

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

B.C.A.M. van Esch^{a,*}, J.H.M. van Bilsen^b, P.V. Jeurink^c, J. Garssen^a, A.H. Penninks^d, J.J. Smit^e, R.H.H. Pieters^e, L.M.J. Knippels^c

^a Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

^b TNO, Zeist, The Netherlands

^c Danone Research Centre for Specialised Nutrition, Wageningen, The Netherlands

^d TNO Triskelion, Zeist, The Netherlands

^e Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

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.

© 2013 Elsevier Ireland Ltd. All rights reserved.

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

* Corresponding author at: Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands. Tel.: +31 302537355; fax: +31 302537420.

E-mail address: e.c.a.m.vanesch@uu.nl (B.C.A.M. van Esch).

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

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

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.

2.4. Animals

Four to five week-old specific pathogen-free female C3H/HeOJ 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 (Quadrach 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 (Quadrach 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, Delaware, 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

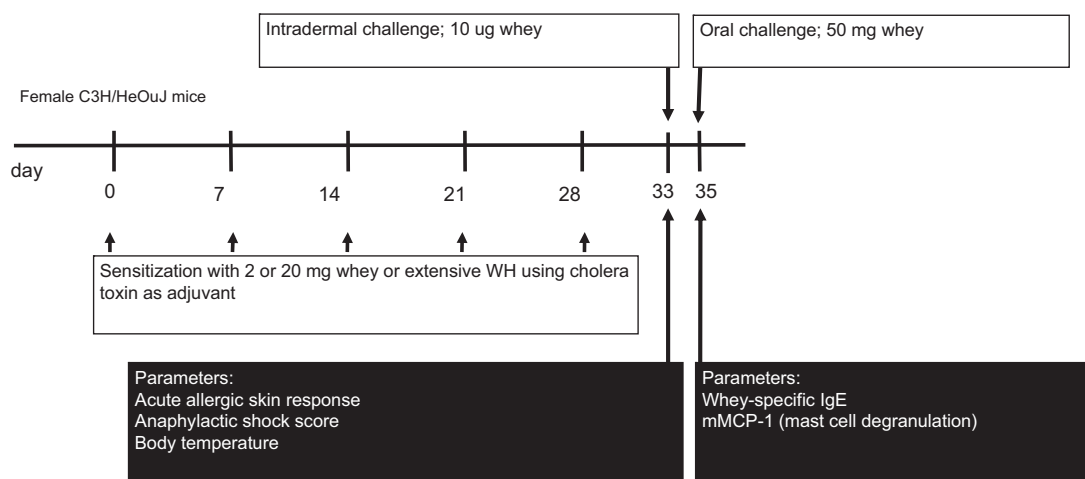


Fig. 1. Mice were sensitized weekly by oral gavage with whey or extensive whey hydrolysate (eWH) in PBS using cholera toxin (CT) as an adjuvant. Non-sensitized mice received CT in PBS only. On day 33 body temperature, anaphylactic shock score and an acute allergic skin response were determined after intradermal whey challenge in the ear pinnae. Whey-specific antibodies (IgE/IgG1) and mMCP-1 were measured on day 35, 30 min after oral whey challenge.

et al., 2010). In short, microtiter plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with 20 µg of whey in coating buffer (Sigma Aldrich, Zwijndrecht, the Netherlands) for 18 h at 4 °C. Plates were washed and blocked for 1 h with buffer containing 50 mM Tris, 2 mM EDTA and 137 mM NaCl/0.05% Tween and 0.5% BSA. Serum samples were applied and incubated for 2 h at room temperature. Plates were washed and incubated with 1 µg/ml biotin labeled rat anti-mouse IgE/IgG1 (Pharmingen, Alphen a/d Rijn, the Netherlands) for 1 h at room temperature. After washing, the plates were incubated with streptavidin-horse radish peroxidase (Sanquin, Amsterdam, the Netherlands) for 1 h, washed and developed with o-phenyldiamine (Sigma Aldrich, Zwijndrecht, the Netherlands). The reaction was stopped after 10 min with 4 M H₂SO₄ and absorbance was measured at 490 nm on a Microplate reader. Results were expressed as arbitrary units (AU). Serum concentrations of mMCP-1 were determined according to the manufacturer's protocol using a commercially available ELISA kit (Moredun Scientific Ltd., Midlothian, UK).

2.9. Statistical analysis

Data were log transformed and analyzed using a one-way ANOVA and post hoc Dunnett's multiple comparison analysis. All statistical analyses were conducted using GraphPad Prism software (version 4.03). *p*-Values are depicted as * for *p* < 0.05, ** for *p* < 0.01, *** for *p* < 0.001.

3. Results of phase I

3.1. Whey-specific antibodies

All participating research centers were able to sensitize mice to whey as shown by elevated levels of whey-specific IgE and IgG1. Minor differences were observed in the magnitude of the antibody response between the two different whey concentrations used for sensitization.

3.1.1. Whey-specific IgE

Elevated levels of whey-specific IgE (Fig. 2a) were observed in mice sensitized with whey (2 mg) in all research centers. Three out of four research centers showed elevated levels of whey-specific IgE in 20 mg-sensitized mice, and one research center did not find an increase in IgE levels at this high dose.

3.1.2. Whey-specific IgG1

Significantly elevated levels of whey-specific IgG1 (Fig. 2b) were measured in whey-sensitized mice compared to non-sensitized mice. No difference in the magnitude of the antibody response was observed between mice sensitized with 2 mg or 20 mg whey with respect to whey-IgG1 concentrations.

3.2. Anaphylactic shock score

Anaphylactic shock reactions were measured after intradermal whey challenge (Fig. 3) in whey-sensitized and non-sensitized mice. In research centers 1, 3 and 4, most whey sensitized mice showed moderate to severe anaphylactic shock reactions, which was fatal for one mouse sensitized with whey (2 mg) in research center 1. Research center 2 observed anaphylactic shock symptoms in 1 out of 10 mice sensitized with 2 mg or 20 mg whey. No anaphylactic shock reactions were observed in non-sensitized mice upon whey challenge.

3.3. Acute allergic skin response

Previous studies suggested that the acute allergic skin response correlates with whey-specific IgE and IgG1 levels (Schouten et al., 2008). In the current study, acute allergic skin responses were observed in center 1, 3 and 4 after intradermal whey challenge in all whey-sensitized IgE-positive mice (Fig. 4). In research center 2, none of the mice showed an acute allergic skin response upon intradermal whey challenge, despite the fact that whey-specific IgE was measured in 5 out of 10 whey (2 mg)-treated mice and in 1 out of 10 whey (20 mg)-treated mice. The moderate allergic skin response measured in research center 1, in the high dose whey (20 mg)-sensitized mice is probably caused by the superseding anaphylactic shock in these mice (Fig. 4) (van Esch et al., 2011).

3.4. mMCP-1 concentrations in serum

To investigate mucosal mast cell degranulation as a clinically relevant symptom, mMCP-1 serum concentrations were measured 30 min after oral whey challenge (Fig. 5). All research centers showed elevated concentrations of mMCP-1 in mice sensitized with 2 mg whey indicating that sensitized mucosal mast cells were triggered. In mice sensitized with whey (20 mg), elevated serum levels of mMCP-1 were observed in research centers 1 and 2 only.

4. Results of phase II

In phase II of the multicenter ring trial, a second study was initiated to investigate the discriminatory power of the mouse model on the sensitizing properties of whey and an extensively hydrolysed formula (eWH). Therefore, the phase I protocol was expanded by including eWH as a test substance. The acute allergic skin response,

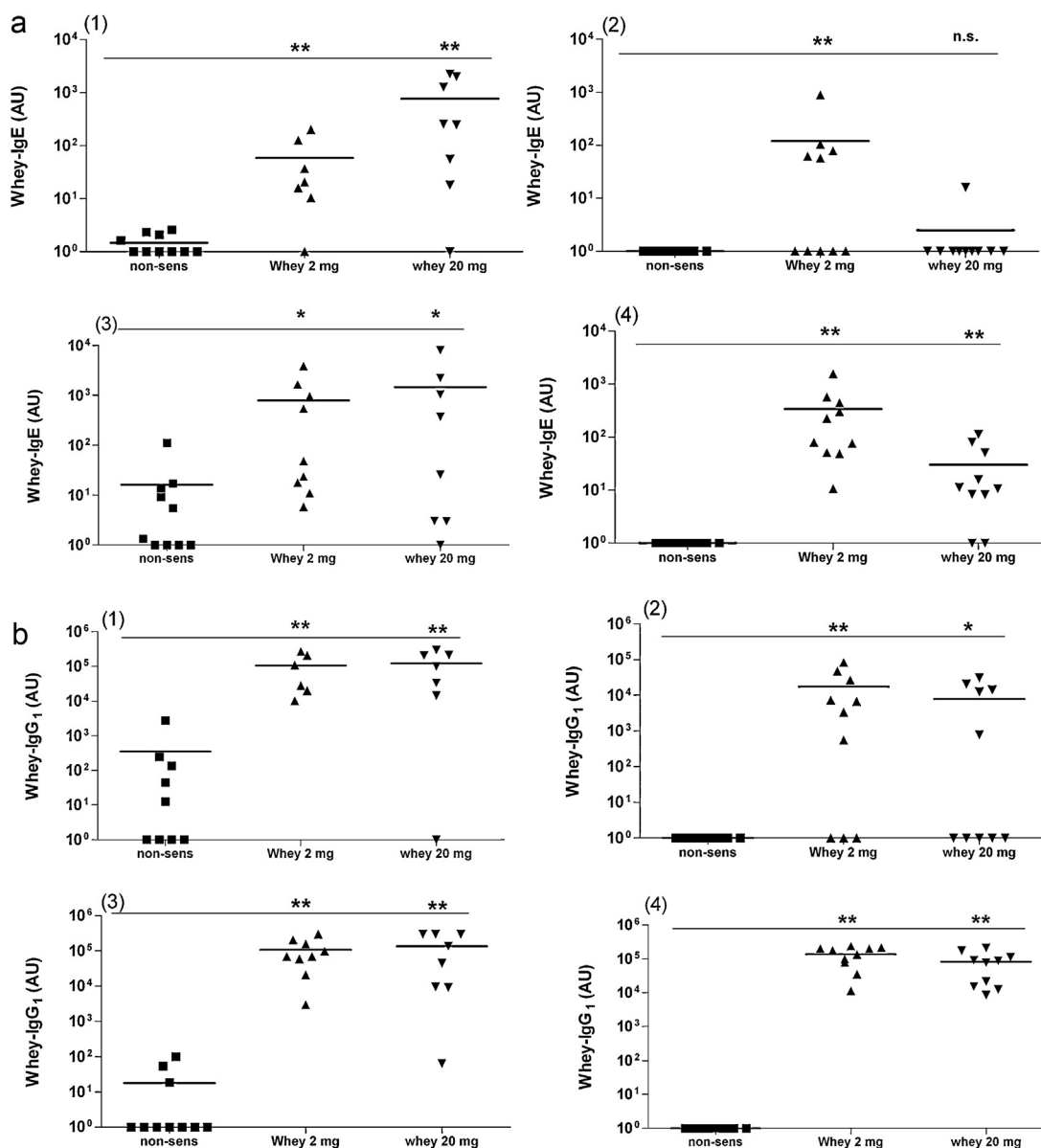


Fig. 2. Phase I: concentrations of whey-specific IgE (a) and whey-IgG1 (b) were determined in sera of non-sensitized or whey-sensitized mice collected on day 35 (data are expressed as individual values; * $p < 0.05$, ** $p < 0.01$ compared to non-sensitized mice). Numbers (1)–(4) indicate different research centers.

anaphylactic shock symptoms and mast cell degranulation (mMCP-1) upon challenge with the native protein were measured as a reflection of sensitization. Moreover, body temperature was determined as an additional parameter of anaphylaxis upon intradermal challenge of the mice.

4.1. Whey-IgE and -IgG1

In accordance with phase I, all four research centers showed whey-IgE (Fig. 6a) and whey-IgG1 (Fig. 6b) in 20 mg whey sensitized mice, whereas sensitization with the eWH did not induce a whey-specific IgE response (Fig. 6a) and strongly reduced whey-specific IgG1 levels (Fig. 6b) in all centers.

4.2. Anaphylactic shock symptoms, acute allergic skin response and mMCP-1 concentrations

As observed in phase I, anaphylactic shock symptoms, acute allergic skin response and increased mMCP-1 concentrations were

observed in sensitized mice in centers 1, 3 and 4. No clinical related symptoms upon challenge were observed in center 2 (Table 2).

In contrast to whey sensitized mice, no anaphylactic shock symptoms, acute allergic skin response or elevated mMCP-1 serum concentrations upon whey challenge were measured in mice sensitized with eWH (Table 2). At center 1, a small but significant acute allergic skin response was observed in mice sensitized with eWH compared to non-sensitized mice, due to a relatively low response in the non-sensitized mice compared to the other research centers.

4.3. Body temperature

Anaphylactic shock upon allergen challenge is accompanied by a drop in the body temperature. A rapid decline in body temperature was observed in whey-sensitized mice which reached its minimum at 60 min after intradermal whey challenge in research centers 1, 3 and 4 (Fig. 7). At center 2, no drop in body temperature was observed upon whey challenge in whey-sensitized mice (Fig. 7). In contrast to mice sensitized to whey,

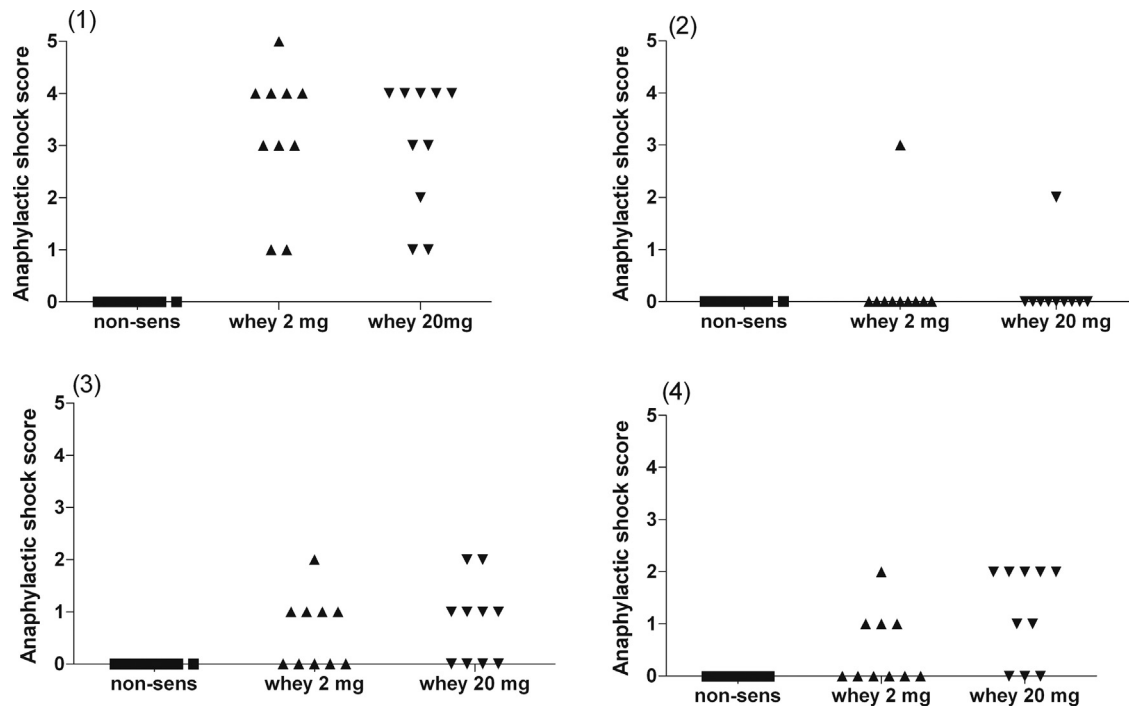


Fig. 3. Phase I: anaphylactic shock symptoms were determined after intradermal whey challenge in non-sensitized or whey sensitized mice on day 33 (data are expressed as individual values). Numbers (1)–(4) indicate different research centers.

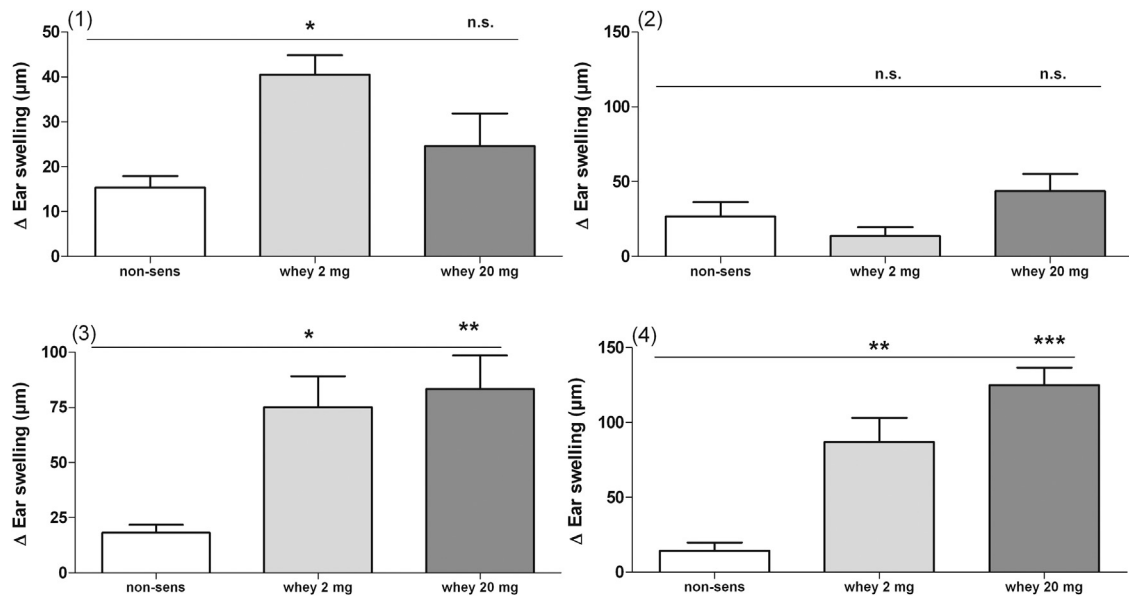


Fig. 4. Phase I: an acute allergic skin response was determined after intradermal whey challenge in non-sensitized or whey sensitized mice on day 33 (data are expressed as mean $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001 compared to non-sensitized mice). Numbers (1)–(4) indicate different research centers.

Table 2

Phase II of the multicenter ring trial of the mouse model for cow's milk allergy. C3H/HeOJ mice (n = 8/group) were sensitized by oral administration of whey (2 mg or 20 mg) or extensively hydrolysed cow's milk (eWH; 2 mg or 20 mg) after which multiple clinical allergy parameters were analyzed.

	Center 1		Center 2		Center 3		Center 4	
	Whey 2/20 mg	eWH 2/20 mg	Whey 2/20 mg	eWH 2/20 mg	Whey 2/20 mg	eWH 2/20 mg	Whey 2/20 mg	eWH 2/20 mg
Ear swelling ^a	+/+	+/-	-/-	-/-	-/+	-/-	+/+	-/-
Anaphylactic shock symptoms ^a	+/+	-/-	-/-	-/-	+/+	-/-	+/+	-/-
Drop in temperature ^a	+/+	-/-	-/-	-/-	$\pm/\pm$	-/-	-/+	-/-
mMCP-1 after oral challenge	+/+	-/-	-/-	-/-	+/+	-/-	+/+	-/-

(+) Elevated levels compared to non-sensitized animals and (-) comparable levels to non-sensitized animal levels.

^a After intradermal challenge.

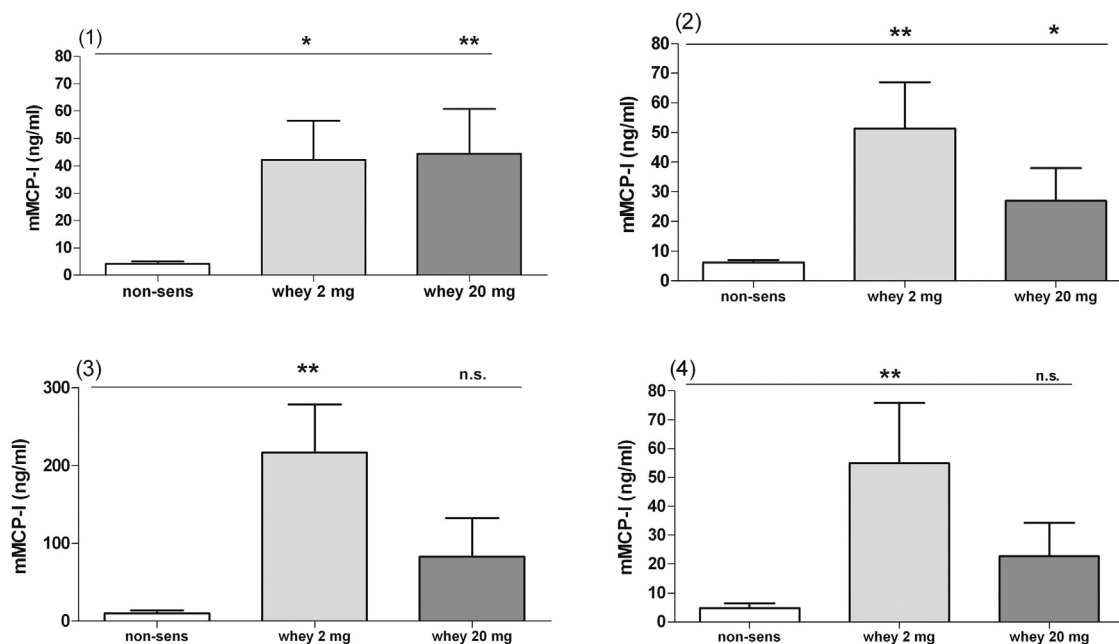


Fig. 5. Phase I: mMCP-1 was measured in serum of non-sensitized or whey-sensitized mice collected 30 min after oral whey challenge on day 35 (data are expressed as mean $\pm$ SEM; * p < 0.05, ** p < 0.01 compared to non-sensitized mice). Numbers (1)–(4) indicate different research centers.

no drop in body temperature was induced in mice sensitized with eWH. At 15 min, a drop in body temperature due to the anesthetic procedure is observed in all centers.

5. Discussion

According to the European Commission-directive 2006/141/E on infant formulas, 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. To assess the safety (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. However, until now no extensive validated animal models exist to perform this requested analysis.

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 to allergens (Boner et al., 1992). In literature, 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 and Penninks, 2005; Ladics et al., 2010), but hypoallergenicity testing was not performed in this model so far.

In recent studies, we and others have described the use of a mouse model for allergenicity testing (Lopez-Exposito et al., 2012; van Esch et al., 2011). In the present study, the suitability of this mouse model to determine the residual allergenicity of new infant and follow-on formulas was examined. This was done as a part of a multi-phase multicenter project by comparing the performance of the model in 4 different research centers with experience with food allergy mouse models. In phase I of the project, whey-specific IgE/IgG1 levels were significantly enhanced in mice orally sensitized to whey in all four research centers. From the results obtained on whey-specific IgE in this first phase, we conclude that the model has a 100% transferability for the 2 mg whey-dose and a 75% transferability for the 20 mg whey-dose. Based on these results, elevated whey-specific IgE levels in combination with elevated levels of

whey-specific IgG1 (100% transferability for 2 and 20 mg whey) are proposed as biomarkers to be used to set performance criteria for this test.

To assess the safety (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 the current study, anaphylactic shock symptoms, a drop in body temperature, acute allergic skin responses and increased concentrations of mMCP-1 were observed in whey-sensitized mice in 3 of the 4 research centers. Except for mMCP-1, no clinical related allergic symptoms were observed in center 2, despite the increased presence of whey-specific IgE antibodies showing a 75% transferability of the clinical related symptoms. Currently, it is not clear which center-specific factors account for this lack of symptoms. Although elevated levels of IgE were measured in centers 3 and 4, no significant increase in mMCP-1 levels was measured. In patients it is known that presence of allergen-specific IgE does not always result in allergic clinical symptoms (van Ree and Aalberse, 1999; Schouten et al., 2010). If the latter also applies to mice, then this finding underscores that combined measurement of whey-specific IgE/IgG1 and clinical related parameters is necessary for accurate prediction of residual allergenicity of cow's milk hydrolysates.

Also in phase II increased levels of whey-specific IgE and IgG1 were found upon whey sensitization in all centers. But importantly, major parameters of allergic sensitization, i.e. whey-specific IgE, and clinical related allergic symptoms to whey (anaphylactic shock symptoms, body temperature drop, increased mMCP-1 serum levels), were not increased in mice exposed to the eWH at the chosen dosages. An extended dose response curve with whey and eWH might improve the predictive value of the food allergy mouse model but this was not part of the current study. Exposure to 2 mg and 20 mg eWH showed low levels of whey-specific IgG1 in a few treated mice and a minor acute allergic skin response was observed in mice treated with eWH (2 mg). This skin response may be due to aspecific ear thickening as result of injection, as response was not observed in mice treated with 20 mg eWH. Thus, although an IgG1 response was observed to the eWH, no allergic sensitization occurred to eWH in the current mouse model.

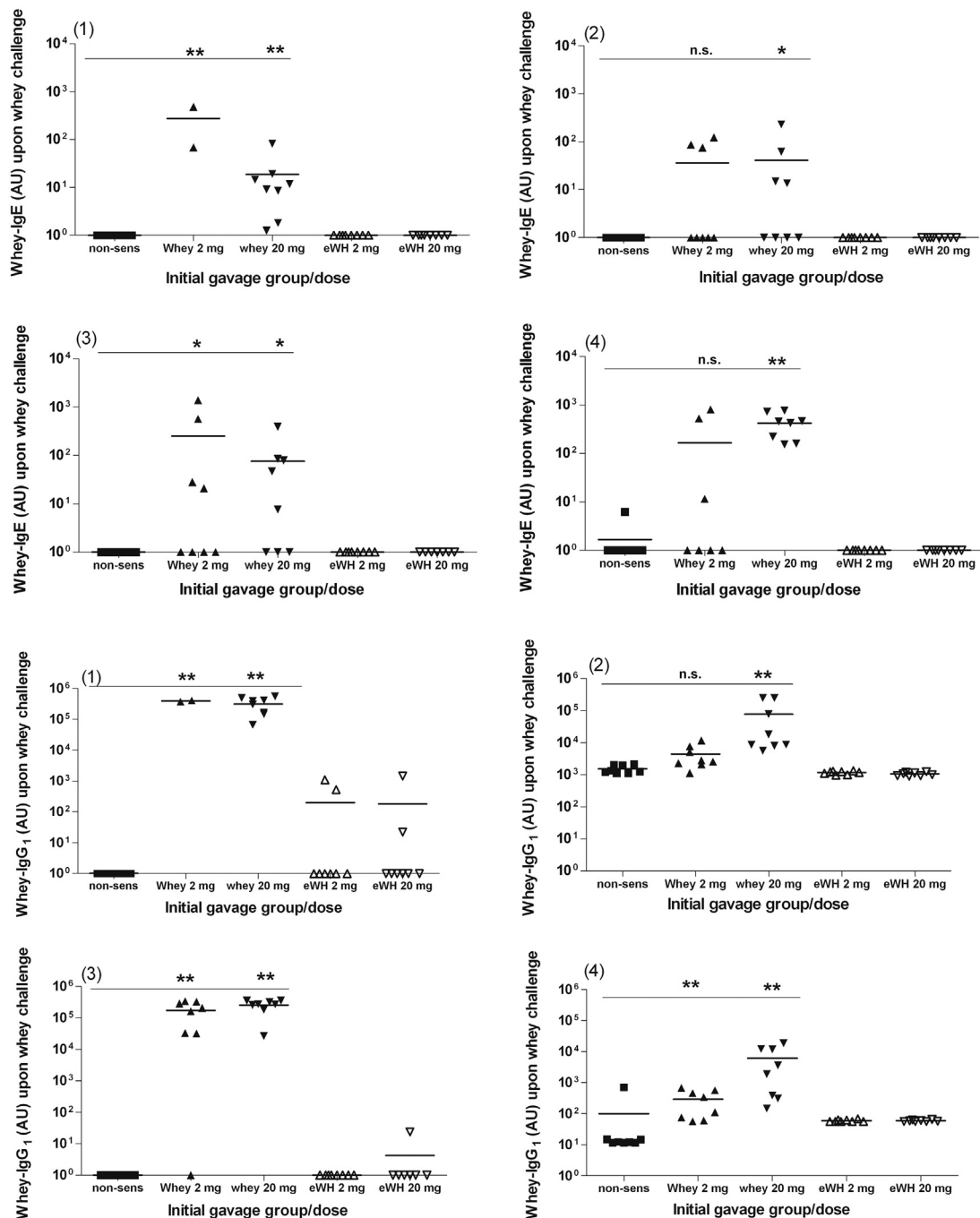


Fig. 6. (a) Phase II: concentrations of whey-specific IgE (a) and whey-IgG1 (b) were determined in sera of non-sensitized, whey-sensitized or eWH-sensitized mice collected on day 35 (data are expressed as individual values; * $p < 0.05$, ** $p < 0.01$ compared to non-sensitized mice). Numbers (1)–(4) indicate different research centers.

In summary, the presented interlaboratory evaluation study demonstrates the transferability of the model, in particular based on allergen specific IgE and IgG1 levels. Hence, specific IgE and IgG1 levels may thus serve as performance criteria. The other more clinical parameters including mast cell degranulation (mMCP-1) are measured in all centers, and only one lab failed to detect truly clinical parameters seen during anaphylaxis showing a 75% transferability of clinical related allergic symptoms. Importantly, all research centers were able to discriminate

the allergenic properties of eWH protein, demonstrating that the mouse model can distinguish between whey and an eWH protein. Based on these results we propose the food allergy mouse model as a new in vivo model for the screening of new hypoallergenic cow's milk formulas. In our opinion, a combination of antibody levels with mMCP-1 and body temperature as a sensitive and quantitative method for measuring clinical related symptoms is necessary to minimize false positive or negative conclusions.

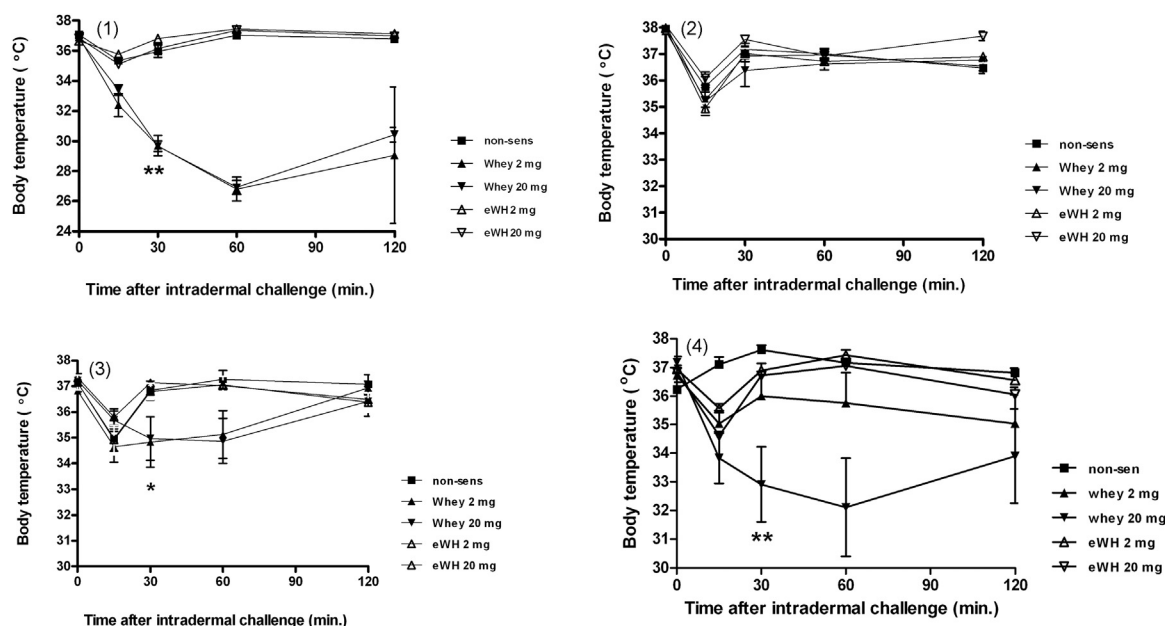


Fig. 7. Phase II: body temperature was measured at different time points after intradermal whey challenge in non-sensitized mice and mice sensitized to whey or eWH (data are expressed as mean $\pm$ SEM; * p < 0.05, ** p < 0.01 measured at 30 min after challenge compared to non-sensitized mice). Numbers (1)–(4) indicate different research centers.

Conflict of interest

No competing interest.

References

- Commission Directive 96/4/EC of 16th February 1996 amending Directive 91/321/EEC on infant formulas and follow-on formulas. Official Journal of the European Communities No L 49, pp. 12–16.
- Boner, A.L., Benedetti, M., Spezia, E., Piacentini, G.L., Bellanti, J.A., 1992. Evaluation of the allergenicity of infant formulas in a guinea pig model. *Annals of Allergy* 68, 404–406.
- Fritsche, R., 2003. Animal models in food allergy: assessment of allergenicity and preventive activity of infant formulas. *Toxicology Letters* 140–141, 303–309.
- Knippels, L.M., Penninks, A.H., 2005. Recent advances using rodent models for predicting human allergenicity. *Toxicology and Applied Pharmacology* 207, 157–160.
- Ladics, G.S., Knippels, L.M., Penninks, A.H., Bannon, G.A., Goodman, R.E., Herouet-Guicheney, C., 2010. Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. *Regulatory Toxicology and Pharmacology* 56, 212–224.
- Li, X.M., Schofield, B.H., Huang, C.K., Kleiner, G.I., Sampson, H.A., 1999. A murine model of IgE-mediated cow's milk hypersensitivity. *Journal of Allergy and Clinical Immunology* 103, 206–214.
- Lopez-Exposito, I., Chicon, R., Belloque, J., Lopez-Fandino, R., Berin, M.C., 2012. In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of beta-lactoglobulin are immunologically inert. *Journal of Dairy Science* 95, 541–548.
- Mayer, L., 2003. Mucosal immunity. *Pediatrics* 111, 1595–1600.
- Niggemann, B., Nies, H., Renz, H., Herz, U., Wahn, U., 2001. Sensitizing capacity and residual allergenicity of hydrolyzed cow's milk formulae: results from a murine model. *International Archives of Allergy and Immunology* 125, 316–321.
- Schouten, B., van Esch, B.C., Hofman, G.A., van den Elsen, L.W., Willemsen, L.E., Garssen, J., 2008. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. *International Archives of Allergy and Immunology* 147, 125–134.
- Schouten, B., van Esch, B.C., van Thuijl, A.O., Blokhuis, B.R., Groot Kormelink, T., Hofman, G.A., Moro, G.E., Boehm, G., Arslanoglu, S., Sprickelman, A.B., Willemsen, L.E., Knippels, L.M., Redegeld, F.A., Garssen, J., 2010. Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins. *Journal of Allergy and Clinical Immunology* 125, 1308–1314.
- van Esch, B.C., Knipping, K., Jeurink, P., van der Heide, S., Dubois, A.E., Willemsen, L.E., Garssen, J., Knippels, L.M., 2011. In vivo and in vitro evaluation of the residual allergenicity of partially hydrolysed infant formulas. *Toxicology Letters* 201, 264–269.
- van Esch, B.C., Schouten, B., Hofman, G.A., van Baalen, T., Nijkamp, F.P., Knippels, L.M., Willemsen, L.E., Garssen, J., 2010. Acute allergic skin response as a new tool to evaluate the allergenicity of whey hydrolysates in a mouse model of orally induced cow's milk allergy. *Pediatric Allergy and Immunology* 21, e780–e786.
- van Ree, R., Aalberse, R.C., 1999. Specific IgE without clinical allergy. *Journal of Allergy and Clinical Immunology* 103, 1000–1001.