Review

Utility of animal models for evaluating hypoallergenicity

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The goal of this article is to describe the use of *in vitro* methods as well as *in vivo* and *ex vivo* animal models to assess residual allergenicity of hypoallergenic (HA) foods, especially infant formulas. These animal models are also used for testing the oral tolerance-inducing capacities of infant formula; thus, they are very helpful in predicting allergenicity and the tolerogenic potential of HA infant formulas, and in helping to prevent adverse reactions in sensitive infants.

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1 Introduction

Food allergy affects as many as 5 – 8% of young children and 3 – 4% of adults [1]. Cow's milk protein allergy (CMPA) is the most common in young infants, with a 2 – 3% incidence [1, 2]. When breastfeeding is not possible, hypoallergenic (HA) cow's milk-based formulas are usually given during the first month of life for prevention of CMPA. Depending on primary (sensitization) or secondary (triggering) prevention, the requested quality of HA formulas may be different. Besides *in vitro* methods, *in vivo* and *ex vivo* animal models are helpful in assessing residual allergenicity and the preventive effect of HA formulas. The oral sensitizing capacity of a formula can be examined by either the guinea pig (IgG1a mediated) or the mouse (IgE) models. The triggering IgE-mediated allergenicity is tested by a parenteral rat model with oral gavage for intestinal mast cell protease II (RMCPII) release. These animal models are also used for testing the oral tolerance inducing capacities of formulas. Together with cellular *in vitro* assays, animal models are very helpful in predicting allergenicity and the tolerogenic potential of HA infant formulas.

2 Results and discussion

For reducing the allergenicity of cow's milk proteins, IgE binding and T-cell epitopes have to be destroyed or inactivated. A number of processes are available to achieve this. For example, heating cow's milk proteins above 80°C will destroy the globular structure of proteins, and consequently conformational epitopes but not sequential ones. Enzymatic hydrolysis is by far the most efficient process for disrupting sequential and conformational epitopes and therefore allergenicity reduction is best achieved by this method. Depending on the type of enzymes used and the conditions of hydrolysis, peptides of different length may be obtained carrying more or less allergenicity. Porcine trypsin/chymotrypsin are frequently used for producing HA formulas, but proteases extracted from bacteria or of fungal origin are increasingly also used.

Physico-chemical methods are often helpful for examining the amount of proteins/peptides obtained after heat and enzymatic hydrolysis processing. SDS-PAGE and peptide profiles give usually a good picture on allergenicity reduction. Immunologic *in vitro* tests are often used for the evaluation of residual allergenicity. ELISA inhibition or uptake (ELISA sandwich) methods are very adequate for the determination of IgG- and IgE-binding epitopes using, respectively, allergen-specific polyclonal animal sera or human patient sera. On the other hand, the IgE-mediated mast cell triggering capacity of allergenic epitopes can be measured with a functional *in vitro* assay. We have set up such an assay in our laboratory, based on peritoneal rat mast cells passively sensitized with specific rat IgE antibodies and labeled with 3H-Serotonin. Cells are triggered for mediator release with standard dilutions of the allergen or test formula dilutions [3].

For the determination of the immunologic *in vivo* allergenicity, animal models are very helpful. The IgE-dependent allergic reaction is composed of two phases: an inducing step, where the immune system of the host is sensitized by

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Abbreviations: CMPA, cow's milk protein allergy; **CT,** cholera toxin; **HA,** hypoallergenic; **RMCPII,** rat mast cell protease II

Table 1. Animal models for allergenicity evaluation

	Protocol	Strengths	Weaknesses
Rats (Brown–Norway, Sprague–Dawley)	Day 1: inj. s.c. Ag + $Al(OH)_3$, day 14: gavage with Ag, bleeding analyses: IgE, RMCPII in serum	Determination of sensitizing, triggering, and tolerizing potentials of Ag (*e.g.*, HA formulas)	Use of adjuvants (alhydrogel) for optimal immune response (IgE)
Guinea pigs (Dunkin–Hartley)	Day 1–14: oral *ad libitum* administration of Ag, day 15: bleeding analyses: IgG1a antibodies	Oral sensitization; no adjuvants necessary in this animal model	Reaginic antibodies are IgG1a, not IgE; lack of sensitivity
Mice (Balb/c, C3H/HeJ)	Week 1–5, once a week: oral administration of Ag and cholera toxin Week 6: challenge with Ag analyses: clinical scores, IgE, cytokines, and MMCP-1	Oral sensitization and recording of clinical scores	Use of cholera toxin as oral adjuvant

For oral tolerance induction, animals are fed preventively *ad libitum* with Ag: Rats: days 5–14 of the protocol. Guinea pigs: 3–5 wk before oral challenge. Mice: 2 wk before start of sensitization. Ag, antigen/modified allergen/hypoallergenic infant formula; s.c, subcutaneous.

the allergen, ending up in specific IgE antiallergen antibodies production which bind to specific cell surface receptors on mast cells in target organs; the second step is a triggering phase mediated by the allergen binding to these IgE and stimulating mediator (histamine) release from mast cells. For evaluating allergenicity of food antigens, both phases should be examined by appropriate tests. Historically, guinea pigs [4] and rats [3] have been used to investigate allergenicity of food proteins. The Brown–Norway rat has been reported to be a useful model for the investigation of food allergy because intraperitoneal sensitization generates IgG and IgE antibodies to a range of milk proteins that are of similar specificity to those produced by humans [5]. Recently, oral mouse models have also been reported which use cholera toxin (CT) as an adjuvant.

There is a significant obstacle to the development of oral murine models of food allergy, namely the strong innate tendency to develop oral tolerance to ingested antigens. I will now discuss each of these models in more detail and elaborate on some of the protocols that are used in our laboratory (Table 1).

2.1 Rat parenteral model

The rat parenteral model appears to be adequate for measuring both the IgE-specific sensitizing (production of IgE antibodies) and triggering (IgE-mediated RMCPII release) capacities of food allergens. It further allows the determination of the tolerizing capacity of an infant formula.

2.1.1 Sensitization/allergic triggering

We use the following protocol: Brown–Norway high IgE-responder rats are injected subcutaneously with the allergen in the presence of $Al(OH)_3$. Fourteen days later, the primary IgE response is determined by ELISA in animal sera. For the evaluation of the *in vivo* triggering activity of a product containing the allergen, above-sensitized rats are gavaged with the test product, bled after 2 h and the level of RMCPII determined in the serum. After a booster injection of the allergen, the secondary IgE response as well as spleen/lymph node lymphocyte proliferation and culture supernatant cytokine determinations are done. This parenteral rat model provides a good indication on the IgE inducing capacity of standard and hypopallergenic infant formulas: moderately and extensively hydrolyzed cow's milk formulas induced, respectively, 100–10 000 times less IgE antibodies than a standard milk formula [3]. Further, intestinal mast cells are primary targets of food allergens in IgE-dependent hypersensitivity. In the rat model, the specific protease (RMCPII) is released into the blood after intestinal mast cell triggering. This protease, determined by ELISA in serum, is a good indicator of the IgE-mediated allergic triggering capacity of infant formulas at the intestinal level. Standard formulas stimulated the highest specific release in our model, whereas HA formulas triggered low or no RMCPII release (Fig. 1)

2.1.2 Induction of oral tolerance

Oral administration of protein antigens induces specific immunologic hyporesponsiveness (tolerance) to these antigens. Induction of oral tolerance with intact proteins has been well documented with a number of antigens in several animal models. Oral tolerance to BSA, for example, has been shown in a Black Norwegian rat model [6]. Immune regulation by the induction of oral tolerance is thought to prevent food allergy [7]. It has been shown that induction of oral tolerance is dependent on the age of the host, the dose of antigen administered [8], and the nature of the antigen. It has further also been shown in animal models that oral tolerance can be induced *in utero* by feeding pregnant mothers

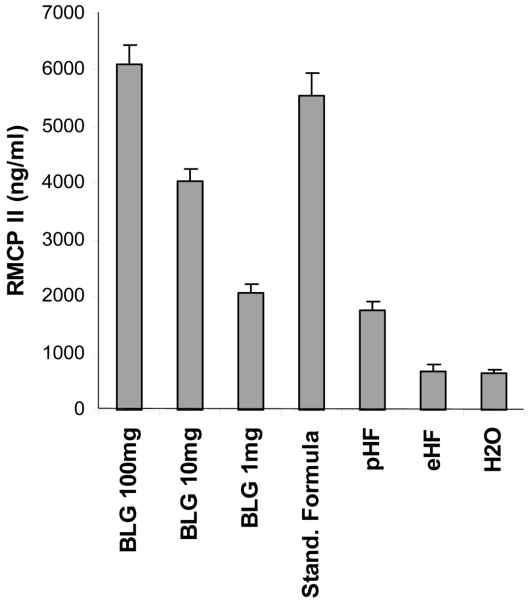

Figure 1. RMCPII release from BLG-sensitized rats challenged orally with BLG or infant formula.

with an antigen-specific tolerizing diet. Although oral tolerance to dietary proteins has been extensively investigated with intact antigens, few studies with antigen fragments or digests have been done.

The protocol we use is the following: for induction of oral tolerance to cow's milk proteins, Sprague – Dawley rats are given different experimental liquid milk formulas *ad libitum* in their drinking bottles and a solid"milk-free" pellet diet from days 1 to 19 of the experiment. For challenge, rats are immunized on day 5 by subcutaneous injection of a selected cow's milk protein (*e.g.*, βLG) in the presence of aluminum hydroxide. Animals are gavaged with intact whey proteins and sacrificed on day 20; sera were analyzed for specific IgE antibodies and RMCPII release.

We have shown with the help of this model that partially hydrolyzed whey proteins (pHF) are able to induce oral tolerance to intact whey proteins whereas extensively hydrolyzed whey proteins (eHF) are unable to achieve this [9]. This was demonstrated at the levels of the IgE response and intestinal mast cell secretion (Fig. 2). Similarly, moderately hydrolyzed soy proteins (with pancreatic enzymes) could be shown with this model to induce oral tolerance to intact soy proteins whereas strongly hydrolyzed soy proteins are unable to achieve this.

2.2 Oral mouse model

Several tests in mice, using adjuvants like CT, have been published which succeeded in inducing oral IgE-mediated sensitization to cow's milk proteins [10]. Briefly, 3-wk-old C3H/HeJ mice are sensitized orally with the allergen in the presence of CT, a mucosal adjuvant, once a week for five successive weeks, followed by an oral challenge with a large dose of the allergen 1 wk later. The model allows, beside clinical symptom scoring, the determination of IgE, T-cell cytokine measurement and determination of mouse mast cell protease 1 (MMCP-1) as markers of allergic sensitization.

Another oral mouse model was recently published [11] where Balb/c mice were orally sensitized with peanut proteins in the presence of CT. There appears, however, to be a strain selectivity for optimal oral sensitization to different allergens [12]. Depending on the protocol used for sensitization, these mouse models allow to mimick either gastrointestinal, dermatologic or respiratory symptoms, which may be helpful for preclinical studies of defined allergen modulation.

We have further used this oral mouse model for the evaluation of the tolerizing capacity of an antigen. We have shown that orally sensitized mice mounted a βLG-specific IgE response when gavaged with βLG in the presence of CT. A single gavage or 2 wk *ad libitum* feeding of whey proteins

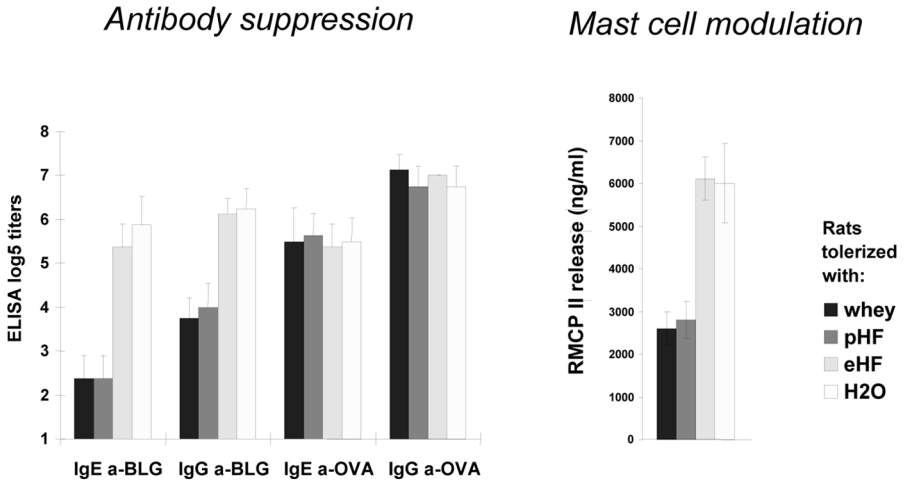

Figure 2. Oral tolerance induction with HA formulas. IgE and RMCPII suppression following oral tolerance induction to BLG with intact or moderately hydrolyzed whey-based infant formula.

given prior to the onset of oral sensitization resulted in the suppression of both specific and bystander IgE [13].

2.3 Guinea pig oral *ad lib* model

Guinea pigs can be sensitized by the oral route without adjuvants making it a good model, close to the human situation. Difficulties associated with passive cutaneous anaphylaxis (PCA) testing, and the fact that the reaginic antibody response is of the IgG1a subtype, limit the use of guinea pigs as a suitable model with which to study CMPA [14]. It is nevertheless a helpful model for the determination of the natural "spontaneous" capacity of a product to induce sensitization or oral tolerance. We have, for example, shown that oral *ad libitum* administration of moderately hydrolyzed whey proteins to Dunkin – Hartley guinea pigs during 5 wk before an oral challenge during 2 wk with intact whey proteins prevents the induction of specific anticow milk protein reaginic IgG1a antibodies. This demonstrated that oral tolerance could be induced by prefeeding with a tolerogenic nonsensitizing formula, in a similar approach as the one occurring in infant feeding.

2.4 Other models

Besides above small laboratory animals, dogs and swine have been reported as useful models for investigations involving food allergy. The advantage of both models lies in their propensity to develop clinical symptoms of food allergy, primarily gastrointestinal and dermatologic reactions.

The atopic dog model is based on an inbred colony of high IgE-producing dogs; to elicit sensitization, dogs are immunized with a live virus vaccine, followed by subcutaneous injections with food antigens over a course of weeks. Skin test titrations were used to assess the allergenicity of processed foods [15].

The neonatal swine model has been used mainly because these animals resemble humans in gastrointestinal physiology and in the development of mucosal immunity. Yound piglets have been used as models for sensitization/tolerance to cow's milk and soy proteins [16].

3 Conclusions

While *in vitro* and cellular assays are very helpful tools for evaluating residual allergenicity in food products (infant formulas, processed foods), their sensitizing capacity as well as their tolerogenic potential can be evaluated only by using *in vivo* animal models. There exists up to now no ideal model of food allergy. The parenteral route leads to stronger but less physiologic sensitizations than oral administration with adjuvants. However, the bias introduced with oral adjuvants in such models limit their use. Therefore, for preclinical studies, it may be optimal to use several animal models, the selection of which may depend on the expected outcome of the studies.

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