Toxicology Letters 220 (2013) 95–102

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Interlaboratory evaluation of a cow's milk allergy mouse model to assess the allergenicity of hydrolysed cow's milk based infant formulas

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HIGHLIGHTS

- Mouse model to assess allergenicity of hydrolysed cow's milk based infant formulas.
- Two phases of a multicenter project performed in four independent research centers.
- Good transferability and discriminatory power of the mouse model for cow's milk allergy.

ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form 11 April 2013
Accepted 15 April 2013
Available online 22 April 2013

Keywords:
Allergenicity
Safety
Sensitization
Mouse model
Hydrolysed infant formulas
Cow's milk allergy

ABSTRACT

This study describes two phases of a multi-phase project aiming to validate a mouse model for cow's milk allergy to assess the potential allergenicity of hydrolysed cow's milk based infant formulas (claim support EC-directive 2006/141/E). The transferability and the discriminatory power of this model was evaluated in 4 research centers. Mice were sensitized by oral gavage with whey or extensively hydrolysed whey (eWH) using cholera toxin as an adjuvant. Whey-specific antibodies, mMCP-1 levels, anaphylactic shock symptoms, body temperature and the acute allergic skin response were determined upon whey challenge. In phases I and II, all 4 centers detected elevated levels of whey-specific IgE/IgG1 in whey sensitized animals. Elevated levels of mMCP-1, anaphylactic symptoms, body temperature drop and acute allergic skin response were scored upon whey challenge in 3 out of 4 research centers. In contrast, none of the evaluated parameters were elevated in eWH orally exposed groups. The cow's milk allergy mouse model is capable to distinguish the sensitizing capacity of complete or hydrolysed cow's milk protein. The model uses straightforward parameters relevant to food allergic responses and can be effectively transferred between different laboratories. We propose this mouse model as a new strategy for the screening of new hypoallergenic cow's milk formulas.

1. Introduction

Infants that are diagnosed with cow's milk allergy commonly use hypoallergenic (HA) formulas as an alternative for standard infant milk formulas. The hypoallergenicity of these formulas needs to be confirmed and European guidelines on HA and follow-on formulas require objective and scientifically verified data as proof of the hypoallergenicity of HA formulas. In essence, this means that hypoallergenicity needs to be assessed by showing that the proteins in the HA formulas are not able to sensitize animals to the protein source they are derived from.

Because of the immunological complexity of the allergic response, animal models are considered indispensable to predict the safety of HA formulas. To assess the residual allergenicity of hydrolysed cow's milk formulas it is inevitable to measure allergic clinical symptoms upon challenge to the native protein, in addition to the induction of allergen-specific antibodies. In vitro assays, e.g. mast cell assays, may only serve as a first indication of the residual capacity of HA formulas to induce clinical symptoms in already sensitized individuals (van Esch et al., 2011).

However, animal models to assess the allergenicity of HA formulas are available but not validated. Until now, active systemic

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http://dx.doi.org/10.1016/j.toxlet.2013.04.008

anaphylaxis (ASA) assays in orally sensitized guinea pigs have been commonly used for this purpose. But besides ethical concerns with systemic anaphylactic responses as main readout, another disadvantage is that guinea pigs generate anaphylactic antibodies of the IgG1a subclass instead of IgE antibodies. This makes the suitability of the guinea pig model questionable with regard to the extrapolation to the human situation. Besides the guinea pig model, both rat and mouse models for cow's milk allergy have been used to test allergenicity of cow's milk hydrolysates (Niggemann et al., 2001; Fritsche, 2003). However, in these studies, IgE antibodies are generated upon systemic instead of oral sensitization. This systemic sensitization will induce a different immunologic response, again hampering direct extrapolation of outcomes to the human situation (Mayer, 2003).

About a decade ago, Li et al. (1999) developed an oral cow's milk allergy model in the mouse using an adjuvant to break oral tolerance to harmless food proteins. This model was adapted by Schouten et al. (2008) to further study mechanisms underlying cow's milk allergy and test new concepts for prevention or treatment of cow's milk allergy. In a recent study, the cow's milk allergy model was used to assess the allergenicity of experimental hydrolysed whey proteins (van Esch et al., 2011). Hydrolysates were not capable of inducing IgE, nor allergic clinical symptoms upon challenge to the native protein. In an in vitro assay they were not capable of cross-linking IgE on mast cells and hereby eliciting clinical symptoms in allergic animals. Results of this study provided a solid base to start validating this mouse model for pre-clinical safety evaluation of HA formulas.

As a first step of validation we examined the transferability of the animal model by setting up the model in 4 different research centers with native whey protein as allergen. In the second phase, whey as well as an extensively hydrolysed formulation of whey was tested in the same 4 centers. Extensively hydrolysed whey (eWH) is tested for its sensitizing ability to intact whey proteins when animals are subsequently challenged with unhydrolysed whey and elicitation immune parameters are used to determine allergic response. Read out parameters in this study included allergen-specific IgE, as well as parameters to determine clinically relevant symptoms (anaphylactic shock symptoms, body temperature, ear swelling upon local challenge and mast cell activity).

2. Materials and methods

2.1. Participating institutes

This multicenter ring trial was performed independently at four research centers in the Netherlands: The Institute for Risk Assessment Sciences, Utrecht University, Utrecht; TNO/TNO Triskelion, Zeist; Danone Research Centre for Specialised Nutrition, Wageningen and the Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht.

2.2. Phases I and II of the validation

In the first phase, the transferability of the mouse model for cow's milk allergy to all participating research centers was evaluated by determining whey-specific IgE, anaphylactic shock symptoms, the acute allergic skin response and serum mMCP-1 as a reflection of intestinal mast cell degranulation. In the second phase, the capacity of the mouse model for cow's milk allergy to distinguish between the sensitizing properties of whey and the eWH protein was evaluated using the same read-outs as in phase I. Body temperature was included as an additional parameter.

The animal experiment was standardized as much as possible. Mice were from the same supplier, delivered on the same day at each center and housed under similar conditions (number of animals per cage, cage bedding, temperature and humidity). Synthetic diets, whey proteins and cholera toxin were derived from the same batch. The samples were analyzed by each center by using the same protocols (with the same standards if applicable).

2.3. Test materials

Whey protein concentrate 80 (indicated as whey) was obtained from DMV International (Veghel, the Netherlands). Whey was hydrolysed with an established mixture of endopeptidases and exopeptidases and ultra-filtrated (confidential enzyme composition used by Danone) resulting in eWH proteins. This is an experimental hydrolysate solely produced for these experiments and not for usage in an end product. All participating centers used whey and eWH (containing less than 0.01% of native protein) from the same batches.

Table 1
Anaphylactic symptom scoring table.

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erect; 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

2.4. Animals

Four to five week-old specific pathogen-free female C3H/HeOuJ mice (Charles River Centers, Saint Germain sur l'Arbresle, France) were used in all studies. The animals were raised and bred on a milk-free diet for at least two generations. Food and water were available ad libitum. The animals were maintained on semi-purified cow's milk protein-free mouse chow (AIN-93G-soja, Research Diet Services, Wijk bij Duurstede, The Netherlands). The ambient temperature was maintained between 20 °C and 24 °C and relative humidity was maintained between 45% and 65% with a 12 h light/dark cycle. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

2.5. Sensitization procedure

Phase I: to investigate the transferability of the cow's milk allergy mouse model to four different research centers, $n = 10$ mice per group were orally sensitized with a blunt needle on days 0, 7, 14, 21 and 28 with 2 mg or 20 mg whey homogenized in 0.5 ml PBS mixed with 10 μg cholera toxin (Quadratech Diagnostics, Epsom, UK) as an adjuvant. Non-sensitized mice received cholera toxin in PBS only (Fig. 1). The 2 mg whey-dose used in the study is based on the daily intake of a child up to 6 months of age. From a safety prospective a 10-fold higher dose (20 mg) was included as well.

Phase II: the aim of phase II was to assess the discriminatory capacity of the cow's milk allergy mouse model. eWH was tested for its sensitizing ability to intact whey proteins when animals are subsequently challenged with unhydrolysed whey and elicitation immune parameters are used to determine the allergic response. 8 mice per group were orally sensitized with a blunt needle on days 0, 7, 14, 21 and 28 with 2 mg or 20 mg whey, or treated with eWH homogenized in 0.5 ml PBS mixed with 10 μg cholera toxin (Quadratech Diagnostics, Epsom, UK) as an adjuvant. Non-sensitized mice received cholera toxin in PBS only (Fig. 1).

2.6. Intradermal whey challenge

On day 33, whey sensitized mice and eWH treated mice were intradermally challenged with 10 μg whey (in PBS) in the right ear pinnae. As control, the left ear pinnae were challenged with PBS. After intradermal challenge, anaphylactic shock symptoms (1 h after challenge), drop in body temperature (at various time points until 2 h after challenge; phase II) and the acute allergic skin response (1 h after challenge) were determined as clinically relevant symptoms. To establish the severity of a shock, a validated anaphylactic scoring table (Table 1) was used, as adapted from Li et al. (1999). To measure changes in body temperature, a programmable temperature transponder (IPTT-300, Biomedical data systems, Delware, USA) was subcutaneously implanted in all mice on day 16 (phase II). To determine the acute allergen-specific skin response, 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 for the basal ear thickness.

2.7. Oral challenge

On day 35, whey sensitized mice and eWH treated mice received an oral challenge of 50 mg whey in 0.5 ml PBS. Thirty minutes after oral challenge, blood samples were collected. Blood samples were centrifuged at room temperature for 15 min at 13,500 rpm and sera were stored at −20 °C until further analysis for antibodies and mouse mast cell protease-1 (mMCP-1) as a reflection of mast cell activity.

2.8. Measurement of whey-specific antibodies and mMCP-1

Concentrations of whey-specific IgE and whey-specific IgG1 were determined in serum collected at day 35 by means of ELISA as described previously (van Esch