B. Proust[1], C. Astier[1], J. M. Renaudin[1], E. Magueur[2], D. Maurice[2], C. Belcourt[2], F. T. Yen[2], G. Kanny[1], B. E. Bihain[2], S. Jacquenet[2]

# A murine model of cow's milk protein-induced allergic reaction: use for safety assessment of hidden milk allergens

[1]EA 3999 "Allergic Diseases: Diagnosis and Therapeutics", Department of Internal Medicine, Clinical Immunology and Allergology, University of Nancy, France; [2]Genclis SAS, Vandoeuvre-lès-Nancy, France
Source of support: Saint Hubert, France

**Key words**

*Cow's milk allergy, anaphylaxis, mouse model, LOAEL, NOAEL, margarine*

**Summary**

***Background:*** *Masked allergens in processed food products can lead to severe allergic reactions following unintentional ingestion. We sought to develop a murine model for the detection of hidden cow's milk proteins (CMP). This study aimed to induce cow's milk allergy in mice, to characterize the anaphylaxis induced by CMP in this model, and to validate its reliability using three margarines manufactured with (A) or without (B, C) milk, sharing the same production line.* ***Materials and Methods:*** *Three-week-old BALB/c mice were sensitized intragastrically with CMP plus cholera toxin and boosted 6 times at weekly intervals. CMP-sensitization status was monitored by skin tests, and measurement of CMP-specific IgE and IgG1 levels. On day 44, the minimal threshold of clinical reactivity to CMP in terms of anaphylaxis was determined by performing a dose response of intraperitoneal CMP challenge. Under the same conditions, anaphylaxis was evaluated in CMP-sensitized mice after challenge with protein extracts of margarines A, B or C.* ***Results:*** *Sensitization to CMP was demonstrated by positive skin tests and increased CMP-specific IgE and IgG1. The minimal clinical reactivity threshold corresponding to 0.1 mg CMP elicited detectable anaphylaxis evidenced by clinical symptoms, a decrease in breathing frequency, and increased plasma histamine upon challenge. Similarly, challenges with margarine A containing CMP demonstrated anaphylaxis, whereas those with B or C did not elicit any detectable allergic reaction.* ***Conclusion:*** *This study shows that our murine model of CMP-induced anaphylaxis is useful for investigating the allergenic activity and the assessment of margarines with respect to milk.*

**Abbreviations:** CMP: cow's milk proteins; CT: cholera toxin; i.g.: intragastrically; i.p.: intraperitoneal; LOAEL: lowest observed adverse effect level; MOS: margin of safety; NOAEL: no-observed adverse effect level

## Background

Food allergy is an important public health problem in industrialized countries. To date, strict dietary avoidance is the only way to manage food allergy, which implies careful labelling of manufactured products. Nevertheless, masked allergens in food can lead to severe accidents such as fatal food-induced allergic reactions following unintentional ingestion [1]. The total absence of any or all allergen in foods is often difficult to achieve because of manufacturing practices. Any remaining allergens are due primarily to different products sharing the same production line.

The relationship of any given food to allergy can be considered as two main components. 1) Allergenicity is defined as the likelihood of a given protein to induce *de novo* sensitization in a non-allergic individual [2]. The determination of allergenicity requires models of allergic sensitization primarily conducted in animals. Several models developed in mice [3-8] and rats [5, 9, 10] have been helpful in investigations of allergic sensitization and humoral immune responses. 2) Allergenic activity reflects the propensity of a substance to induce allergic reactions in sensitized individuals [2]. This activity is usually evaluated in allergic patients by oral challenge tests. The clinical objective is to determine whether the component in question induces allergic response in allergic individuals and to estimate the magnitude and the risk related to this reaction. Several rodent [11-13] and non-rodent models such as swine [14] or canine [15] have been developed to mimic food allergies similar to those seen in humans. A major advantage of these models is that a protein induces not only an immune response but, also clinical symptoms as well after allergenic challenge in sensitized animals. These models are useful for the investigation of allergenic activities of allergens and the immunopathological mechanisms involved, as well as for the exploration of potential immunotherapeutic approaches. Despite these interesting and valuable models, none have been used for the study of the allergenic activity of finished food products before their marketing. *In vitro* assays are commonly used to detect proteins in food products [16-21]. These tests provide information for safety assessment, but do not determine the allergenic activity of finished products. It is clear that clinical studies are the gold standard tests, but in practice, they cannot be implemented on a routine basis for detection of allergens in foods. Genetically Modified Foods by the Food and Agricultural Organization of the United Nations (FAO)/World Health Organization (WHO) [22, 23] has identified a need for the development of well-defined food allergy animal models that can serve as predictive tools for the determination of the allergenic activity of finished food products.

Cow's milk allergy is one of the most common food allergies in infants. Most patients outgrow this by the age of 5 years, but cow's milk allergy can persist in some adults [24]. Contamination of food products with milk proteins have been reported to be unsafe in children allergic to cow's milk [1, 25]. The wide use of cow's milk proteins (CMP) in various food products complicates the application of dietary avoidance. This is most notably the case for fats used as cooking oils or spreads such as margarines. These products are defined as foodstuffs other than butter whatever their origin or their composition, that present the same aspect as that of butter and are intended for the same use. Margarines are composed of two major fractions: fat (83 %) and an aqueous fraction (17 %) which includes water and/or milk, emulsifiers, conservatives, aromas and coloring agents. Some margarines therefore contain cow's milk allergens when milk is included in their manufacturing, while others prepared without milk can be contaminated due to manufacturing practices.

This study aimed (i) to induce cow's milk allergy in mice and characterize the anaphylactic reaction induced by CMP in this model, and (ii) to validate the suitability and the reliability of this model for the testing of margarines manufactured with or without milk, yet sharing the same production line.

## Materials and Methods

Three-week-old female BALB/c mice were purchased from Charles River Laboratory (Lyon, France). Animals were maintained on milk-free chow (Harlan Teklad, Gannat, France) under specific pathogen-free conditions on a 12 h light/dark cycle in a room maintained at a mean temperature of 21 ± 2°C with a relative humidity of 50 ± 20%. Drinking water and standard laboratory animal food pellets were provided *ad libitum*. Animals were handled in accordance with French State Council guidelines for the use and care of laboratory animals (decree N° 87-848, October the 19, 1987 and decree 2001-464, May the 29, 2001).

Commercially available powdered cow's milk (355 mg CMP/g, Régilait, Saint-Martin-Belle-Roche, France)

was used. Three margarines referred to as A, B and C were provided by a manufacturer without any indication on their composition. Detection antibodies for ELISAs, i.e. HRP-labeled goat anti-mouse IgE and IgG1, were purchased from Serotec Ldt (Kidlington, Oxford, UK) and Southern Biotech (Southern Biotechnology Associates Inc., Birmingham, AL, USA), respectively. Compound 48/80, red blood cell lysis buffer and concanavalin A were obtained from Sigma (Saint Louis, MO, USA).

BALB/c mice were sensitized intragastrically (i.g.) with cow's milk administered together with cholera toxin (CT) and boosted 6 times at weekly intervals. To determine the optimal sensitizing dose, 3 groups of mice received 0.1, 1 or 10 mg of CMP in PBS containing 4 μg CT per mouse (200 μL per mouse) through oral administration. Control mice were sensitized i.g. with 4 μg CT alone. Naive mice never exposed to CMP or CT were used as second controls. Immediately prior to each boosting, individual blood samples from each group of mice were obtained from the retro-orbital venous plexus under isoflurane anaesthesia, centrifuged and the sera were stored at - 20°C until use. Two skin tests were performed: an ear swelling test and an intradermal skin test (see below). Forty four days after the initial boosting, mice were challenged intraperitoneally with 15 mg CMP in 150 μL of PBS per mouse, and anaphylaxis was assessed by monitoring clinical symptoms, rectal temperature, breathing frequency, and by measuring plasma histamine levels.

Ear swelling test was performed as previously described (Proust et al., 2008). Briefly, CMP (10 μL, 5 mg/mL) was intradermally injected into the dorsal aspect of a mouse ear and ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan). Ear swelling response was determined as the incremental increase in thickness above baseline control values. Compound 48/80 (5 mg/mL) and PBS were used as positive and negative controls, respectively.

Intradermal skin tests were carried out as previously described (Proust et al., 2008). Briefly, before testing, the abdominal skin was shaved. Evan's blue dye (100 μL, 0.25 %) was intravenously injected and five minutes later, CMP (10 μL, 2.5 mg/mL) was injected intradermally under isoflurane anaesthesia. Compound 48/80 (30 μg/mL) and PBS were used as positive and negative controls, respectively. A blue wheal with a diameter > 0.3 cm appearing within 5 minutes after the injection of allergen was considered as positive.

CMP-specific antibodies were assayed by ELISA. Plates (MaxiSorp, Nunc Immunoplate, Roskilde, Denmark) were coated overnight with CMP (0.5 μg/mL for specific IgE and 1 μg/mL for specific IgG1) diluted in carbonate buffer (50 mM, pH 9.6). Plates were incubated with diluted serum samples (1:10 for IgE; 1:5000 for IgG1) at 37°C for 2 h. CMP-specific IgE were detected by HRP-labeled goat anti-mouse IgE (1:5,000). CMP-specific IgG1 were detected by HRP-labeled goat anti-mouse IgG1 (1:1,000). Plates were developed with tetramethyl benzidine substrate (Pierce, Rockford, IL, USA) and read at 450 nm with an automated microplate reader (Biorad, Hercules, CA, USA). The specificity of HRP-labeled goat anti-mouse IgE was verified in preliminary experiments. IgE detection was not modified after removing IgG from mouse pooled sera with protein-G (Sigma) (data not shown).

Anaphylactic symptoms were assessed by 2 independent investigators within 0-45 minutes after the intraperitoneal (i.p.) challenge; this study was conducted in a blind manner. Disease severity was evaluated by using a scoring system as previously described (Proust et al., 2008) with slight modifications and scored as follows: 0, no symptoms; 1, reduced activity; 2, scratching and rubbing around the nose, the ears and eyes, partial immobility; 3, prostration, pilar erection, total immobility; 4, edema around the mouth and the eyes, puffiness around the eyes; 5, no activity after prodding, convulsion, and death.

Rectal temperature was measured before and 30 minutes after the i.p. challenge using a thermal probe (Anritsu meter CO., LTD, Tokyo, Japan).

Breathing rate (breaths per minute, bpm) was assessed in conscious unrestrained mice following evaluation of anaphylactic symptoms after the i.p. challenge using a barometric plethysmography method (EMKA Technologies, Paris, France).

Blood was collected 60 minutes after the i.p. challenge and plasma histamine concentrations were measured with an ELISA kit (Immunotech, Marseille, France) according to the manufacturer's instructions.

Spleens were harvested from mice allergic to CMP after challenge under sterile conditions. After lysis of red blood cells with buffer (Sigma) and several washes, splenocytes were resuspended in complete culture medium (RPMI-1640 plus 10 % fetal calf serum, 1 % penicillin/streptomycin and 1 % L-Glutamine). Cells were incubated in 24-well plates (4 x $10^6$ cells/mL) in the presence or absence of CMP (5 μg/mL) or Concanavalin A (2 μg/mL, positive control) for 72 h at 37°C (5 % CO2). Supernatants were then removed and stored at -

80°C until use. Levels of IL-4, IL-5 and IFN-γ were assayed using CytoSetsTM kits (BioSource International Europe, Nivelles, Belgium) according to the manufacturer's instructions. The limits of detection for IL-4, IL-5 and IFN-γ were < 5 pg/mL, 3 pg/mL and 1 pg/mL, respectively.

To determine the clinical reactivity threshold in CMP-sensitized and -challenged mice, i.e. the minimal dose of CMP leading to anaphylactic symptoms, mice sensitized with the optimal sensitizing dose of CMP as previously determined, as well as CT mice, were blind challenged intraperitoneally at day 44 either with 0, 0.01, 0.1, 1, 5 or 15 mg CMP per mouse.

Protein extracts of each of the margarines (A, B and C) were freshly prepared by treating 10 g melted margarine with di-isopropyl ether. Samples were mixed for 30 min at room temperature on a circular rotator (30 rpm) and then centrifuged 5 min at 2500 rpm. The aqueous phase was collected and organic solvent was evaporated by $N_2$(g). A negative internal control, i.e. PBS alone, was prepared similarly and simultaneously with the different samples of protein extracts. Mice sensitized to CMP, as well as CT mice were blind challenged by i.p. injection with 150 µL of protein extracts of either margarines A, B or C or with 150 µL PBS alone in order to determine for presence of CMP in margarines.

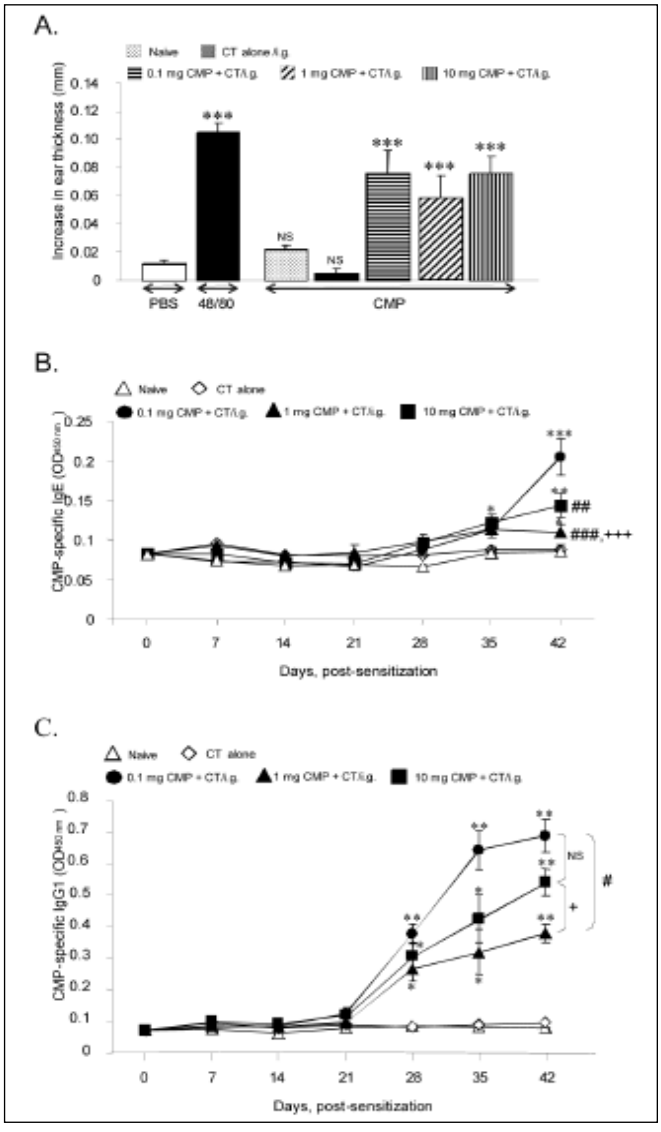
Margarine extracts were prepared as described in the previous paragraph. CMP content of these extracts was measured by sandwich ELISA using polyclonal antibodies directed against all CMP (Neogen, Ayr, Scotland).

Results are expressed as mean ± SEM. Statistical analyses were determined using Student's t test and one-way ANOVA. A p value < 0.05 was considered as statistically significant.

## Results

To characterize the relationship between the dose of CMP administered and sensitization status, we performed skin tests (ear swelling and intradermal skin tests) and monitored sera CMP-specific IgE. On day 42, significant increases in ear thickness in response to intradermal injection of CMP were observed with all sensitizing doses of CMP (Figure 1A). No increase in ear thickness was obtained in control mice (naive and CT alone). Similarly, positive skin responses with intradermal skin test were observed in all CMP-sensitized mice as compared to control mice (data not shown). Animals sensi-

***Figure 1*** - CMP-sensitization following oral exposure to CMP plus CT (A) Ear swelling response after CMP intradermal injection at day 42 post-sensitization. Forty minutes after intradermal injection of PBS, compound 48/80 or CMP (10 µL, 5 mg/mL), increase of ear thickness (mm) was measured in CMP-sensitized mice and control (naive and CT alone). Results are expressed as mean ± SEM of 6 mice per group. ***p < 0.001: CMP or compound 48/80 *versus* PBS treatment for each group. NS: non significant. Sera CMP-specific IgE (B) and IgG1 (C). Pooled sera from each group of mice (n=6 mice/group) as indicated were obtained weekly just before each boosting. CMP-IgE and IgG1 levels were assessed by ELISA. Results are expressed as mean ± SEM of 6 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001: CMP-sensitized *versus* naive or CT mice. #p < 0.05, ##p < 0.01, ###p < 0.001 *versus* 0.1 mg CMP + CT. +p<0.05, +++p < 0.001 *versus* 10 mg CMP + CT. NS: non significant



tized with 0.1, 1 and 10 mg CMP plus CT produced significant increase in CMP-specific IgE and IgG1 levels from 35 and 28 days, respectively, after the initial boosting in contrast to control mice (Figures 1B, 1C). On day 42, both 1 and 10 mg doses of CMP plus CT induced an increase in levels of CMP-specific IgE that were significant, but lower than that observed for 0.1 mg CMP plus CT (Figure 1B). CMP-specific IgG1 levels were significantly increased on day 42 at the dose of 1 mg CMP plus CT, but lower than that observed for 0.1 and 10 mg CMP plus CT (Figure 1C).

We next evaluated the anaphylactic reaction in CMP-sensitized mice at day 44 upon an i.p. challenge of 15 mg CMP per mouse. CMP-sensitized mice expressed severe anaphylactic symptoms reaching a clinical score of 4 to 5 irrespective of the sensitizing dose (Figure 2A). However, the dose of 10 mg CMP elicited a more consistent anaphylactic response in all CMP-sensitized mice as compared to those sensitized to lower doses of CMP. In contrast, control mice obtained a clinical score of 0 (Figure 2A). Measurement of changes in body temperature and breathing frequency were consistent with clinical score and provided an assessment of anaphylactic responses that was significantly more pronounced in mice sensitized with 10 mg CMP plus CT (Figures 2B and C). We therefore selected 10 mg CMP plus CT as the optimal sensitizing dose for BALB/c mice. This dose was used in the remainder of the study.

We next determined the production of Th1 and Th2 cytokines by spleen cells stimulated *in vitro* with CMP and collected from BALB/c mice (sensitized with 10 mg CMP plus CT) allergic to CMP. Seventy-two hours post-culture, Th2 cytokine production was significantly increased in CMP-stimulated cultures, 8 ± 0.6 pg/mL ($p<0.01$) and 140 ± 35.8 pg/mL ($p<0.001$) for IL-4 and IL-5, respectively, when compared to unstimulated cells (undetectable). In contrast, IFN-γ levels in CMP-stimulated and unstimulated spleen cells (35 ± 3.4 pg/mL and 31 ± 2.9 pg/mL, respectively) were essentially the same (non significant). IL-4, IL-5 and IFN-γ levels for concanavalin A were 28 ± 1.1 pg/mL, 547 ± 15.4 pg/mL and 695 ± 1.6 pg/mL, respectively.

At day 44, CMP-sensitized BALB/c were challenged intraperitoneally either with 0, 0.01, 0.1, 1, 5 or 15 mg CMP, respectively in order to determine the clinical reactivity threshold. CMP-sensitization status was also confirmed. Dose response curve of Figure 3A shows that a significant increase in anaphylactic clinical score was observed with 0.1 mg CMP challenge and reached a plateau with higher doses. Figure 3 also revealed that a dose of 1 mg CMP induced a maximal decrease in body temperature (Figure 3B) and in breathing frequency (Figure 3C). Although relatively less pronounced, both parameters remained significantly modified at higher

***Figure 2*** - Anaphylactic response depending on the amount of CMP used for oral sensitization CMP-sensitized (n = 6/group), naive (n = 6/group) and CT (n = 6/group) mice were challenged intraperitoneally with 15 mg CMP at day 44 post-sensitization. (A) anaphylactic symptoms, (B) change in body temperature and (C) change in breathing frequency were evaluated after i.p. challenge. Data are given as mean ± SEM. *p<0.05, **p < 0.01, ***p < 0.001: CMP-sensitized *versus* naive or CT mice after CMP challenge. #p < 0.05, ##p < 0.01 *versus* 10 mg CMP + CT. NS: non significant

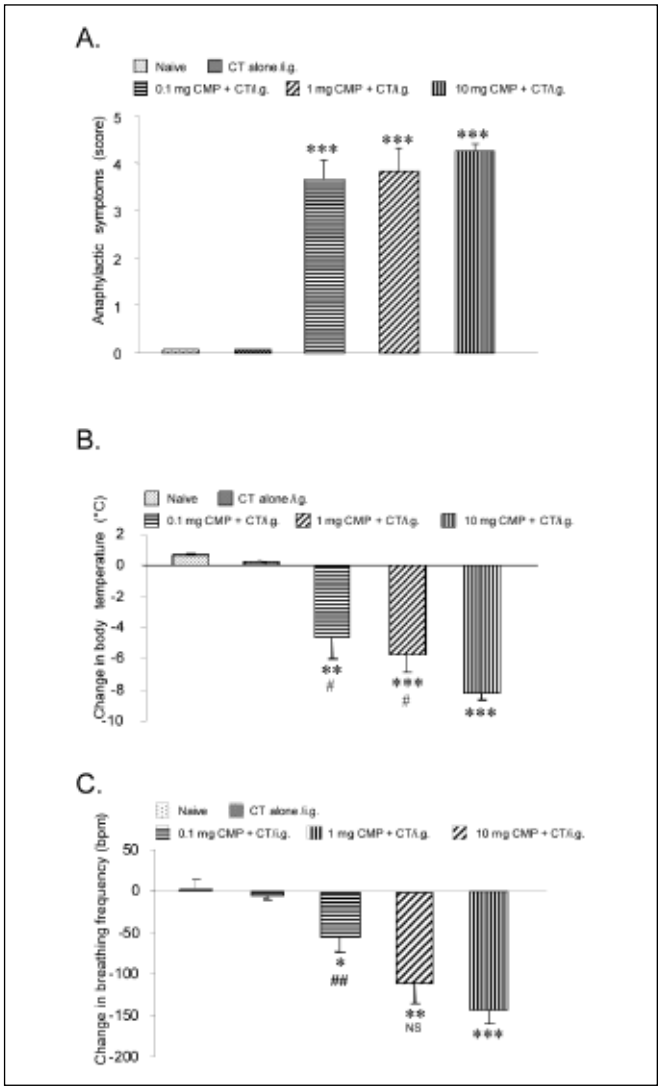
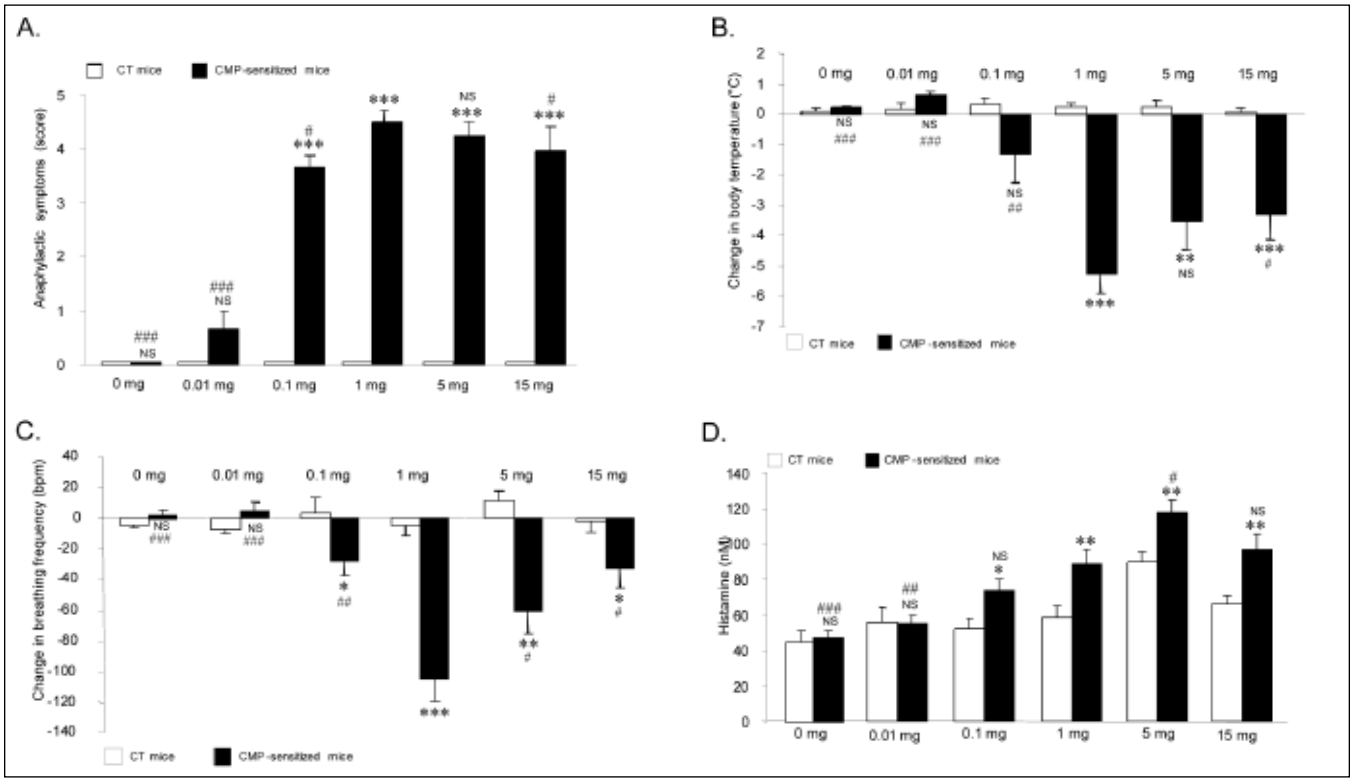


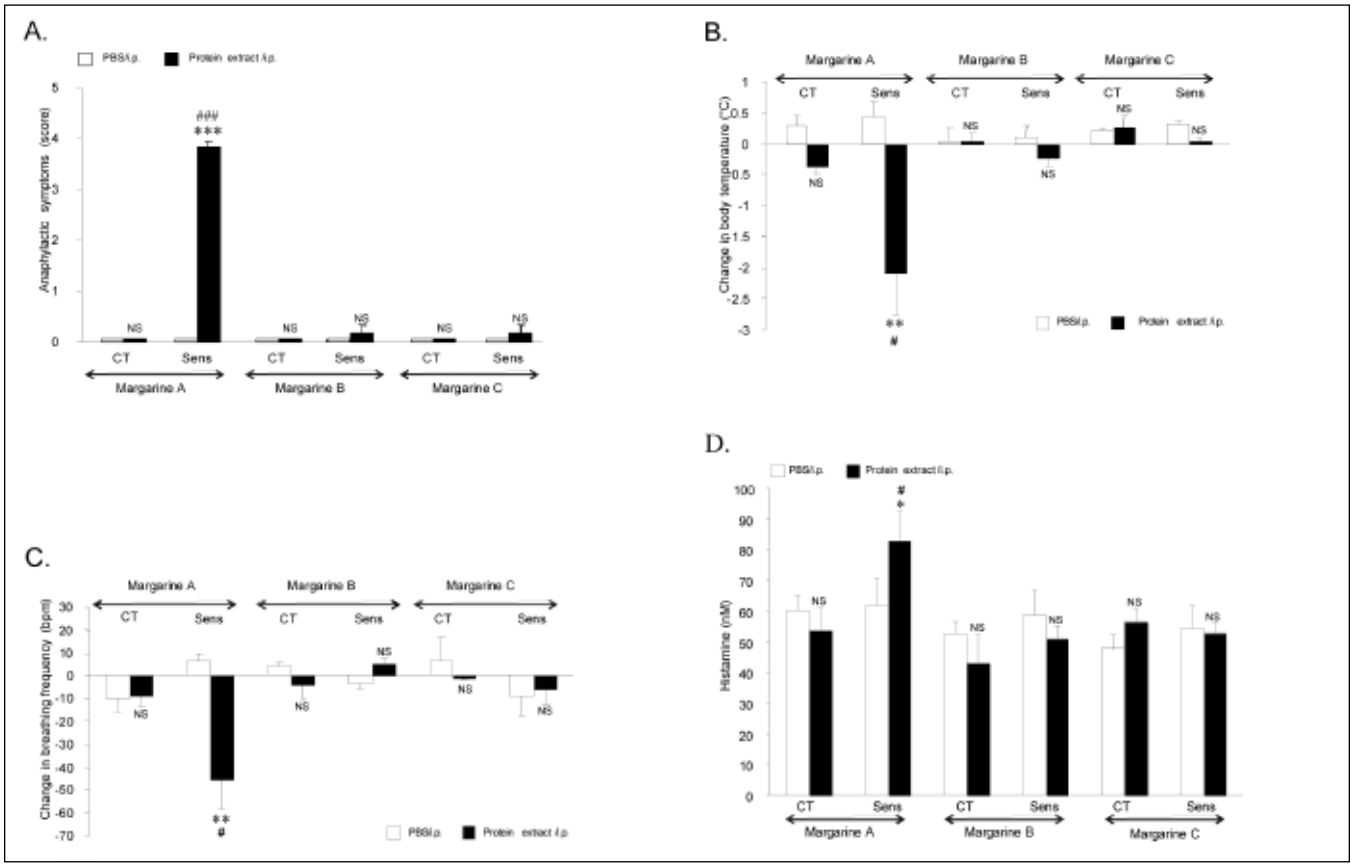
***Figure 3*** – Threshold of clinical reactivity to CMP in CMP-sensitized mice. Sensitized mice with 10 mg CMP plus CT (n = 6/group) and CT mice (n = 4/group) were challenged intraperitoneally either with 0, 0.01, 0.1, 1, 5 and 15 mg CMP per mouse at day 44 post-sensitization. The clinical reactivity threshold for CMP was determined by monitoring (A) anaphylactic symptoms, (B) change in body temperature, (C) change in breathing frequency and measuring (D) plasma histamine concentrations, after i.p. challenge. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001: CMP-sensitized *versus* CT mice after CMP challenge. #p < 0.05, ##p < 0.01, ###p < 0.001 *versus* 1 mg CMP. NS: non significant




challenge doses (5 and 15 mg CMP). Histamine release was significantly increased with a challenge dose of 1 mg but further increased with 5 mg CMP (Figure 3D). Therefore, the minimal dose of 0.1 mg CMP elicited clinically detectable allergic reaction. However, a dose of 1 mg was necessary to obtain objective measures of anaphylactic reaction.

BALB/c mice sensitized with 10 mg CMP plus CT leading to positive skin tests and significant increase of CMP-specific IgE in serum were blind challenged intraperitoneally at day 44 either with protein extracts from margarines (A, B, or C) or PBS. CT mice treated either with PBS or protein extracts of margarines did not develop anaphylactic reactions in terms of clinical symptoms, decrease in body temperature and in breathing frequency (Figures 4A, B and C). Among the 3 tested margarines in CMP-sensitized mice, the extract from margarine A led to anaphylaxis with a clinical score in a range of 3 to 4 associated with a statistically significant 1) drop in body temperature, 2) decrease in breathing frequency and 3) release of plasma histamine (Figure 4). Margarines B or C failed to induce any detectable anaphylactic reactions. These results indicated that only margarine A contained CMP in quantity sufficient to provoke an allergic reaction. We next estimated the concentration of proteins in the extracts of margarines A, B and C. Immunobiochemical analysis revealed that CMP concentrations of extracts of margarines A, C and B were 10.5 μg/μL, 0.0035 μg/μL and undetectable, respectively. Consequently, we estimated the quantity of CMP administered intraperitoneally per mouse in a final volume of 150 μL to be 1.6 mg and 525 ng per mouse for margarines A and C, respectively.

***Figure 4*** *- In vivo* assessment of allergenic activity of margarines. On day 44 post-sensitization, mice sensitized with 10 mg CMP plus CT and CT mice were challenged intraperitoneally either with protein extracts of margarines (A, B, C) (Sens n = 6, CT n = 4 per margarine) or with PBS (Sens n = 6, CT n = 4 per margarine) used as the negative internal control. (A) anaphylactic symptoms, (B) change in body temperature, (C) change in breathing frequency and (D) plasma histamine concentrations were evaluated after i.p. challenge. Data are given as mean ± SEM. *p<0.05, **p<0.01, ***p < 0.001: protein extract *versus* PBS challenge within each group. #p < 0.05, ###p < 0.001: CMP-sensitized *versus* CT mice after challenge with protein extract. NS: non significant




## Discussion

This is the first report of the application of animal model towards detection of the allergenic activity of hidden milk allergens extracted from food using a murine model of cow's milk-induced allergy. However, this is not the first description of CMP-induced anaphylaxis. Previous interesting and valuable models of CMP-induced allergy have been reported [12, 27], but none of them have determined the clinical reactivity threshold doses to CMP in order to define the lowest observed adverse effect level (LOAEL) and the no-observed adverse level (NOAEL). Similarly to the experimental approach reported in these studies, mice were sensitized using several oral exposures of milk plus CT. Increased CMP-specific IgE and IgG1 levels and positive skin tests to CMP demonstrated sensitization to CMP in BALB/c mice. In the context of providing a sensitive model for the detection of CMP, the i.p. route was used for allergenic challenge to elicit anaphylaxis [26, 28]. This route offers the advantage of minimizing variations of allergen bioavailability. Indeed, we demonstrated recently that this route was much more sensitive than the i.g. route in terms of anaphylactic response [28]. The determination of the threshold clinical reactivity to CMP is based on the assessment of anaphylactic reaction by monitoring the clinical symptoms and quantifiable parameters (body temperature, breathing frequency, histamine). In our model, the LOAEL was found to be 0.1 mg CMP. This dose was demonstrated to be favourable towards eliciting a detectable allergic reac-

tion including anaphylactic symptoms scored in a range of 3 to 4 associated with a significant decrease in breathing frequency and increased release of plasma histamine compared to 0.01 mg CMP. This latter dose that failed to lead to anaphylactic reaction corresponds to the threshold CMP dose below which no adverse effects occur and thus is defined as the NOAEL in our model [29]. As shown in our study, the 1 mg CMP dose was necessary to obtain objective measures of anaphylactic reaction including body temperature, breathing frequency and plasma histamine release. In case of 0.1 mg CMP dose, no global significant change in body temperature was observed suggesting that the temperature is related to the variability of response in mice. Indeed for this dose, a marked decrease in body temperature was only recorded in some individuals (n = 2 mice/6) indicating that a drop in body temperature is nevertheless a sign of disease severity [30-32]. According to our results, clinical tests were required to evaluate the allergenic activity of milk allergens in terms of anaphylaxis, because they clearly evidenced a severe sign of anaphylactic shock. Moreover, the combination of these clinical tests with a biologic assay such as the measurement of plasma histamine release is important in order to confirm the involvement of mast cells in CMP-specific anaphylaxis.

In practice, the reliability of our model as CMP detection tool was tested by assessing the allergenic activity of 3 different margarines A, B and C sharing the same production line, manufactured with or without milk. Indeed, only CMP-sensitized mice challenged with margarine A exhibited an anaphylactic reaction similar to that observed with CMP challenge, indicating the presence of CMP at levels sufficient to provoke anaphylaxis. On the other hand, no anaphylactic reaction was developed with margarines B and C, suggesting that either the finished margarines did not contain CMP, the concentration of CMP was below the limit of detection, or that the margarines contained proteins without allergenic activity. We then evaluated the levels of CMP in the margarine extracts that led to the appearance of anaphylactic symptoms in order to compare these clinical data to the murine LOAEL or NOAEL. This allowed us to evaluate the feasibility of this model as a tool for determining the allergenic risk of the analyzed margarines. The lack of allergenic activities of margarines B or C was supported by the immunobiochemical evaluation of the CMP amounts in the extracts of margarines. Indeed, in contrast to margarine A (1.6 mg CMP per mouse), under the same conditions, margarine C did not lead to anaphylaxis probably due to the fact that 525 ng CMP per mouse is largely below the LOAEL and the NOAEL, nor did margarine B due to the absence of any detectable protein. The LOAEL described in human varies between 0.6 and 180 mg CMP, whereas no NOAEL for milk has been reported [33]. Interesting and valuable existing *in vitro* assays are more sensitive for protein detection, but their major inconvenience is lack of information on the allergenic activity of food products in contrast to *in vivo* detection tools [16-21]. The application of our model to margarines confirms the fact that this murine model of CMP-induced anaphylaxis may be used as a tool to assess the safety of a finished food product for people with cow's milk allergy. Mice did not exhibit allergic reactions with 525 ng CMP in margarine C, which is 1150 times below the human LOAEL. The harmlessness of the margarine C is confirmed with a margin of safety (MOS) > 100, which is established from the mouse NOAEL [29]. Since a MOS > 100 is considered to be without risk for human subjects [29]. Thus, this margarine could be considered as being of no risk to CMP-allergic patients. CMP detected in margarine C are most likely contaminants resulting from the use of CMP on the same production line. On the other hand, the margarine A would be prohibited to patients allergic to CMP because of the wide overlap between the murine and human LOAEL values. The knowledge of a NOAEL for milk obtained from animal studies could provide the food industry with a much needed MOS to establish good manufacturing practices and allergenic risk control programs. This model could be used as a supplement to the biochemical tests in order to investigate a potential allergenic activity when a biochemical risk with respect to milk has been detected in a food product intended for allergic consumers before its marketing.

## Conclusions

We report here the development and characterization of a BALB/c model of CMP-induced anaphylaxis that represents a potential *in vivo* CMP detection tool for the safety assessment of finished food products such as margarines. Additional studies are required to determine the capacity of our model to evaluate the CMP allergenic activity of other finished food products, i.e. to analyze whether the food product contains specific CMP allergens at levels that could potentially induce an allergic reaction in sensitized individuals.

## Acknowledgments

The authors thank Pierre Verdellet, Sylvie Breton and Béatrice Lemois for their support of this study (Saint Hubert, France). None of the authors have any conflict of interest.

## References

1. Moneret-Vautrin DA, Morisset M, Cordebar V, Codreanu F, Kanny G. Probiotics may be unsafe in infants allergic to cow's milk. Allergy 2006; 61:507-8.
2. Spök A, Gaugitsch H, Laffer S, Pauli G, Saito H, Sampson HA, Sibanda E, Thomas W, van Hage M, Valenta R. Suggestions for the assessment of the allergenic potential of genetically modified organisms. Int Arch Allergy Immunol 2005; 137:167-80.
3. Dearman RJ, Stone S, Caddick HT, Basketter DA, Kimber I. Evaluation of protein allergenic potential in mice: dose-responses analyses. Clin Exp allergy 2003; 33:1586-94.
4. Kimber I, Stone S, Dearman RJ. Assessment of the inherent allergenic potential of proteins in mice. Environ Health Perpect 2003; 111:227-31.
5. Knippels LMJ and Penninks AH. Recent advances using rodent models for predicting human allergenicity. Toxicol Appl Pharmacol 2005; 207:157-60.
6. Foss N, Duranti M, Magni C, Frokiaer H. Assessment of lupin allergenicity in the cholera toxin model. Induction of IgE response depends on the intrinsic properties of the conglutins and matrix effects. Int Arch Allergy Immunol 2006; 141:141-6.
7. Gizzarelli F, Corinti S, Barletta B, Lacovacci P, Brunetto B, Butteroni C, Afferni C, Onori R, Miraglia M, Panzini G, Di Felice G, Tinghino R. Evaluation of allergenicity of genetically modified soybean protein extract in a murine model of oral allergen-specific sensitization. Clin Exp Allergy 2006; 36:238-48.
8. Dearman RJ and Kimber I. A mouse model for food allergy using intraperitoneal sensitization. Methods 2007; 41:91-8.
9. Knippels LMJ, Penninks AH, Spanhaak S, Houben GF. Oral sensitization to food proteins: a Brown Norway rat model. Clin Exp Allergy 1998; 28:368-75.
10. Knippels LMJ and Penninks AH. Assessment of the potential of food protein extracts and proteins on oral application using the Brown Norway rat model. Environ Health Perspect 2003; 111:233-8.
11. Knippels LMJ, Penninks AH, Smit JJ, Houben GF. Immune-mediated effects upon oral challenge of ovalbumin-sensitized Brown Norway rats: further characterization of a rat food allergy model. Toxicol Appl Pharmacol 1999; 156:161-9.
12. Li XM, Schofield BH, Huang CK, Kleiner GI, Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 1999; 103:206-14.
13. Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, Stanley JS, Burks AW, Bannon GA, Sampson HA. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol 2000; 106:150-8.
14. Helm RM, Furuta GT, Stanley JS, Ye J, Cockrell G, Connaughton C, Simpson P, Bannon GA, Burks AW. A neonatal swine model for peanut allergy. J Allergy Clin Immunol 2002; 109:136-42.
15. Teuber SS, del Wal G, Morigasaki S, Jung HR, Eisele P, Frick O, Buchanan BB. The atopic dog as a model of peanut and tree nut food allergy. J Allergy Clin Immunol 2002;110:921-7.
16. Poms RE, Klein CL, Anklam E. Methods for allergen analysis in food: a review. Food Addit Contam 2004; 21:1-31.
17. Poms RE, Anklam E, Kuhn M. Polymerase chain reaction techniques for food allergen detection. J AOAC Int 2004; 87:1391-7.
18. Stephan O, Vieths S. Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (Arachis hypogaea) in processed foods. J Food Agric Chem 2004; 52:3754-60.
19. Matsuda R, Yoshioka Y, Akiyama H, Aburatani K, Watanabe, Y, Matsumoto T, Morishita N, Sato H, Mishima T, Gamo R, Kihira Y, Maitani T. Interlaboratory evaluation of two enzymes-linked immunosorbent assays kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. J AOAC Int 2006; 89:1600-8.
20. Careri M, Elviri L, Mangia A, Mucchino C. ICP-MS as novel detection system for quantitative element-tagged immunoassay of hidden peanut allergens in foods. Anal Bioanal Chem 2007; 387:1851-4.
21. Koppelman SJ, Vlooswijk R, Bottger G, Van Duijn G, Van der Schaft P, Dekker J, Van Bergen H. Development of an enzyme-liked immunosorbent assay method to detect mustard protein in mustard seed oil. J Food Prot 2007; 70:179-83.
22. FAO/WHO. Evaluation of allergenicity of genetically modified Foods: Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods derived from Biotechnology. Food and Agriculture Organization of the United Nations (FAO), Rome, 2001.
23. Taylor SL. Protein allergenicity assessment of foods produced through agricultural biotechnology. Annu Rev Pharmacol Toxicol 2002; 42:99-112.
24. Sicherer SH, Sampson HA. Food allergy. J Allergy Clin Immunol 2006; 117:S470-5.
25. Lee TT, Morisset M, Astier C, Moneret-Vautrin DA, Cordebar V, Beaudouin E, Codreanu F, Bihain BE, Kanny G. Contamination of probiotic preparations with milk allergens can cause anaphylaxis in children with cow's milk allergy. J Allergy Clin Immunol 2007; 119:746-7.
26. Proust B, Astier C, Jacquenet S, Ogier V, Magueur E, Roitel O, Belcourt C, Morisset M, Moneret-Vautrin DA, Bihain BE, Kanny G. A single oral sensitization to peanut without adjuvant leads to anaphylaxis in mice. Int Arch Allergy Immunol 2008; 146:212-8.
27. Adel-Patient K, Bernard H, Ah-leung S, Créminon C, Wal JM. Peanut- and cow's milk-specific IgE, Th2 local and anaphylactic reaction are induced in Balb/c mice orally sensitized with cholera toxin. Allergy 2005; 60:658-64.
28. Poulsen OM, Hau J, Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. Clin Allergy 1987; 17:449-58.
29. Madsen C. Where are in risk assessment of food allergens? The regulatory view. Allergy 2001; 56:91-3.
30. von Garnier C, Astori M, Kettner A, Dufour N, Heusser C, Cor-

radin G, Spertini F. Allergen-derived long peptide immunotherapy down-regulates specific IgE response and protects from anaphylaxis. Eur J Immunol 2000; 30:1638-45.
31. Li XM, Zhang T.F, Huang CK, Srivastana K, Teper AA, Zhang L, Schofield B, Sampson HA: Food allergy herbal formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. J Allergy Clin Immunol 2001; 108:639-46.
32. Srivastana KD, Kattan JD, Zou ZM, Li JH, Zhang L, Wallenstein S, Goldfarb J, Sampson HA, Li XM: The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. J Allergy Clin Immunol 2005; 115:171-8.
33. Taylor SL, Hefle SL, Bindslev-Jensen C, Bock SA, Burks AW, Christe L, Hill DJ, Host A, Hourihane JO, Lack G, Metcalfe DD, Moneret-Vautrin DA, Vadas PA, Rance F, Skrypec, DJ, Trautman TA, Yman IM, Zeiger RS. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? J Allergy Clin Immunol 2002; 109:24-30.