



Research paper

A shorter and more specific oral sensitization-based experimental model of food allergy in mice

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ABSTRACT

Cow's milk protein allergy (CMPA) is one of the most prevalent human food-borne allergies, particularly in children. Experimental animal models have become critical tools with which to perform research on new therapeutic approaches and on the molecular mechanisms involved. However, oral food allergen sensitization in mice requires several weeks and is usually associated with unspecific immune responses. To overcome these inconveniences, we have developed a new food allergy model that takes only two weeks while retaining the main characters of allergic response to food antigens.

The new model is characterized by oral sensitization of weaned Balb/c mice with 5 doses of purified cow's milk protein (CMP) plus cholera toxin (CT) for only two weeks and posterior challenge with an intraperitoneal administration of the allergen at the end of the sensitization period. In parallel, we studied a conventional protocol that lasts for seven weeks, and also the non-specific effects exerted by CT in both protocols.

The shorter protocol achieves a similar clinical score as the original food allergy model without macroscopically affecting gut morphology or physiology. Moreover, the shorter protocol caused an increased IL-4 production and a more selective antigen-specific IgG1 response. Finally, the extended CT administration during the sensitization period of the conventional protocol is responsible for the exacerbated immune response observed in that model.

Therefore, the new model presented here allows a reduction not only in experimental time but also in the number of animals required per experiment while maintaining the features of conventional allergy models. We propose that the new protocol reported will contribute to advancing allergy research.

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1. Introduction

Cow's milk protein allergy (CMPA) is one of the major causes of food hypersensitivity in children. Approximately 2 to

6% of infants exhibit cow's milk hypersensitivity in the first year of life (Sampson, 2004). CMPA is associated with a broad spectrum of IgE-mediated and non-IgE-mediated hypersensitivity disorders. The clinical features of allergy to cow's milk proteins (CMP) involving IgE-mediated reactions are expressed mostly as immediate symptoms, which may affect the skin (e.g., urticaria and eczema), respiratory tract (e.g., asthma and rhinoconjunctivitis), gastrointestinal tract (e.g., vomiting, diarrhea and colic) or systemic anaphylactic shock (Sampson, 1997; 2004). Although most patients outgrow CMPA by 3 years of age, patients with IgE-mediated hypersensitivity have a

Abbreviations: CMP, cow's milk protein; CMPA, cow's milk protein allergy; CT, cholera toxin; IP, intraperitoneal

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lower rate of outgrowing CMPA and higher susceptibility to develop allergic reactions to other foods than patients with non-IgE-mediated disorders.

The incidence of CMPA is rising in the western world (Bjorksten, 2001). This increase may not be an isolated phenomenon but may herald the beginning of an “atopic career”. Infants and children with CMPA may also develop additional atopic diseases such as other food allergies, rhinitis, asthma and early atopic dermatitis, the latter affecting up to 15–20% of children (Broberg et al., 2000; Oehling et al., 1998; Ozol and Mete, 2008; Skripak et al., 2007).

Significant advances in our understanding of the mechanism behind food allergy and its aetiology have been achieved from the study of animal models. These models allow not only experiments that are not feasible in humans but also the study of the molecular mechanisms involved in atopy and the development and testing of potential new treatments. For this purpose, several rodent models have been developed, these using distinct sensitization routes, such as intraperitoneal (Hsieh et al., 2003; Thang et al., 2011) or subcutaneous (Gonipeta et al., 2010; Strid and Strobel, 2005; Valeur et al., 2009) injection of allergen or genetically modified bacteria that express food antigens (Adel-Patient et al., 2005). However, these models have several limitations that significantly diminish their utility and they do not reflect the pathogenic mechanism that leads to food allergy, and thus could underestimate the involvement of the mucosal immune system. In this regard, the desired model should mimic human food allergy by provoking food hypersensitivity by oral ingestion. To accomplish this prerequisite, it is necessary to bypass the tendency of mice to develop oral tolerance and to ensure a Th2 response after antigen administration. For this reason, weaned Balb/c mice have commonly been used (Gonipeta et al., 2010; Lara-Villoslada et al., 2004, 2005; Thang et al., 2011) because this strain of mice is more susceptible to developing Th2 responses than other strains (Sun et al., 2001). Moreover, toxins such as cholera toxin (CT) are required to stimulate a Th2 response and the production of IgG1 antibodies (Marinaro et al., 1995; Snider et al., 1994). Recently, other bacterial toxins, such as staphylococcal enterotoxin B (Ganeshan et al., 2009) or approaches based on anti-acid treatment (Diesner et al., 2008; Untersmayr et al., 2010) have been described. Moreover, a desirable food allergy model should provoke systemic reactions with a specific antigen response that is not observable in some allergy models (Ito et al., 1997).

Despite the variety of models currently available, they are flawed by two main common drawbacks. First, they require long periods of sensitization, which extend for 6 to 8 weeks to obtain a sustained and measurable systemic immune response. And second, the adjuvants of bacterial origin or anti-acid treatments used to ensure disruption of the tolerogenic potential of the oral route for such a long period could mislead the results obtained as a result of the immune-stimulatory properties of adjuvants and also the lack of specificity of the approach, which allows the generation of allergic response to other food components of the diet not related to the antigen of interest.

With the goal to solve these two main drawbacks of current allergy models, here we present a shorter murine allergy model consisting of only 2 weeks of oral sensitization but requiring

intraperitoneal challenge to achieve a consistent immune response and compare it with a conventional model of milk allergy consisting of 6 weeks of oral sensitization, which has previously been described (Lara-Villoslada et al., 2004, 2005; Li et al., 1999). Both models used CT as adjuvant, but the immune stimulatory effects induced by this bacterial toxin were abolished in the shorter protocol, thereby allowing a more selective and specific response than that observed in the conventional model.

2. Materials and methods

2.1. Reagents and preparation of CMP

CT was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for ELISA were purchased from Bethyl Laboratories (Montgomery, TX) for immunoglobulins (Igs) or from Biosource (Camarillo, CA) for cytokines. All other products were of the highest grade available and were purchased from Sigma Chemical Co.

To obtain CMP, homogenized fat free cow's milk was obtained from Puleva Food SA (Granada, Spain) and centrifuged at 5000×g for 10 min at 4 °C and the upper layer of fat was discarded to obtain skimmed milk, which was stored at –20 °C in 1-ml aliquots and defrosted immediately before use. Protein content in cow's milk was measured by the Kjeldahl method, as previously described (Lara-Villoslada et al., 2004).

2.2. Animals

Female Balb/c mice (3-wk-old, immediately after weaning) were purchased from Harlan (Barcelona, Spain) and housed under a temperature (22 °C) and light-controlled (12 h) cycle. Animals ($n = 10$ per group) were maintained on a plant-based chow diet (Harlan Laboratories, Indianapolis, IN) under specific-pathogen-free conditions. These studies were carried out following the European Union Directive (86/609/EEC) for the protection of vertebrates used for experimental and other scientific purposes, in compliance with the Helsinki Declaration.

2.3. Sensitization and challenge

2.3.1. Original protocol

Mice were sensitized intragastrically with CMP (1 mg of CMP per gram of body weight) plus CT (0.3 µg/g) as an adjuvant in a total volume of 200 µL of PBS, and boosted 7 times at weekly intervals (Fig. 1A). Oral gavage was performed using a polyvinyl chloride tube-feeding needle purchased from Vygon (Ecoue, France). To analyze the effect of the adjuvant *per se*, a group of mice received the same dosage of CT in PBS (CT 6w control). Moreover, a control of non-sensitized animals (sensitized with only PBS and challenged with PBS and/or CMP) was also used (non-sensitized). Seven weeks after the first oral gavage, all groups were fasted overnight and challenged intragastrically with a high dose of CMP (35 mg/mouse).

2.3.2. Shorter protocol

Mice were also sensitized intragastrically with CMP plus CT at the same doses but boosted only 5 times at three to four

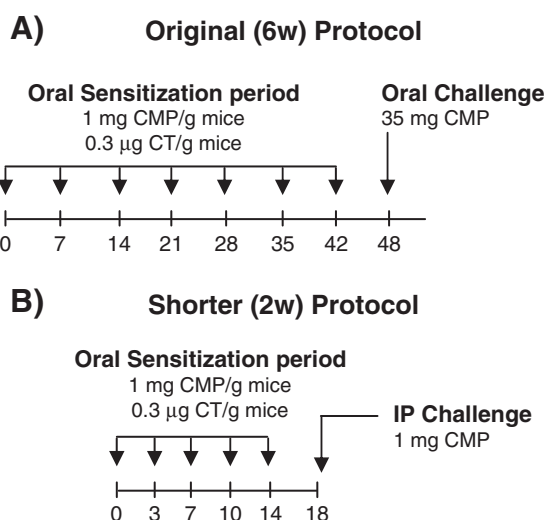


Fig. 1. Protocol diagram. A) Original protocol adapted from Li et al., 1999, and consisting of oral sensitization and challenge. B) Shorter protocol, comprising oral sensitization but intraperitoneal challenge.

day intervals (Fig. 1B). Equivalent control groups as in the original protocol were also used. Eighteen days after the first oral gavage, mice were challenged intraperitoneally with a 1 mg dose of CMP (100 µl).

In both protocols, 30 min after the last challenge, mice were killed by IP administration of sodium pentothal (50 mg/kg), and blood was collected by cardiac puncture in tubes containing EDTA. Spleen and intestine were removed from each mouse and weighed.

2.4. Evaluation of symptoms

Immediately after challenge, hypersensitivity responses were evaluated using a scoring system modified slightly from previous reports (Lara-Villoslada et al., 2004, 2005; Li et al., 1999) and scored as shown in Table 1. An extra half point for each score was obtained when the symptom became visible at 10 min or less after the challenge. Symptom evaluation

Table 1

Score system to evaluate systemic hypersensitivity response (modified from Li et al., 1999).

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the nose and head
2	Puffiness around the eyes and mouth and pilar erecti
3	Decreased activity with increased respiratory rate
4	Wheezing and labored respiration
5	Cyanosis around the mouth and the tail
6	Death

Symptoms were evaluated by two independent researchers who were blinded to the study treatments. An extra half-point for each score was assigned to mice when symptoms appeared in the first 10 min after the CMP challenge.

was performed by 2 independent researchers who were blinded to the study treatments.

Gastrointestinal symptoms were evaluated as diarrhea and small intestine and colon inflammation, as previously described (Bailón et al., 2011). Animals that suffered from diarrhea on at least 2 consecutive occasions were considered positive cases. To evaluate intestinal inflammation, intestinal samples were placed on ice-cold plates, cleaned of fat and mesentery, and then blotted on filter paper. Each small intestine or colon specimen was weighed and the length was measured under a constant load (2 g).

2.5. Determination of histamine and cytokine levels

Blood was centrifuged (3500×g) for 10 min at 4 °C, and plasma aliquots were collected and frozen at −80 °C. Colon homogenates were obtained as previously described (Garrido-Mesa et al., 2011).

Plasma histamine levels were determined using an enzyme immunoassay kit (IBL Laboratories, Hamburg, Germany) following the manufacturer recommendations. IL-4 and Ig levels were measured in plasma and colon homogenates by ELISA, following the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). The dilutions performed were 1:10,000 for the determination of total IgG1, IgG2a and IgA in plasma and at 1:2 for IgE. In the colon determinations, dilutions used were 1:1000 (IgG1), 1:500 (IgG2a), 1:5000 (IgA) or no dilution for IgE determination.

Levels of specific IgG1 to CMP were measured as previously described (Li et al., 1999) in non-diluted plasma. Briefly, 96-well plates were coated with 20 µg/mL of CMP in coating buffer (0.5 M Na₂CO₃). After overnight incubation at 4 °C, plates were washed 3 times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20) and blocked with a solution (50 mM Tris, 0.14 M NaCl, 1% BSA). Plasma samples were then added to the plates and incubated for 1 h at room temperature (25 °C). Plates were washed 3 times, and 100 µL of goat antimouse IgG1 antibody conjugated with peroxidase (Bethyl Laboratories) was added for 1 h at 25 °C. Staining was performed with 3,3'-5,5'-tetramethyl-benzidine (TMB) (Sigma Chemical Co.) for 30 min at 25 °C in the dark, stopped with 0.1 N H₂SO₄, and plates were then read at 450 nm. All analyses were performed in triplicate.

2.6. Statistical analysis

Statistical significance was calculated by the Student's *t*-test in the case of parametric parameters, such as the ELISA results. All tests were performed with one tail following the 2-sample equal variance model (homoscedastic). For non-parametric parameters (score and number of mice with diarrhea), the Mann-Whitney *U*-test and Fisher's exact test were used to determine statistical significance. In all cases data are represented as the mean ± SEM and figures correspond to representative data from one of at least three independent experiments.

3. Results

Two CMPA protocols were tested in this study with the objective to evaluate the non-selective effect of the adjuvant

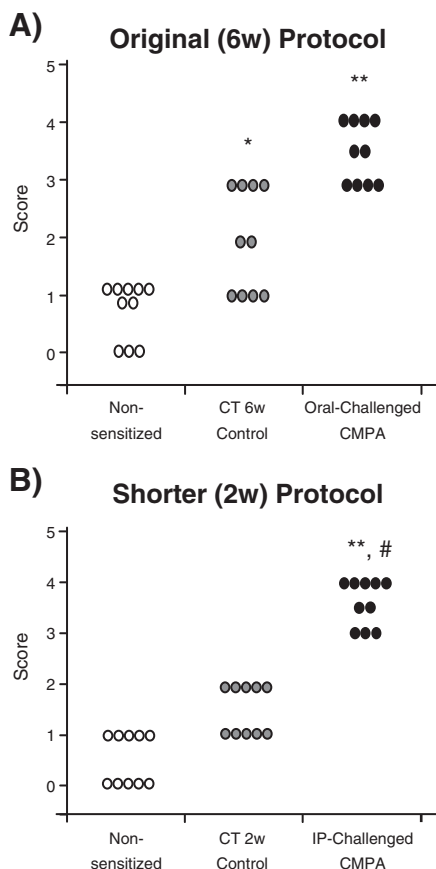


Fig. 2. Score of hypersensitivity symptoms. Mice ($n = 10$ per group) were challenged with cow's milk. During the first 30 min, the symptoms of hypersensitivity were scored on a scale from 0 (no symptoms) to 6 (death) as described in Table 1. Black circles indicate individual mice from one independent and representative experiment. A) Original protocol, where non-sensitized animals correspond to animals orally challenged with CMPA; CT 6w Control, correspond to those receiving only CT during sensitization (7 doses in weekly intervals during 6 weeks) and orally challenged with CMPA; while oral-challenged CMPA animals correspond to those receiving CMP and CT during oral sensitization (6 weeks) and orally challenged with CMP, as indicated in the Materials and methods section. B) Shorter protocol, where non-sensitized animals correspond to those only IP challenged with CMP; CT 2w Control correspond to animals receiving only CT during sensitization (5 doses in 2 weeks) and IP challenged with CMP; and finally, IP-challenged CMPA corresponds to animals orally sensitized with CT and CMP during 2 weeks and IP challenged with CMP. Mann–Whitney U -test was performed to determine statistical significance. * ($p < 0.05$), ** ($p < 0.01$) compared with non-sensitized animals; # ($p < 0.05$), compared with CT control.

(CT) and the effect of the length of sensitization. The two protocols used are summarized in Fig. 1. Although the shorter protocol consists of two fewer sensitizing doses containing CT, the main difference between them is that in the shorter one the 5 sensitizing doses are administered in only two weeks while 6 weeks are required in the original protocol. Nevertheless, to obtain a measurable response, the shorter protocol requires that the challenge be made by IP administration since no allergic response was obtained after oral challenge (data not shown).

In order to examine the non-selectivity of CT effects, the study included three types of control groups per protocol. However, no differences were observed between the control group of animals in which both sensitization and challenge were performed only with PBS and with the second control group corresponding to animals sensitized with only PBS but challenged with CMP. For this reason, the data corresponding to these two non-sensitized groups were analyzed together (as a unique group) and referred as “non-sensitized” group in order to simplify the understanding of the work. The third control performed corresponds to the adjuvant treatment, where a group of mice were sensitized only with CT in the absence of CMP during 6 weeks (CT 6w Control) or 2 weeks (CT 2w Control) and challenged with CMP. All these groups were compared with the oral- and IP-Challenged CMPA groups.

Hypersensitivity symptoms became evident in most cases within 15 to 30 min after challenge. The severity of the reaction was scored as indicated in Table 1 and shown in Fig. 2. As expected, no major allergic symptoms were observed in non-sensitized animals. The most severe allergic reactions were observed in both protocols (oral and IP) of CMPA-challenged mice (Fig. 2). However, although no significant differences on the allergy symptoms in CMPA-challenged animals were observed between both protocols, a significant difference from each particular Control group was only observable in the shorter protocol, since the CT 2w Control animals showed a more moderate symptomatology than CT 6w Controls.

Moreover, the number of animals with diarrhea was higher in the original protocol, especially in oral-challenged CMPA animals but clearly observable also in CT 6w Control animals (Table 2). Nevertheless, diarrhea in the two protocols was never as severe as to cause a significant decrease in body weight or in food intake (data not shown). Thirty minutes after the challenge, mice were killed under anesthesia and the intestine of each mouse was removed and weighed. In concordance with the incidence of diarrhea, alterations in gut morphology, consisting of modifications in

Table 2
Intestinal and immunological macroscopic symptoms of atopy.

	Original (6w) protocol			Shorter (2w) protocol	
	Non-sensitized	CT 6w Control	Oral-challenged CMPA	CT 2w Control	IP-challenged CMPA
Diarrhea	0/10	4/10 *	7/10 **, #	1/10	2/10 ††
Small intestine (weight/length) (mg/cm)	17.66 ± 1.67	14.88 ± 1.08 *	20.88 ± 1.71 #	16.33 ± 1.53	17.96 ± 1.83
Colon (weight/length) (mg/cm)	19.08 ± 1.81	26.32 ± 0.92 **	27.78 ± 2.19 **	18.28 ± 1.34 ††	17.18 ± 1.96 ††
Spleen (mg/g mice)	3.94 ± 0.42	4.65 ± 0.08 *	5.21 ± 0.1 **, #	4.86 ± 0.17 *	5.97 ± 0.4 **, #

Values represent the mean ± SEM of a representative experiment. Animals that suffered from diarrhea on at least 2 consecutive occasions were considered positive cases. Fisher's exact test was used for the statistical analysis of diarrhea. All the other parameters were evaluated using Student's t -test with one tail following the 2-sample equal variance model. * ($p < 0.05$), ** ($p < 0.01$) compared with non-sensitized animals; # ($p < 0.05$), ## ($p < 0.01$) compared with each particular CT control; † ($p < 0.05$), †† ($p < 0.01$) compared with similar group (CMPA or CT control) between both protocols.

the weight/length ratio of small or large intestine was found only in groups using the original protocol. Again, similar values than those observed in non-sensitized animals were obtained in CT 2w Control animals and in the IP-challenged CMPA group (Table 2). Taken together, these results suggest that CT administration for 6 weeks, but not for 2, has an impact on gut physiology and this effect is not exacerbated by the presence of CMP during the sensitization period.

The allergy process with systemic effects was confirmed not only for the symptoms observed in whole animals but by the increased size of the spleen (Table 2). In this case, although sensitization with CT alone enlarged the spleen in both protocols, a significant exacerbation was observed in CMPA groups of both protocols, thereby demonstrating that the addition of the target protein during sensitization increased the immune response observed after challenge with the same protein.

These results were further supported by histamine and IL-4 levels (Fig. 3). In this regard, a significant increase in plasma histamine was observed in CMPA groups of the two protocols in comparison with non-sensitized and CT Control groups. Nevertheless, the amounts of histamine found in groups belonging to the original protocol were significantly higher than those obtained in the shorter protocol. This difference was due to the effect of CT treatment, since no differences in plasma histamine levels were observed between non-sensitized animals and CT 2w Controls, while a four-fold increase was detected in the CT 6w Control group (Fig. 3A). Similarly, in the CT groups, plasma and colonic IL-4 levels were increased only in the CT 6w Controls in comparison with non-sensitized animals. However, in this case, the levels of IL-4 were exacerbated only in the IP-challenged CMPA group and no further increase was detected in the oral-challenged CMPA group (Fig. 3B–C).

Allergy response was further characterized by measuring Ig production in plasma and colon. Although CMPA is an IgE-mediated hypersensitivity; an increase in IgE response is not easily observable in mouse allergy models while an increase in IgG1 is more frequently detected (Lara-Villoslada et al., 2004, 2005; Thang et al., 2011). Despite this consideration, total IgG1 and IgE levels increased in the oral-challenged CMPA group but not in the IP-challenged CMPA one (Fig. 4A–B). However, when the CMP-specific IgG1 response was analyzed, a similar increased response was detected in the two CMPA groups, independently of the protocol used (Fig. 4C). This observation demonstrates that a specific Ig response was induced in both allergy models and suggests that the immune response detected in the shorter protocol is more antigen-specific than that induced by the original protocol. Regarding other plasma Ig levels, the amount of IgG2a (more indicative of a Th1 response) was maintained or slightly increased in the original protocol but reduced in the IP-challenged CMPA group (Fig. 4D). In contrast, an increase in IgA production was observed only in plasma samples from the oral-challenged CMPA mice (Fig. 4E).

Regarding colon Ig levels, the two groups of the original protocol showed an increased production of IgE and IgG1 compared with non-sensitized animals while only an increase in IgG1 was observed in groups subjected to the shorter protocol. No difference between CT Control group and the CMPA group was observed in any protocol

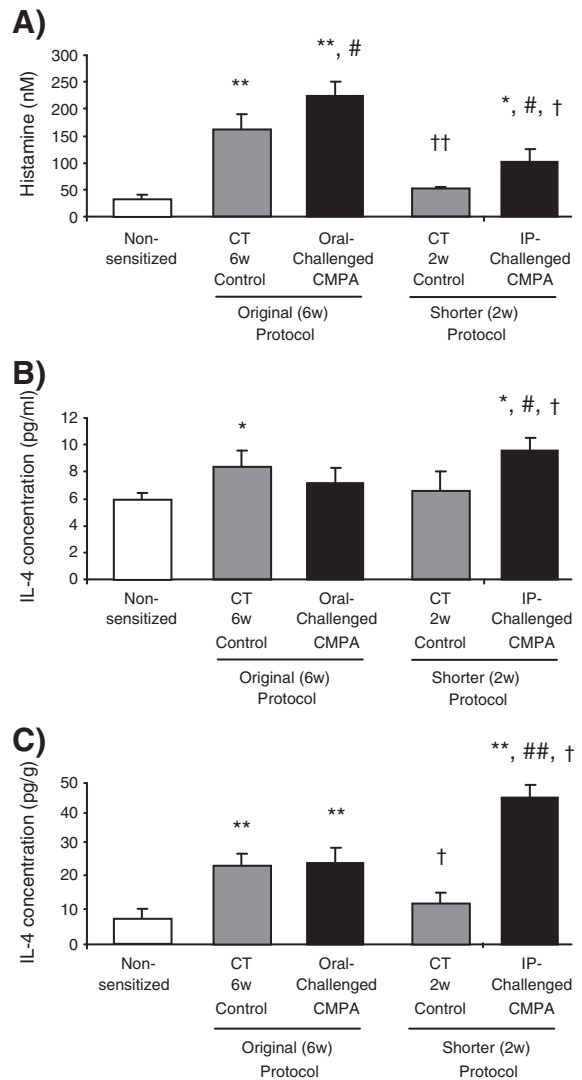


Fig. 3. Histamine and IL-4 levels. Plasma and colon homogenates from all mice ($n = 10$ per CT or CMPA groups of each protocol, and 20 animals in non-sensitized group) were collected after sacrifice. A) Histamine concentration was determined by enzyme immunoassay kit and represented as plasma concentration (nM). IL-4 concentration measured by ELISA in plasma (B) or colon homogenates (C) was also measured in samples from all animals. All results are expressed as the median $\pm$ SEM. Student's *t*-test was used to determine statistical significance. * ($p < 0.05$), ** ($p < 0.01$) compared with non-sensitized animals; # ($p < 0.05$), ## ($p < 0.01$) compared with each particular CT control; † ($p < 0.05$), †† ($p < 0.01$) compared with similar group (CMPA or CT control) between both protocols.

(Fig. 5A–B). In contrast to observations in plasma, colon IgG2a was increased in the CT 6w Control group and further exacerbated in the oral-challenged CMPA animals, while no differences were observed in groups sensitized for only two weeks (Fig. 5C). Similarly, colonic IgA was increased in the oral-challenged CMPA group but decreased in IP-challenged CMPA mice (Fig. 5D). This observation is consistent with the macroscopic manifestations of the gut previously described (Table 2).

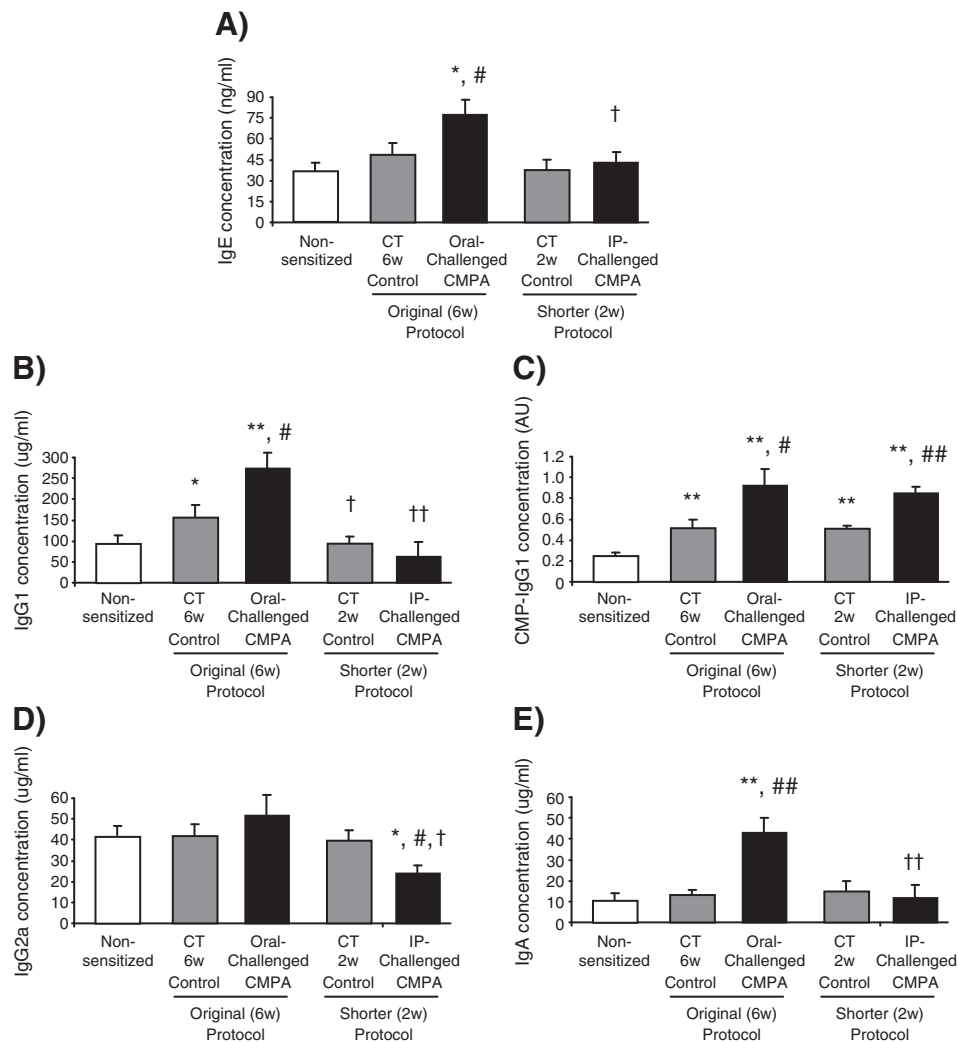


Fig. 4. Plasma Ig levels. Plasma from all mice was collected after sacrifice and used to measure IgE (A), IgG1 (B) CMP-specific IgG1 (C), IgG2a (D) and IgA (E) by ELISA as described in the [Materials and methods](#) section. Results are expressed as the mean plasma concentration $\pm$ SEM except for C) where CMP-specific IgG1 levels were represented as Absorbance units (AU) $\pm$ SEM. Student's *t*-test was used to determine statistical significance. * ($p < 0.05$), ** ($p < 0.01$) compared with non-sensitized animals; # ($p < 0.05$), ## ($p < 0.01$) compared with each particular CT control; † ($p < 0.05$), †† ($p < 0.01$) compared with similar group (CMPA or CT control) between both protocols.

4. Discussion

Research into human pathology has been widely supported by laboratory data thanks to the use of diverse experimental animal models. Mouse and rat models have been used satisfactorily in studies of various allergic diseases, such as asthma (Bates et al., 2009; Kucharewicz et al., 2008; Stevenson and Birrell, 2011). However, no fully acceptable mouse model of CMPA or other food allergies has been established. As a result, studies use distinct animal models, thereby hindering the comparison of results. An experimental model of food allergy should be characterized by the robustness and reproducibility of the symptoms, the selectivity and antigen-specificity of the response, the involvement of the mucosal immune system but with systemic dispersion of the effects, and the easiness of the protocol, both regarding time and resources.

Several models of food allergy have been described, these differing in the route of sensitization and challenge, the strain of mouse used, the use of adjuvant or co-administered drugs, the type of antigen or the doses required and the duration of the sensitization period (Diesner et al., 2008; Ganeshan et al., 2009; Gonipeta et al., 2010; Hsieh et al., 2003; Strid and Strobel, 2005; Thang et al., 2011; Untersmayr et al., 2010; Valeur et al., 2009). However, for all these models the most significant obstacle to oral sensitization is the strong innate tendency to develop oral tolerance to ingested antigens. Consequently, long sensitization periods and the introduction of adjuvants such as CT are common practice (Lara-Villoslada et al., 2004, 2005; Li et al., 1999; Schouten et al., 2009).

Here we tested the immune consequences of this sensitization approach and compared it with the allergy response obtained with a novel shorter CMPA protocol, requiring only two weeks of sensitization. Our results suggest

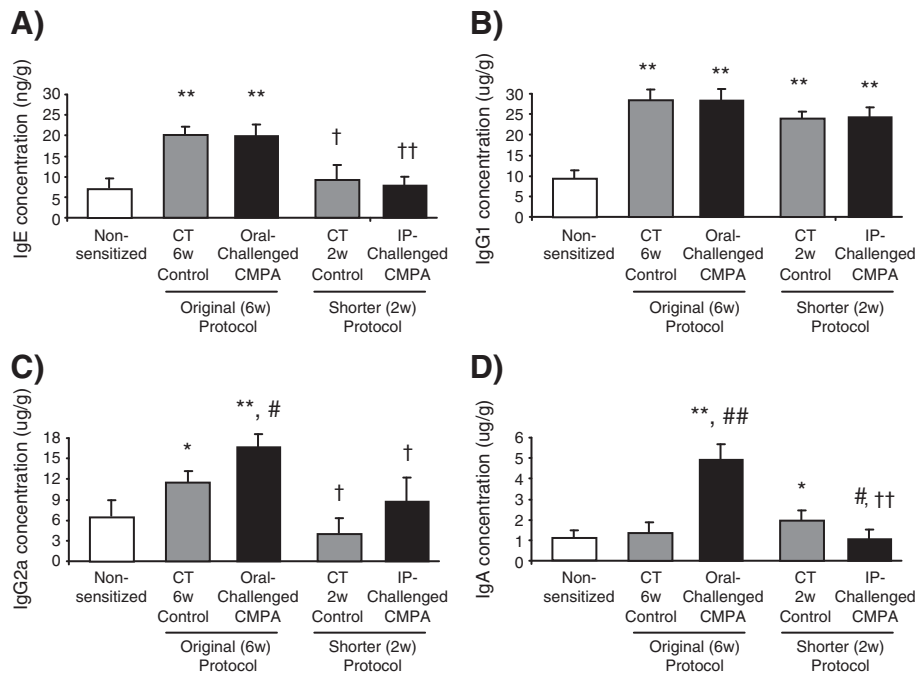


Fig. 5. Igs levels in colonic samples. Colon homogenates obtained from all mice were obtained after sacrifice and used to measure IgE (A), IgG1 (B), IgG2a (C) and IgA (D) by ELISA as described in the [Materials and methods](#) section. Results are expressed as the mean colonic concentration per gram of colon specimen $\pm$ SEM. Student's *t*-test was used to determine statistical significance. * ($p < 0.05$), ** ($p < 0.01$) compared with non-sensitized animals; # ($p < 0.05$), ## ($p < 0.01$) compared with each particular CT control; † ($p < 0.05$), †† ($p < 0.01$) compared with similar group (CMPA or CT control) between the two protocols.

that the long-term use of CT rather than its dosage seems to be responsible for the unselective immune response induced by this adjuvant. In this regard, weekly administration of CT for 6 weeks without administration of CMP during sensitization led to a significant increase in several allergy response markers, such as diarrhea, colon edema, spleen enlargement, histamine levels, plasma and colon IL-4, plasma and colonic IgG1 or colonic IgE or IgG2a. Although CT stimulates a Th2 response and the production of IgG1 antibodies (reviewed by [Berin and Shreffler, 2008](#)), the mechanisms through which this adjuvant promotes immune responses remain controversial ([Cox et al., 2006](#)). Indeed, allergy-suppressing rather than stimulating effects have been described in mice through the induction of secretory IgA ([Smits et al., 2009](#)) or Th1-associated IgG2a responses ([Grdic et al., 1999](#); [Kroghsbo et al., 2003](#); [Snider et al., 1994](#)). Although CT treatment clearly increased IgG2a levels in colon, in no case was this related to a suppressive effect on CMPA, an effect previously observed in respiratory mucosa ([Smits et al., 2009](#)).

The reduced effect on the same parameters induced by 5 doses of CT administered in only 2 weeks (CT 2w Control in shorter protocol) suggests that the immune-enhancing effects of CT are not direct but related to the induction of an allergic response to other food antigens present in the diet, a process that would probably require more than 2 weeks of treatment to become evident. In this regard, oral challenge with CMP after 2 weeks of sensitization was not sufficient to induce an allergic response (unpublished data) and at least 5 weeks was required ([Lara-Villoslada et al., 2004, 2005](#)). Unfortunately, no samples were obtained to evaluate the

immune status before CMP challenge nor was a third control group consisting of CT sensitization without challenge included in this study. Consequently, a potential effect of the oral challenge cannot be discarded on the final response observed in CT Control groups despite the fact that no effect was observed in non-sensitized animals. The reason we cannot discard this possibility is that oral challenge was performed using a considerable amount of protein and volume of administration that could induce mucosal distension (as observed in small intestine specimens from original protocol samples), thereby facilitating the extravasation of intact food-borne proteins in the gut that are not related to CMP. Moreover, the high amount of CMP during challenge could induce cross-reaction with other food antigens. In this regard, cross-reactions between caseins and soy-derived proteins have been reported in allergic individuals ([Chandra et al., 1989](#); [Hill et al., 1984](#); [Perkio et al., 1981](#)). This cross-reactivity could explain the presence of a higher plasma level of CMP-specific IgG1 antibodies in 6w CT Control animals after challenge despite the fact that these mice did not ingest this antigen through diet.

The strong immune response observed in CT 6w Control animals does not preclude the fact that CMPA was successfully induced using the original protocol, as demonstrated by the fact that a trend to increase clinical score was observed in oral-challenged CMPA group and a significant increase was observed in parameters such as spleen size, plasma histamine and plasma Igs such as IgE, IgG1, IgA and CMP-specific IgG1 or colonic IgG2a and IgA. However, of note, the differences observed between the CT 6w Control group and the oral-challenged CMPA animals as a result of the immune-

enhancing potential of CT were reduced. Therefore, an increase in sample size of the oral-challenged CMPA animals is required to demonstrate significance. Moreover, the CT effect explains why no differences were observed in critical allergy markers such as the clinical score or IL-4 production in plasma and colon. Finally, our results suggest that the extended use of CT also induces some alterations – such as those detected in the intestine – that may modify the final outcome of CMPA. In this regard, we have previously described that the alternation in intestinal permeability induced during an active intestinal inflammation exacerbates the immune response against ingested food antigens (Cueto-Solà et al., 2010).

Our data demonstrate that the novel protocol has several advantages over published methods. In summary, it reduces the experimental time required by two thirds, CT Control groups do not interfere with the data interpretation, thereby allowing significance to be reached in key allergy parameters such as the clinical score or IL-4 levels, and the response obtained is more allergen-specific than that achieved with the original protocol, since it does not increase the total amount of Igs in plasma or colon, while clearly increasing with similar potency the amount of CMP-specific IgG1, a common occurrence in allergic response (Lara-Villoslada et al., 2004, 2005).

Despite these benefits, the short CMPA protocol described here also has some drawbacks, the first regarding the IP challenge required to induce a measurable response. Although the CMPA protocol could be considered a non-physiological route of antigen contact, it is the best option to internalize minimal amounts of antigen, thereby allowing the maintenance of a specific and selective response to the target antigen. In order to ensure internalization of minimal amounts of intact protein sufficient to induce the allergy response, oral challenge with huge amounts of protein is required (Lara-Villoslada et al., 2004; 2005; Thang et al., 2011). Alternatively, the use of inhibitors of acid secretion in the gut with the same objective (Diesner et al., 2008; Untersmayr et al., 2010) presents other drawbacks, such as the loss of selectivity in response to other food antigens. Moreover, no detectable increase in IgE levels in plasma or colon is detected, this Ig being a hallmark of allergy. However, the lack of IgE response against CMP and other food-borne antigens has been reported (Lara-Villoslada et al., 2004, 2005; Thang et al., 2011). In this regard, serum concentrations of allergen-induced IgE are highly dependent on the type of allergen administered and cow milk caseins do not appear to be effective at inducing IgE (Thang et al., 2011). This observation also suggests that the increase in IgE induced in the original protocol could be triggered by food antigens other than CMP. Finally, the short duration of the modified protocol and the IP challenge probably explain why no IgA response was induced in this protocol despite targeting intestinal mucosa. Nevertheless, the lack of IgA response could also be an advantage since the blocking effect of the Igs (Smits et al., 2009) is avoided in our model.

5. Conclusions

Current available experimental models of food allergy are highly time-consuming and the robustness and sensitivity of the effects induced by the food-borne antigen are sometimes

diminished due to the immune-enhancing properties of the adjuvant required to overcome oral tolerance in mice. Here we present a novel CMPA model in mice that requires only 2 weeks of sensitization and that allows a more robust, specific and selective immune response to the target antigen. These characteristics thus favor the consistency of the results and a reduction in the number of animals required.

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