



In vivo and in vitro evaluation of the residual allergenicity of partially hydrolysed infant formulas

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ABSTRACT

Hypoallergenic infant formulas are commonly used for genetically predisposed children and infants diagnosed with cow's milk allergy. This study describes both in vitro and in vivo approaches to assess residual allergenicity of partially hydrolysed infant formulas. Electrophoretic patterns indicated that β-lactoglobulin and other whey proteins were largely degraded. For safety reasons, according to the European commission-guidelines, it is required that the sensitizing capacity of hypoallergenic formulas is tested in an animal model. In contrast to whey sensitization, no elevated levels of whey-specific IgE, anaphylactic reactions or drop in body temperature were observed in sensitized mice exposed to whey hydrolysates. This indicates that the whey hydrolysates lost their putative sensitizing capacity in a mouse model using oral sensitization, which is highly relevant in relation to the human situation. In combination with the lost capacity of hydrolysed infant formulas to cross-link human IgE antibodies on RBL-huFcεRI in vitro, both the sensitization and the challenge phase of the allergic response were studied. This combination of assays is proposed as a strategy for the screening of new hypoallergenic formulas aimed at preventing sensitization in atopic children and avoiding clinical symptoms in infants suffering from cow's milk allergy.

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1. Introduction

Food allergens, aeroallergens, medications and insect venoms are the most common allergens encountered that are responsible for inducing Type I or IgE-mediated hypersensitivity reactions (Johansson et al., 2004). Type I allergic responses to cow's milk proteins leading to cow's milk allergy are characterized by a T helper 2 (Th2) response resulting in the production of allergen-specific IgE (sensitization phase). Binding of IgE to the high affinity receptor FcεRI on mast cells or basophils followed by cross-linking of the IgE-antibodies by the food allergen elicits degranulation and the release of mediator (effector/challenge phase). Clinical symptoms may occur in the skin, gastrointestinal tract or airways. Cow's milk allergy is considered to be the first and most common type of allergy during early infancy.

While breastfeeding is considered to be the golden standard for infant nutrition, hypoallergenic formulas are a good alternative for infants at high risk or infants diagnosed with cow's milk allergy. Hypoallergenic formulas are processed by enzymatic treatment, heat treatment and/or ultra filtration of cow's milk proteins. These Hydrolysed formulas are generally categorized as partial and extensive hydrolysates based on the degree of hydrolysis and can be characterized by assessing the molecular weight distribution of the residual proteins. However, residual allergenicity cannot be ascertained solely based on peptide size distribution. Several clinical studies have been performed to address the sensitizing capacity of partial and extensive hypoallergenic formulas in high risk children (Zeiger and Heller, 1995; Halken et al., 2000; Chan et al., 2002; von Berg et al., 2003). Differences in peptide size, variations in protein sources and hydrolysis methods of hypoallergenic formulas can modify the preventive effect of these formulas (Hays and Wood, 2005) stressing the importance of adequate pre-clinical testing of hypoallergenic formulas.

Residual allergenicity of Hydrolysed formulas can be determined in vitro by measuring the binding of the hydrolysed proteins to serum antibodies of sensitized animals or humans using an in vitro cell based degranulation assay reflecting the effector phase of the allergic response. According to the EC-directive on

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infant formulas and follow-on formulas, it is required that objective and scientifically verified data are available as proof of the claimed hypoallergenicity. For safety reasons, the hypoallergenicity of hydrolysed infant formulas needs to be assessed by showing that the hypoallergenic formulas are not able to sensitize animals to the protein source they are derived from (Commission Directive 96/4/EC of 16th February 1996 amending Directive 91/321/EEC on infant formulae and follow-on formulae. Official Journal of the European Communities No L 49: 12–16). Guinea pigs sensitized by the oral route have been used as a common model for the identification of the residual sensitizing capacity of new hypoallergenic formulas due to their innate responsiveness. A main disadvantage of the guinea pig model is the generation of IgG1a subclass anaphylactic antibody responses instead of IgE antibody responses, which are the main physiological antibody responses in allergic humans. This makes the suitability of the guinea pig model questionable with regard to the potential extrapolation to the human situation, although this model was frequently used due to the absence of other suitable oral sensitization models. Recently, however, a predominantly IgE-mediated mouse model of orally induced cow's milk allergy was introduced (Schouten et al., 2008). Using this model, the residual allergenicity of partially and extensive hydrolysed whey proteins was determined (van Esch et al., 2009b), indicating that this model can potentially be used to study the sensitizing capacity of hydrolysed whey formulas.

In the present study, a strategy for comprehensive and detailed analyses of the potential allergenicity of whey-based hydrolysed infant formulas is described. By assessing the sensitizing capacity in an in vivo mouse model using oral sensitization in combination with the capacity to cross-link human IgE antibodies on RBL-huFcεRI cells in vitro, both the sensitization and challenge phase of the allergic response can be studied.

2. Material and methods

2.1. Materials

Whey protein concentrate 80 (WPC80; indicated as whey) was obtained from DMV International (Veghel, The Netherlands) and hydrolysed with an established mixture of endopeptidases and exopeptidases (confidential enzyme composition used by Danone) resulting in partially hydrolysed whey proteins (hWhey). This is an experimental hydrolysate solely produced for these experiments and not for usage in an end product. The partially whey based Hydrolysed formula CS pre Aptamil HA (Hydrolysed formula) (Nutricia, Zoetermeer, The Netherlands) and an amino acid based formula (AA based formula) were also included, the latter serving as a negative control.

2.2. Sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The pattern of the residual proteins in the hWhey and the Hydrolysed formula was analyzed with SDS-PAGE. A Bio-Rad (Hercules, CA, U.S.A.) Mini-PROTEAN II

system was used with a 15% Tris–HCl gel. Molecular weight markers were used as a reference. The proteins were diluted in a 1:3 ratio with reducing sample buffer (6.05 g Tris, 8.0 g SDS, 3.2 g dithiothreitol, 20 mg bromophenol blue in 60 ml H₂O and 40 ml glycerol 87%, pH 6.8), 40 µg of protein was loaded on the gel. The proteins were either stained in the gel using silver staining according to Wray et al. (1981) or blotted to a PVDF membrane (Roche Diagnostics, Basel, Switzerland). The membranes were incubated with monoclonal antibodies against α-lactalbumin or β-lactoglobulin monoclonal antibody (1:30,000; Bethyl Laboratories Montgomery, TX, USA). The binding of antibody was visualized by using Lumi-light Plus Western blotting substrate (Roche Diagnostics) and chemiluminescence signal was measured with the Chemidoc XRS (BioRad).

2.3. Measurement of residual whey proteins in hWhey and the Hydrolysed formula

For the determination of residual whey proteins in the hWhey and the Hydrolysed formula, a sandwich-type ELISA was performed according to the manufacturers' protocol (Vitrochemie, Nijmegen, The Netherlands).

2.4. Degranulation of rat basophil leukemia cells expressing human FcεRI (RBL-huFcεRI)

To investigate the cross-linking capacity of the Hydrolysed formula, the release of β-hexosaminidase was measured using the RBL-huFcεRI cell line. Degranulation of RBL-huFcεRI cells sensitized with a pooled serum of cow's milk allergic (CMA) patients was performed as described previously (Knipping et al., 2008). In short, confluent growing RBL-huFcεRI cells were sensitized with hulgE or with a human serum pool of 10 CMA patients. The human serum sensitized cells were incubated with Whey, Hydrolysed formula and AA based formula for 1 h at 37 °C. For this assay a valuable unique serum pool obtained from truly cow's milk allergic patients as assessed by double blind placebo controlled food challenge was used and because of ethical reasons we decided to use these sera solely for testing of real end product hydrolysates and not experimental hydrolysates and therefore decided not to include the hWhey group in the RBL-assay. The cross-linking capacity of the hWhey was therefore not determined in this assay. The β-hexosaminidase activity released into the medium was expressed as percentage of the maximum release observed with purified hulgE and α-hulgE.

2.5. Oral sensitization of mice with whey and whey hydrolysates

Three-week-old specific pathogen-free female C3H/HeOJ mice were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France), and maintained on semi-purified cow's milk protein-free mouse chow (Research Diet Services, Wijk bij Duurstede, The Netherlands). Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. To investigate the residual sensitizing capacity of hWhey and the Hydrolysed formula, mice were orally sensitized with a blunt needle on day 0, 7, 14, 21, 28 and 35 with 20 mg whey, hWhey or an adjusted amount of Hydrolysed formula homogenized in PBS (0.5 ml, Cambrex Bio Science, Verviers, Belgium) mixed with 10 µg cholera toxin (Quadrachet Diagnostics, Epsom, UK) as an adjuvant. (The amount of hydrolysed whey was equal for the hWhey and the Hydrolysed formula.) Non-sensitized mice received cholera toxin in PBS only. One week after the last oral sensitization, anaphylactic reactions, drop in body temperature and acute allergic skin response were determined after an intradermal whey challenge (10 µg whey/10 µl PBS per ear). After 24 h, mice were challenged orally with 50 mg whey and 30 min later blood samples were collected. Blood samples were centrifuged for 15 min at 13,500 rpm and sera were stored at –20 °C (Fig. 1).

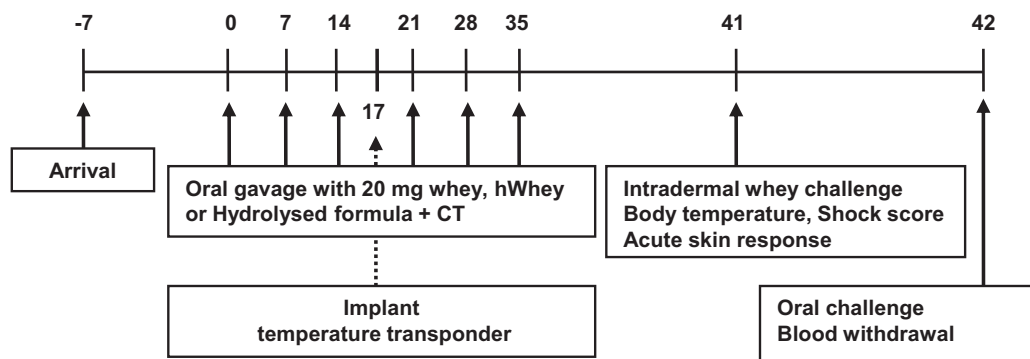


Fig. 1. Mice were sensitized weekly by oral gavage with whey, hydrolysed whey (hWhey) or Hydrolysed formula in PBS using cholera toxin (CT) as an adjuvant. Non-sensitized mice received CT in PBS only. One week after the last sensitization the body temperature, anaphylactic shock score and an acute allergic skin response were determined after intradermal whey challenge in the ear pinnae.

2.6. Measurement of whey-specific serum immunoglobulins

Concentrations of whey-specific IgE and IgG1 were determined in serum by means of ELISA as described previously (van Esch et al., 2009a). In short, Microlon plates (Greiner Bio-one, Monroe, USA) were coated with whey for 18 h at 4 °C. Plates were washed after each incubation step. Serum samples were applied and incubated for 2 h at room temperature (RT), followed by incubation with biotin-labeled rat anti-mouse IgE or IgG1 for 90 min at RT. The plates were incubated with streptavidin-horseradish peroxidase for 1 h at RT and developed with o-phenyldiamine (Sigma–Aldrich). The reaction was stopped with 4 M H₂SO₄ and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA).

2.7. Anaphylactic shock score and body temperature

To evaluate the residual sensitization capacity of hWhey and Hydrolysed formula, the anaphylactic reactions and body temperature were determined as a clinical related symptom after intradermal whey challenge. To establish the severity of the shock, a validated anaphylactic scoring table (Table 1) was used, as adapted from Li et al. (1999). To measure changes in body temperature, all mice were given an implantable electronic ID transponder (Bio Medic Data Systems, Delaware, USA) on day 16 to measure the individual body temperature.

2.8. Acute allergic skin response

An acute allergic skin response in whey sensitized mice was determined 1 h after intradermal challenge with 10 µg whey in the ear pinnae. As a negative control, non-sensitized mice were challenged in the ear pinnae with whey. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). The allergen-specific net ear swelling was calculated by correcting the allergen-induced ear thickness with the basal ear thickness. The delta ear swelling is expressed as µm.

2.9. Statistical analysis

Statistical analysis of the whey-specific immunoglobulins and β-hexosaminidase release of RBL-huFcεRI cells and body temperature were performed using a 1-way ANOVA and post hoc Bonferroni's multiple comparison test. The anaphylaxis scores were statistically analyzed using the Kruskal–Wallis test followed by the Dunn's multiple comparison test. All statistical analyses were conducted using GraphPad Prism software (version 4.03). *p*-Values are depicted as **p* < 0.05, ***p* < 0.01 and ****p* < 0.001. Animal studies were performed with *n* = 7 mice per group based on power analyses.

3. Results

3.1. SDS-PAGE and Western blotting

Electrophoretic patterns indicated that β-lactoglobulin (β-Lg), a major allergen in whey, was completely degraded after hydrolysis in both hWhey and Hydrolysed formula. A small residual band of α-lactalbumin (α-La) was seen in hWhey, but not in the Hydrolysed formula. In the Hydrolysed formula, a large band of low molecular weight peptides was found. In the AA based formula, no protein bands were detected (Fig. 2). Positive staining on the Western blot after incubation with anti-α-La and anti-β-Lg antibodies of the bands corresponding to α-La and β-Lg were detected in whey, but not in the hWhey, Hydrolysed formula or in the AA based formula (data not shown).

Table 1
Anaphylactic symptom scoring table.

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erecti; reduced activity; higher breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death by shock

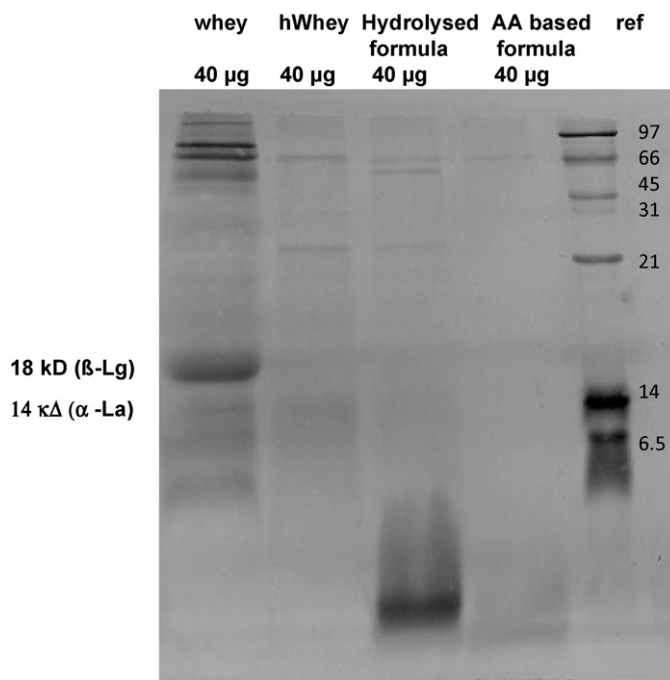


Fig. 2. SDS-PAGE patterns of whey, hWhey, Hydrolysed formula and AA based formula. The reference (ref) is a standard broad-range marker: phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa) and aprotinin (6.5 kDa). The band at 66 kDa in all lanes reflects the non-specific coloring from the sample buffer.

3.2. Residual whey proteins in hWhey and Hydrolysed formula

The whey powder contained 159.2 ± 14.7 µg/mg whey proteins, after hydrolysis only 1.8 ± 0.3 µg/mg and 0.9 ± 0.1 µg/mg of residual whey protein was detected in the hWhey and Hydrolysed formula respectively (Fig. 3). This is a reduction of 97% (hWhey) and 98.4% (Hydrolysed formula) in whey proteins when compared to whey. The whey protein content of the AA based formula was below the detection limit of the assay.

3.3. Degranulation assay with RBL-huFcεRI cells

The capacity of the Hydrolysed formula to elicit degranulation of RBL-huFcεRI cells was investigated (Fig. 4). The degranulation of the RBL-huFcεRI cells which were sensitized with purified huIgE and cross-linked with α-huIgE was considered as the maximum release (100%). Incubation of the serum sensitized RBL-huFcεRI cells with whey resulted in a release of $88.7 \pm 3.8\%$. The Hydrolysed formula showed no enhanced degranulation ($46.7 \pm 6.2\%$) as compared to

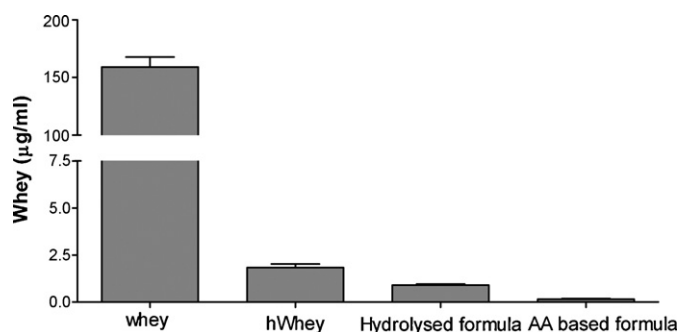


Fig. 3. The residual concentrations of whey were determined in whey, hWhey, Hydrolysed formula and AA based formula by an ELISA assay (data are expressed as mean $\pm$ SD).

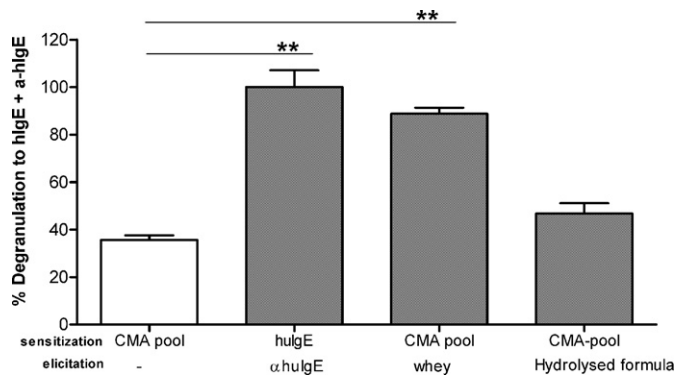


Fig. 4. β -Hexosaminidase release by RBL-huFc ϵ RI cells sensitized with serum from cow's milk allergic (CMA) patients. Degranulation was determined after incubation of the sensitized RBL-huFc ϵ RI cells with whey or Hydrolysed formula. The maximum release (100%) was induced with hulgE and α -hulgE. (Data are expressed as mean $\pm$ SD; * p < 0.05, ** p < 0.01 compared to the control.)

the CMA pool in the absence of anti-IgE or protein ($35.6 \pm 2.7\%$). The AA based formula has been tested in a previous assay and did not induce degranulation (data not shown).

3.4. Whey-specific IgE and IgG1

The whey-specific IgE and IgG1 levels were strongly elevated in the whey-sensitized group compared to the non-sensitized mice, whereas sensitization with the hWhey and the Hydrolysed formula did not induce a whey-specific IgE response (Fig. 5A) and strongly reduced whey-specific IgG1 levels (Fig. 5B). After the oral sensitization period no further increase in whey-specific IgE and IgG1 levels

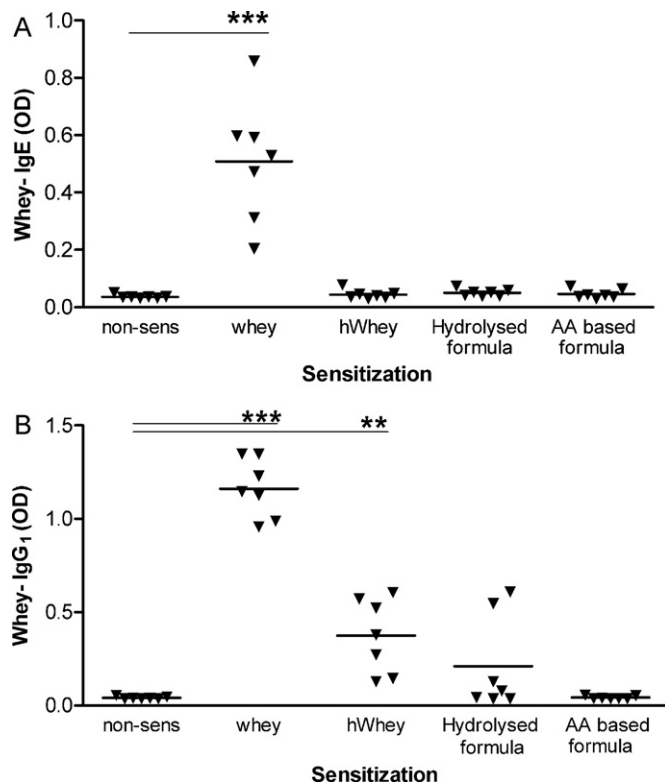


Fig. 5. Levels of whey-specific IgE (A) and IgG1 (B) were determined in sera from whey, hWhey, Hydrolysed formula or AA based formula sensitized mice collected on day 42. (Data are expressed as individual values; ** p < 0.01, *** p < 0.001 compared to non-sensitized mice.)

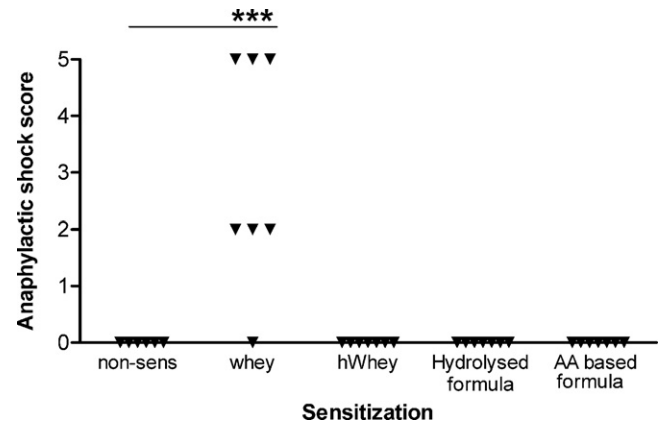


Fig. 6. Anaphylactic symptoms were determined after intradermal whey challenge in mice sensitized to whey, hWhey, Hydrolysed formula or AA based formula. (Data are expressed as individual values; *** p < 0.001 compared to non-sensitized mice.)

were observed due to the intra dermal whey challenge (data not shown).

3.5. Anaphylactic shock score

To study whether challenge with the whole whey protein induced clinical symptoms in the whey, hWhey and the Hydrolysed formula sensitized mice, anaphylactic shock reactions were measured after intradermal whey challenge. Fig. 6 shows the anaphylactic reactions one hour after allergen challenge. Six out of the seven mice sensitized with whey showed mild to severe anaphylactic shock symptoms. However, no shock symptoms were observed in the hWhey, the Hydrolysed formula or the AA based formula sensitized group, indicating that no allergic response to whey was induced in mice exposed to the whey hydrolysates.

3.6. Body temperature

When animals experience an anaphylactic shock upon allergen challenge, a drop in the body temperature can be observed. The starting body temperature of all animals was approximately 36°C before whey challenge. A rapid decline to 32°C was observed in the whey-sensitized mice. In agreement with the anaphylactic shock score, no drop in body temperature after the intradermal whey

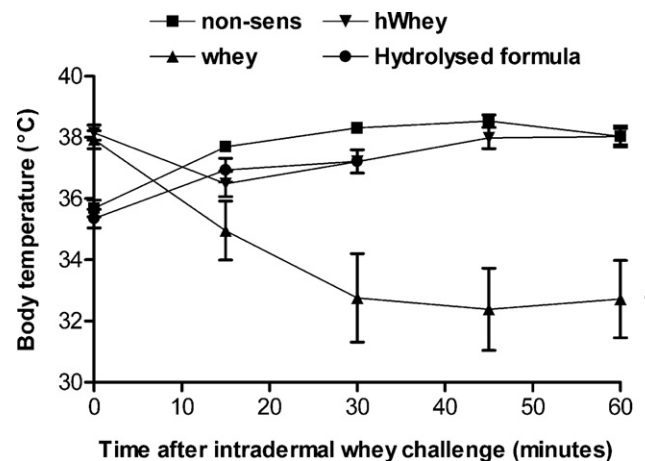


Fig. 7. The body temperature was measured at different time-points after intradermal whey challenge in mice sensitized to whey, hWhey or Hydrolysed formula. (Data are expressed as mean $\pm$ SD; * p < 0.05 compared to non-sensitized mice.)

challenge was induced in mice sensitized with the hWhey or the Hydrolysed formula (Fig. 7).

3.7. Acute allergic skin response

Previous studies have confirmed that the acute allergic skin response correlates with whey-specific IgE levels (Schouten et al., 2008). As expected because of the absence of whey-specific IgE, exposure of mice to the hWhey or the Hydrolysed formula did not induce an acute allergic skin response after intradermal whey challenge. However, although whey-sensitization induced whey-specific IgE antibodies, the whey-sensitized control group failed to show an acute allergic skin response, probably because of the superseding anaphylactic shock in these mice (data not shown).

4. Discussion

Hydrolysed infant formulas are commonly used as an alternative for standard infant milk formulas in genetically predisposed infants or infants diagnosed with cow's milk allergy. The hypoallergenicity of new infant and follow-on formulas must be assessed to confirm that the formulas are not able to sensitize animals as described by the European guidelines. In our opinion, a combination of several techniques, covering both the sensitization phase and the elicitation or effector phase of the allergic response, is necessary to obtain a comprehensive and detailed picture of the hypoallergenicity of Hydrolysed formulas.

Whey, which represents 20% of the total protein content of cow's milk, contains primarily β -Lg, α -Lg and bovine serum albumin. Of these proteins, β -Lg is considered the major allergenic component of the cow's milk derived whey fraction. Complete degradation of β -Lg was observed and no binding of antibodies against β -Lg and α -Lg was observed in the whey hydrolysates giving a first indication of the reduced antigenicity of the hydrolysates. Moreover, the Hydrolysed formula lost the capacity to cause degranulation of the RBL cell-line transfected with the human IgE receptor. This degranulation assay, which may serve as a model for the effector phase of the allergic response (as a reflection for clinical effects in allergic children), is considered a highly relevant in vitro model for the human situation because of predictive value of recognition of residual proteins by the human immune system (Dibbern et al., 2003; Nowak-Węgrzyn et al., 2009).

Besides the obtained in vitro data, it is required to determine the residual sensitizing capacity of hypoallergenic infant formulas in respect to safety. Recently, we positioned a mouse model of cow's milk allergy using oral sensitization to assess both the sensitizing capacity of whey hydrolysates and the residual potency of whey hydrolysates to induce an allergic effector response upon challenge in whey allergic mice using an acute allergic skin response as the major read-out (van Esch et al., 2009b). In the current study, this model was slightly adapted to assess the residual sensitizing capacity of hWhey and the Hydrolysed formula with anaphylactic shock score and body temperature as major read-out parameters for clinical symptoms.

Oral sensitization to whey resulted in the induction of whey-specific IgE and IgG1 antibodies and after challenge acute clinical symptoms were observed, as measured by severe anaphylactic reactions and a rapid drop in body temperature. Oral sensitization to hWhey and the Hydrolysed formula did not result in the induction of allergen-specific IgE responses. As expected by the absence of whey-specific IgE antibodies, no anaphylactic shock reactions and no drop in body temperature were observed after whey challenge in mice sensitized with hWhey or the Hydrolysed formula. Exposure to hWhey or Hydrolysed formula did, however, show low levels of whey-specific IgG1 compared to whey-sensitized mice. Thus, although an immune response was observed to the

whey hydrolysates, no allergic IgE response and clinical symptoms occurred in this model. In allergic mice experiencing a severe anaphylactic reaction and a rapid drop in body temperature no local acute allergic skin response could be determined anymore. It can be hypothesized that after a whey challenge, due to the large systemic anaphylactic response, local vasodilatation is not detectable anymore.

For safety reasons, the hypoallergenicity of hydrolysed infant formulas needs to be assessed by showing that the hypoallergenic formulas are not able to sensitize animals. Although some attempts were made to use the oral sensitization route, in many animal studies no oral sensitization was used (Niggemann et al., 2001; Fritsche, 2003). Although a rat model using oral sensitization with whey has been described which generates both specific IgG and IgE antibodies of similar specificity to those produced by humans (Knippels et al., 2000), hypoallergenicity testing was not performed in this model so far. Guinea pigs sensitized via the oral route are the most commonly used model of reference for testing the safety of new hypoallergenic formulas due to their predisposed responsiveness (Boner et al., 1992). However, a main disadvantage of this guinea pig model is the generation of IgG1a subclass anaphylactic antibody responses instead of IgE antibody responses which is the main physiological antibody response in allergic humans. This discrepancy between IgG1a and IgE makes this model questionable with regard to the potential extrapolation to the human situation. In the described whey based mouse model for cow's milk allergy, which also uses the oral route of sensitization, allergen-specific IgE could be induced and, upon challenge, clinical symptoms were measured to establish the residual allergenicity of whey hydrolysates.

In summary, a mouse model of cow's milk allergy using oral sensitization with relevant clinical read-out parameters was used to determine the residual sensitizing capacity of hydrolysed whey proteins. In combination with in vitro assays determining the amount of intact protein by ELISA and the residual capacity of hWhey and Hydrolysed formula to cross-link human IgE antibodies and subsequent degranulation of the huFc ϵ RI transfected RBL cell-line, a comprehensive and detailed assessment of the hypoallergenicity of Hydrolysed formulas is provided. This combination of assays is proposed as a strategy for the screening of new hypoallergenic formulas aimed at preventing sensitization in atopic children and avoiding clinical symptoms in infants suffering from cow's milk allergy.

Conflict of interest statement

None declared.

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