Assessment of allergenicity of irradiated dairy products in a Balb/c mice model

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Abstract

The objective of the present in vivo study was to analyze the changes in the immune response of the sensitized Balb/c mice against milk allergens in lyophilized cow’s milk (LCM) and whey curd (LC) before and after gamma irradiation (10 kGy). The results showed lower levels of IgE in the group treated with irradiated LCM (ICM) compared to the group treated with non-irradiated LCM (NiCM). Hence, it could be suggested that gamma irradiation influenced the epitopes of the major milk proteins and was associated with lower allergenicity of the lyophilized irradiated milk. The gamma irradiation in the whey curd, however, did not significantly change the level of IgE antibodies in IC (treated with irradiated LC) compared to NiC (treated with non-irradiated LC) group.

Keywords

Cow’s milk hypersensitivity, murine model, irradiation, IgE, ELISA

Introduction

In modern societies food allergy is one of the main problems connected with food safety, currently attracting significant attention due to its increasing frequency and life-threatening potential. On average, it affects 12% of the total population, 8% of the children and 4% of adult people (Nieuwenhuizen and Lopata 2005; Platts-Mills 2015; Tordesillas et al. 2017). Food allergy is defined as an immune-related adverse reaction to food, when the immune system of the organism reacts against exposure (by inhalation, ingestion or skin contact) to harmless food protein with immunopathological process, recognizing it as a harmful one. This includes rapid IgE-mediated reactions, delayed non-IgE-mediated reactions and reactions, triggered by the involvement of both mechanisms (Yu et al. 2016; Tordesillas et al. 2017; Sicherer and Sampson 2018).

So far many food allergens have been identified, but these in milk, eggs, nuts, in particular peanuts, fish, soya, wheat and crustacean are responsible for 90% of the severe reactions (Schülke and Albrecht 2019). Mostly, they are water soluble glycoproteins (usually with molecular weight 10–60 kDa), which are resistant to heat, acids and enzymes, and have a variety of epitopes (sequential and conformational), recognized by specific IgE.

Cow’s milk allergy (CMA) is one of the most common food allergies in infants and children up to 3 years of age, with highest frequency during the first year of the life (Flom and Sicherer 2019). The prevalence of the CMA in Europe is defined as 0.7% of the total population (Schoemaker et al. 2015).

Cow’s milk contains more than 20 proteins which could cause allergic reactions. The main proteins in milk are caseins (80%, Bos d 8) and whey proteins (20%). The casein fraction could be further separated into 4 main proteins: a S₁-casein (Bos d 9), a S₂-casein (Bos d 10), β-casein (Bos d 11) and κ-casein (Bos d 12), while the whey contains β-lactoglobulin (BLG or Bos d 5), α-lact-
albumin (ALA or Bos d 4), immunoglobulins (Bos d 7), bovine serum albumin (BSA or Bos d 6) and traces of lactoferrin. Although each of these proteins has a potential to react as an allergen, the caseins, BLG and ALA are considered the most allergenic (Van Gramberg et al. 2013; Tordesillas et al. 2017).

Currently the mechanism of the allergy to cow’s milk protein (CMP) is not completely clarified. Generally, the allergies to milk are classified as IgE-mediated and non-IgE-mediated disorders, with IgE-mediated allergy being considered as potentially more risky one (Sampson and Anderson 2000). The IgE-mediated allergic reaction to CMP starts with an activation of Th2 lymphocytes, which induce formation of specific IgE antibodies from B lymphocytes after isotype switching. The circulated IgE antibodies are bound with mastocytes and basophils using high affinity receptor – FceR1. The second exposition to the same allergen leads to degranulation of mastocytes and basophils and release of histamine and other mediators, which cause allergic reaction of quick type. Subsequent clustering of eosinophils and basophils in tissues triggers late phase reaction. The released mediators lead to clinical manifestations of allergy (itching, hives, edema, gastrointestinal symptoms such as vomiting, diarrhea and abdominal pain, rhinitis, bronchospasm, laryngospasm, anaphylactic shock) (Liu et al. 2016; Sicherer and Sampson 2018; Schülke and Albrecht 2019).

Animal models in vivo are frequently used in food allergies research (Dearman and Kimber 2009; Van Gramberg et al. 2013; Arámburo-Galvez et al. 2018; Kanagara et al. 2013). They are a mandatory element in the preclinical studies, thus overcoming not only potentially risky and difficult manipulations, but also ethical limitations in clinical studies (Oyoshi et al. 2014). The approaches using experimental animals are characterized with high relevance and the data obtained from animal models could be extrapolated to man.

A large number of experimental models for investigation of immune and allergic reactions to milk proteins exist in literature (Li et al. 1999; Lara-Villoslada et al. 2004; Adel-Patien et al. 2005; Proust et al. 2009; Zhou et al. 2016). Due to their high reactivity and easy accessibility, the mice lines BALB/c, together with C3H/HeJ, C57/BL6 and DBA/2 are the most commonly used animals. In many of the models (together with allergens), adjuvants such as cholera toxin, staphylococcus enterotoxin B, ovalbumin and etc., are used, while sensibilisation is carried out mainly per os or intraperitoneally and rarely epicutaneously.

The development of hypoallergenic form of cow’s milk and dairy products is an area, attracting considerable interest from professionals involved in functional and special-purpose foods production. Over the years, different types of technological approaches have been applied to reduce protein allergenicity in cow’s milk (such as heat or enzyme treatment), without any conclusive result. There are no meta-analyses in the literature based on the randomized controlled studies, to provide convincing evidence of a lower risk of allergy when taking the currently existing partially hydrolyzed (pHF oligopeptides with molecular weight less than 5000 Da) or extensively hydrolyzed (eHF peptides with molecular weight less than 3000 Da) formulas.

To our knowledge, there are no data in the literature concerning the obtaining of a hypoallergenic form of lyophilized cow’s milk and whey curd by radiation with gamma rays. In this study skimmed cow’s milk and whey curd were subjected to irradiation with gamma rays (10 kGy) after lyophilisation which is innovative approach for reducing their allergenicity.

The aim of the work was to analyze the changes in the immune response of sensitized Balb/c mice to milk allergens in irradiated and non-irradiated products – cow’s milk and whey curd.

### Materials and methods

**Reagents:** Cholera toxin beta protein (CTB – Cholera toxin beta protein, NBP2-61449) was bought from Novus Biologicals, USA. Ready- to -use kit – Mouse Immunoglobulin E ELISA Kit, Cat. No E0449Mo from Bioassay Technology Laboratory was used for immunoglobulin assays. All chemicals applied in the experiments were of analytical grade and were obtained from Valerus, Bulgaria.

**Dairy products, lyophilization and gamma irradiation:** The skimmed cow’s milk (3.1% protein, 0.1% fat) and whey curd (13.2% protein, 0.1% fat) were bought from local supermarket. The products were lyophilized for 24 ± 1 h in „Hochvakuum-TG – 16.50“ (Germany) system at the following parameters: drying temperature (–40 °C), temperature of the desublimator (-60 °C), maximal working vacuum – 2 . 10⁻¹ Pa, temperature of complete drying +30 °C. Further the products were vacuum packed and kept at 10 ± 2 °C. Part of them were irradiated in National Center of Radiobiology and Radiation Protection (NCRRP) on gamma- irradiating installation – “NIGU-7”, with Cobalt 60 as a source of gamma-ray, with dosage rate – 2 kGy.h⁻¹. The applied dosage was 10 kGy. Just before use, the samples (irradiated and non-irradiated) were crushed and dissolved in 200/300μl PBS (pH 7.4, Ph Eur.7.0). The protein content in the lyophilized products was determined by kjeldahl method (ISO 8968:1:2014). The content of the protein was 33.58% and 43.58%, for the milk and curd respectively.

**Experimental animals:** The study was conducted with male Balb/c mice (n = 60) with an average weight 20 ± 2 g, bought from the vivarium of the Medical faculty of Medical University- Sofia. For the purpose of the experiment, the mice were kept in standard cages at temperature 22 ± 2 °C and humidity 55 ± 5%, on a 12h light/dark cycle under specific pathogen-free conditions. Drinking water and standard laboratory animal food pellets (TopMix-LTD, Bulgaria) were provided ad libitum. All the procedures on the animals were conducted in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” (Washington,
ton DC, National Academy Press). The experiment was approved by the Ethics Committee of the Bulgarian Food Safety Agency (Permit No. 261/ 09.12.2019).

**Experimental design and protocol**

After 2-week acclimatization period the mice (n = 60) were randomly separated in 6 groups (4 experimental and 2 control), each containing 10 animals. The experimental groups included ICM (irradiated lyophilized cow’s milk + CTB); NiCM (non-irradiated lyophilized cow’s milk + CTB); IC (irradiated lyophilized curd + CTB); NiC (non-irradiated lyophilized curd + CTB). The control groups were CTB (positive control, taking CTB in PBS) and C (control non-sensitized, taking only PBS).

The Balb/c mice were sensitized per os using a metal probe, as the milk proteins were applied together with CTB (0.02 μg/g in 200 μl PBS) five times at weekly intervals (Figure 1). The optimal sensitized dosage was determined based on initial research as an equivalent of 1 mg milk protein (MP) per gram body weight. After a period of six weeks, the mice were fasted over night, after that they were challenged by intraperitoneal (i.p.) administration of 30 mg MP/mouse in 300 μl PBS. The control groups were administered only with 300 μl PBS.

**Evaluation of anaphylactic symptoms**

In the period of sensitization (after 14-th day) and 45 minutes after the last treatment (i.p. challenge) the symptoms of anaphylaxis were evaluated, using a scoring system as described by Liu et al. (2016). The evaluation of the systemic anaphylaxis was scored as follows: 0 – no symptoms; 1– scratching and rubbing around the nose and head; 2 – edema around the eyes and mouth and pilar erection; 3 – wheezing and difficulty in breathing; cyanosis around the mouth and tail; 4 – no activity after pushing, tremor and convulsions; 5– death.

**ELISA analysis of total IgE in serum**

Blood was collected 45 minutes after challenge into chilled microtubes. After being kept at room temperature for about 30 minutes it was centrifuged (1500 rpm) for 20 minutes. The serum was separated and kept at -20 °C until analysis.

The serum concentrations of total IgE was estimated using a ready-to-use ELISA kit (Bioassay Technology Laboratory), following the instructions of the producer. Briefly, the standards (St₀–St₆, 0–64μg/ml in volume 50 μl) and samples (dissolving of serum 1:2 in PBS, pH 7.4, Ph Eur.7.0; in volume 40 μl) were dropped at the bottom of each well for standards and respectively for samples in 48-well plate in duplicates, followed by 50 μl biotin-conjugated mice IgE antibody. After adding 50 μl Streptavidin-HRP the plates were incubated at 37 °C for 60 minutes. After five times washing the wells with washing buffer and addition of substrate solutions, the plates were incubated for 10 minutes in dark at 37 °C. After terminating the reaction with stop-solution, the optical density at 450 nm was estimated on ELISA reader (RT-2100 C, Germany). The concentration of each IgE in μg/ml was determined through standard curve using a specific software (RIDA SOFT Win.net; Z9996, R-Biopharm AG, Germany).

**Statistical analysis**

The results were expressed as Mean ± SD. Statistical analyses were performed through one-way ANOVA on Minitab (Version 17), followed by Turkey’s post –hoc comparisons (p < 0.05).

**Results**

**Concentration of total IgE in serum**

The results for serum total IgE levels are presented on Figure 2.

![Figure 1.](image1.png)  
**Figure 1.** A model of food allergy in Balb/c mice with a period of sensitization, containing five time probe feeding with milk protein (MP) and adjuvant (CTB) at weekly intervals, and challenged after 6 weeks by intraperitoneally (i.p) applied MP.

![Figure 2.](image2.png)  
**Figure 2.** Serum levels of total IgE. Sera from different groups of mice (n = 6) were obtained 45 minutes after challenge. Different letters indicate statistically significant differences (mean ± SD, one-way ANOVA followed by Tukey test, p < 0.05).

Group C had significantly lower values of IgE antibodies compared to the CTB and all the experimental groups (p < 0.05). When comparing ICM and NiCM groups, it was found that the irradiation significantly decreased the level of IgE antibodies (p = 0.006), as in the ICM mice they were closer to those of the positive control CTB. Gamma irradiation of the curd did not significantly affect the level of IgE of IC in comparison to NiC group.
Evaluation of anaphylactic reactions

The analysis of anaphylaxis based on a scoring system, described above, is showed on Figure 3. The symptomatic changes followed not only 10–45 minutes after the challenge, but also in the period of sensitization after the 14-th day. There were cases of severe anaphylaxis in the third week of the experiment in mice from NiCM and CTB groups, evaluated as 5 on the standardized scale. After the challenge no cases of anaphylaxis were recorded.

Discussion

The animal mice models are widely used in evaluation of allergic and immune response to different food antigens. They are a suitable model, due to the possibility to use large experimental groups, the short reproduction cycle, as well as the relatively easy and cheap maintenance. Also they have physiological, genetic and immunological resemblance to man (Liu et al. 2016).

The present study was used as comparative analysis of the allergenic potential of milk proteins in lyophilized cow’s milk and whey curd before and after gamma irradiation. Balb/c line was selected as one of the most frequently used in immunological assays. Also, it has been described as IgE responder (Zhou et al. 2016).

The use of Th2 adjuvant (cholera toxin beta protein, CTB), dosages, regime and way of application of cow’s milk and whey curd were found to be effective for significantly elevated serum levels of the total IgE, demonstrating adequate immune response to allergens in cow’s milk and whey protein. The investigated serum samples (after sensitization and challenge) showed considerably increased levels of IgE in the experimental groups compared to the control, non-sensitized group C. It is known that IgE together with mastocytes and basophils are essential components of the allergenic infection. The antigen specific production and following fixation of IgE to FceR, receptors on mastocytes and basophils are extremely important for the beginning and spreading of the quick reactions of hypersensitivity. The elevated levels of total IgE could be observed not only at food allergies, but also in conditions such as atopic asthma, allergic rhinitis, atopic dermatitis, parasite infections, immunodeficiency illnesses (Hamilton 2010).

The concentration of total IgE in the positive control CTB was significantly higher than the control C, although the B-subunit of the CT that is responsible for weaker immunological response was applied. In many in vitro studies it was estimated, that CT could affect the production of cytokines by Th2 cells and thus upon isotype switching of B- cells to production of IgE. The same activity, but with lower efficiency was reported for CTB (Snider et al. 1994).

The reduced concentration of IgE in ICM vs. NiCM animals treated with lyophilized cow’s milk, before and after irradiation suggests that gamma irradiation (10 kGy) leads to changes in some of the epitopes of the protein molecule and they do not connect with paratopes in the mouse organism. Besides, the values displayed in ICM group are closer to those of the positive control CTB and it is difficult to discriminate between the influence of the milk allergens and those of the used adjuvant. We consider appropriate to reduce the dosage of the adjuvant in future work. No significant difference in the levels of the total IgE in serum samples of IC and NiC groups was detected. It could be suggested that there is no masking of the epitopes after irradiation with gamma-rays in the lyophilized whey curd.

No symptoms of systemic anaphylaxis after challenge by the used model of food allergy were observed. In contrast to our results, symptoms of systemic anaphylaxis in models of C3H/HeJ and Balb/c mice in groups with increased levels of investigated antibodies were reported (Li et al. 1999; Liu et al. 2016; Schülke and Albrecht 2019).

The results of the analysis showed, that the allergenic power of the investigated products could be defined in following sequence:

- Irradiated cow’s milk < irradiated curd < non-irradiated cow’s milk < non-irradiated curd

Hence, we consider that gamma irradiation (10 kGy) affects the epitopes of the main milk proteins and is associated with lower allergenicity of the irradiated products. Previously, Lee et al. (2001) reported structural change in the epitopes of the isolated and purified α-casein and BLG, caused by gamma irradiation.

Conclusion

The present in vivo model could be useful for determination of the allergenic potential and immune response of sensitized mice to proteins in dairy products, subjected to alternative technology for allergenicity reduction. The results showing low levels of IgE in ICM vs. NiCM groups give us reason to believe, that gamma irradiation affects the epitopes of the major milk proteins leading to lower allergenicity of the lyophilized irradiated cow’s milk. The gamma irradiation in the whey curd did not significantly change the level of IgE antibodies in IC, compared NiC.
mice. Future studies on specific IgE, IgG1, interleukins, histamine, will further contribute for the defining of a product as a hypoallergenic.

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