Figure 2A). This model was based on pilot investigations performed to optimise oral

sensitisation, the use of adjuvant, BLG IP concentration, number of challenges, and rat age and sex (data not shown). Since there were no significant differences between a 3 and 6 week desensitisation regimen, the results shown combined data from experiments using both regimens (Figure 2B-G). Only rats with measurable native BLG-specific IgE before treatment were included in the analysis of the desensitisation regimen (Figure 2B-G).

The desensitisation regimens using eHF, pHF, or intact BLG were found to have a limited effect on native BLG-specific IgG1 and IgE levels when comparing before and after treatment (Figure 2B-C). Only the BLG desensitisation regimen was found to significantly increase native BLG-specific IgG1 in serum. However, IP challenges with BLG significantly increased native BLG-specific IgG1 and IgE in rats having received a desensitisation regimen containing water only, eHF or pHF, but not BLG (Figure 2D-E). Furthermore, the post-challenge native BLG-specific IgG1 and IgE levels were significantly lower in rats having received the BLG desensitisation regimen (Figure 2F-G). These findings suggest that desensitisation requires the presence of intact protein and cannot be mediated by hydrolysed products. Liquid consumption was similar in all desensitisation groups (data not shown) indicating that findings cannot be ascribed to differences in consumed dosages.

DISCUSSION

Reduced allergenicity of cow's milk proteins in IFs is important when administered to infants with an established CMA. This property can readily be assessed by *in vitro* assay using materials from allergic humans or sensitised animals [17]. However, it is also of interest to assess a potential tolerogenic capacity of IF for the primary prevention and treatment of CMA. This assessment requires appropriate animal models [14] that can be experimentally sensitised before and after the administration of IF. Such models would be particularly useful for the preclinical screening of new IF product candidates before trials in humans, which is required by recent EU regulation of IF products to allow labelling of health claims. We have previously assessed the allergenicity of hydrolysed IFs in high IgE-responder BN rats, which reflect the findings from other studies in animals and humans [16,18]. Here we developed two new models using this allergy prone rat strain for the assessment of primary preventive and desensitising capacity of whey-based hydrolysed IF.

The BN rats used for these experiments were kept on a milk-free diet for more than 3 generations to ensure complete immunological naivety to cow's milk proteins. Administration of eHF, pHF, or intact BLG for the prevention of CMA was not associated with the development of native BLG-specific IgG1 or IgE, suggesting absence of humoral immune responses to the orally administered proteins. Next, we immunised the animals by IP injections of BLG to assess the degree of established tolerance by the IF. The level of sensitisation increased with increasing numbers of IP immunisations with BLG in rats not having received an active preventive regimen. pHF and BLG induced complete tolerance to BLG, whereas eHF did not. This finding is in line with the assumption that larger peptides are required for IFs to have preventive capacity, likely due to higher immunogenicity of pHF compared to eHF [6]. The mechanisms by which the oral preventive regimens induce tolerance to BLG cannot be determined based on our data, however the absence of native BLG-specific IgG1 before IP immunisations suggest exclusion of tolerance mediated by IgG [19]. Rather the mechanism may involve generation of BLG-specific regulatory T cells, as suggested by a previous study using preventive administration of specific peptides derived from BLG [20].

The model developed for the assessment of desensitising capacity used BN rats orally sensitised to BLG. The 3 to 6-week oral desensitisation regimen of eHF, pHF, or intact BLG had no

effect on the level of native BLG-specific IgE antibodies when comparing levels before and after treatment. However, IP challenges with BLG significantly increased the level of native BLG-specific IgE in rats with a desensitisation regimen consisting of water alone, eHF, or pHF, but not with BLG, leading to higher post-challenge native BLG-specific IgE levels in all regimen groups compared to the BLG regimen. Thus, the hydrolysed IFs had a limited desensitisation capacity, whereas the intact BLG regimen prevented further sensitisation. This indicates that the presence of intact protein is required for desensitisation. Interestingly, we previously found that the sensitising capacity of intact BLG was reduced by co-administration with digested BLG [21], yet the desensitising capacity of such a mixture remains unknown. Previous studies have reported a desensitising capacity of pepsin-hydrolysed cashew nut protein extract [22] and aminopeptidase-hydrolysed egg white [23], however the presence of intact protein in these preparations is unclear. Several studies have demonstrated a therapeutic effect of T cell-directed peptide epitopes from ovalbumin [24], ovomucoid [25], tropomyosin [26], and BLG [27] in sensitised mice. These studies support the concept of a desensitising capacity of hydrolysates and specific peptides. The lack of desensitising capacity of eHF and pHF reported here could be attributed to the specific composition of the hydrolysed IFs, including the absence of T cell epitopes, specific biochemical/biophysical properties, or differences between the specific food allergens. Interestingly, recent clinical trials using Fel d 1 peptide therapy found no therapeutic effect, suggesting that this approach has limited applicability in humans (Circassia Pharmaceuticals plc, 20 June 2016 press release, www.circassia.com). Thus, it may be important that future therapeutic agents and IFs in part contain intact allergens, possibly to ensure immunomodulation of conformational epitopes.

Overall, our findings are in line with emerging clinical data on the prevention and treatment of food allergy. The LEAP study has recently shown, that early introduction of peanut prevent the development of peanut allergy in high-risk infants [28]. Still, high-quality clinical studies are needed on pHF in the prevention of CMA [29]. Oral immunotherapy is emerging as a viable treatment option for food allergy [13], but issues concerning efficacy and adverse effects remain central obstacles, which novel preclinical models may contribute to solving.

The results from the presented models, used to assess primary preventive and desensitisation capacity of IF, highlight that different mechanisms are at play in the induction of

291 tolerance to cow's milk proteins and the desensitisation in CMA. This indicates that different IF
292 products have to be developed for the primary prevention and treatment of CMA, respectively.

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Figure text

Figure 1 – Assessment of primary preventive capacity of whey-based hydrolysed infant formula (IF): Model design with 2 or 4 weeks of administration with whey-based extensively hydrolysed IF (eHF, n = 12), partially hydrolysed IF (pHF, n = 12), intact beta-lactoglobulin (BLG, n = 12), or water alone (water, n = 6) *ad libitum* in drinking bottles to cow's milk naïve Brown Norway rats followed by three IP immunisations with BLG without adjuvant (A; photo/illustration credit: Colourbox.com). Native BLG-specific IgG1 (B) and IgE (C) one week after the prevention regimen and each of the IP immunisations (median with upper IQR, and Kruskal-Wallis test with Dunn's correction for multiple testing). Levels of specific IgG1 to native compared to denatured BLG one week after third IP immunisation (D; Wilcoxon matched-pairs signed-rank test). Liquid consumption by rats during the preventive regimen (E; n = 8 (water group; 2 cages observed for 4 week), n = 12 (eHF, pHF and BLG groups; 2 cages observed for 2 week and 2 cages observed for 4 weeks), mean with SEM, and ANOVA test with Bonferroni's correction for multiple testing).

Figure 2 – Assessment of desensitising capacity of whey-based hydrolysed infant formula (IF): Model design with oral BLG-sensitisation of Brown Norway rats followed by 3 to 6-weeks of administration with whey-based extensively hydrolysed IF (eHF, n = 7), partially hydrolysed IF (pHF, n = 10), intact beta-lactoglobulin (BLG, n = 8), or water alone (water, n = 11) *ad libitum* in drinking bottles, and three IP challenges with BLG (A; photo/illustration credit: Colourbox.com). BLG-specific IgG1 (B) and IgE (C) before and after the desensitisation regimen (Wilcoxon matched-pairs signed-rank test). BLG-specific IgG1 (D) and IgE (E) before and after three IP challenges with BLG (Wilcoxon matched-pairs signed-rank test). Comparison of BLG-specific IgG1 (F) and IgE (G) levels between regimen groups after three IP challenges with BLG (mean with SEM, and ANOVA test with Bonferroni's correction for multiple testing).

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Statement of Ethics

All animal experiments were conducted in accordance to internationally accepted standards, and have been approved by the Danish Animal Experiments Inspectorate.

Disclosure Statement

KLB has ongoing collaboration with the company Arla Foods Ingredients P/S, which supplied the whey-based IF products for this study. Arla Foods Ingredients P/S provided financial support for past and ongoing research projects. The authors have no other conflicts of interest to declare.

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Author Contributions

KLB and CBM conceived the study and designed experiments. LHJ performed experiments and collected the data. KLB, JML, and LHJ analysed and interpreted the data. JML and LHJ wrote the manuscript. RRL and LNJ supplied and characterised BLG and whey-based IF products. All authors provided important intellectual contributions to the study and reviewing of the manuscript.

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FIGURE 1

FIGURE 2