

Experimental Parameters Differentially Affect the Humoral Response of the Cholera-Toxin-Based Murine Model of Food Allergy

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Key Words

Cholera toxin · Animal model · Food allergy ·
Antigen-specific IgE · ELISA

Abstract

Background: Recent studies have developed a murine model of IgE-mediated food allergy based on oral coadministration of antigen and cholera toxin (CT) to establish a maximal response for studying immunopathogenic mechanisms and immunotherapeutic strategies. However, for studying subtle immunomodulating factors or factors effective during response initiation, this maximal response-based model is less suitable due to a lack of sensitivity. Therefore, in attempts to identify essential parameters to fine-tune the immune response towards a submaximal level, potentially more sensitive, we were interested in characterizing the individual effects of the parameters in the CT-based model: CT dose, antigen type and dose, and number of immunizations. **Methods:** BALB/c mice were orally sensitized weekly for 3 or 7 weeks with graded doses of CT and various food anti-

gens (soy-trypsin inhibitor, ovalbumin or ovomucoid). Antigen-specific IgG1, IgG2a, IgA and IgE were monitored by ELISA. **Results:** The CT dose exerted a clear dose-dependent effect on the antigen-specific antibody response whereas the antigen dose tended to affect the kinetics of the developing response. Both the intensity and kinetics of the antibody response depended on the type of antigen and number of immunizations. **Conclusions:** The critical parameters of the CT-based murine allergy model differentially control the intensity and kinetics of the developing immune response. Adjustment of these parameters could be a key tool for tailoring the response to submaximal levels rendering the model potentially more sensitive for evaluating the effect of subtle immunomodulating factors that would be lost in the maximal response-based model.

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Introduction

Food allergy is thought to affect up to 6% of children under 3 years and approximately 1.5% of adults [1]. IgE-mediated immediate hypersensitivity reactions are among the most common types of food allergy [2]. Yet, the underlying mechanisms are poorly understood, leav-

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ing a great need for suitable animal models. Recently, Li et al. [3–5] established a murine model that closely mimics the human clinical features of IgE-mediated food allergy. This model is based on oral immunizations with various food allergens using cholera toxin (CT) as adjuvant. CT is an enterotoxin produced by *Vibrio cholerae*, which possesses a potent Th2-driving mucosal adjuvant activity [6, 7]. The murine CT model of Li et al., in common with most of the other work reported in the literature using CT in mice, consistently employs an optimal dose of CT (approx. 10 µg CT/mouse) to generate a robust allergic response primarily aimed at studies on immunopathogenic mechanisms and development of new therapeutic strategies.

Most of the characteristic features of atopy, especially IgE production, are associated with a Th2 response, and the induction of allergy appears to relate to immune dysregulation promoting Th2 cells [8]. Microenvironmental factors are emerging as important determinants of the regulation of the fine balance between proallergic Th2 cells and the counteracting cells (Th1/T_{regulatory}), most probably by influencing the allergen encountering antigen-presenting cells such as dendritic cells [9]. Consequently, investigations of microenvironmental factors, such as the gut flora or dietary components, apart from the allergen per se, potentially influencing the development of the allergic response, are of utmost importance in understanding allergy and also in approaching allergy-preventive strategies. However, allergy models based on a maximally induced response may result in mistakenly negative results, as delicate preventive/inhibitory and also promoting immunomodulating factors might not affect a robustly established response whilst being critical during initial or weak responses. Therefore, more sensitive animal models reflecting more closely the conditions prevailing during initiation of an allergic response or during weakly manifested allergic reactions are definitely required. One line of approach for such a model could be a modification of the CT model involving controlled induction of a submaximal Th2 response both concerning the intensity and the kinetics. To enable the control of the CT-mediated response, identification and characterization of the essential parameters affecting the pattern of the induced Th2 response are required. Therefore, the primary objective of the present work was to evaluate the effect of the individual parameters forming the CT model, i.e. CT and antigen dose, model antigen and number of immunizations. For this purpose, we measured the antigen-specific humoral response, including IgE, as this was a practicable data output allowing us to monitor Th2 response development

over time within the same animals and to perform a large number of measurements necessary for comparing multiple parameters. The evaluated parameters were found to differentially affect the developing immune response and individual adjustment, especially of the CT dose, seemed a promising approach for tailoring the Th2 response of the CT model to submaximal levels, thus rendering the model potentially more sensitive for evaluating the effect of subtle immunomodulating factors.

Materials and Methods

Animals and Experimental Protocol

Female BALB/c mice, 5–6 weeks old (M&B, Ry, Denmark), were kept in standard animal housing and fed a standard diet (Altromin 1324, Altromin, Lage, Germany) and water ad libitum. The guidelines formulated in 'The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' were followed. All animal studies were approved by The Danish Animal Experiments Inspectorate. Several experiments were performed. Groups of mice (n = 8–9) were orally immunized weekly by gavage for 7 weeks with 0.25 mg/g of body weight of Kunitz soy-trypsin inhibitor (KSTI), ovomucoid (OM) or ovalbumin (OVA) (Sigma, St. Louis, Mo.) together with either none (control), 0.005 µg/g (low dose), 0.05 µg/g (medium dose) or 0.5 µg/g (high dose) of CT (List Biological Laboratories, Campbell, Calif., USA). One group received only 3 weekly oral immunizations of OVA and 0.5 µg/g of CT. Two groups received a low dose of OVA at 0.025 mg/g together with a low or high dose of CT. In addition, a group, serving as a control for the effect of the gavage per se, was given 5 mg of KSTI once a week by adding 1 mg/ml to the drinking water for 24 h (water consumption was measured to be 5 ml/day/mouse). All animals were deprived of food for 2 h before oral immunizations. The antigen/CT mixtures were freshly prepared in sterile PBS and administered at 0.2 ml/mouse.

Antibody Measurements by ELISA

Blood was obtained from the retro-orbital plexus on the day before each immunization and then weekly until 3 or 5 weeks after the last immunization. The blood samples were diluted 1:10 in incubation buffer (0.01 M PBS, 0.1% Triton X-100, pH 7.4) and stored at –20°C until analysis. For detection of antigen-specific IgG1 and IgG2a, 96-well microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with KSTI or OM (1 µg/ml), or OVA (1 mg/ml) in carbonate buffer (0.05 M, pH 9.6). Then, plates were incubated with serially diluted blood samples (starting at 1:64) for 1 h at room temperature (RT) followed by peroxidase-labeled rabbit anti-mouse IgG1 (0.25 µg/ml) or IgG2a (0.5 µg/ml) antibodies (Serotec, Oxford, England) for 1 h at RT. The plates were developed with 3,3'-5,5'-tetramethylbenzidine substrate for 10 min in the dark, stopped with 2 M phosphoric acid and read at 450 nm. For detection of antigen-specific IgA, rabbit anti-mouse IgA antibodies (1 µg/ml; Zymed, San Francisco, CA) were used followed by peroxidase-labeled swine antirabbit immunoglobulin antibodies (1.3 µg/ml; Dako, Glostrup, Denmark).

Because of the much higher level of IgG than IgE interfering with the detection of antigen-specific IgE in an indirect ELISA format like

that described above, a format based on selective IgE capture was established for detection of antigen specific IgE. Plates were coated with rat anti-mouse IgE antibodies (4°C overnight, 2 µg/ml; Zymed) in PBS (0.01 M, pH 7.4). Samples were serially diluted starting at 1:10 and the plates were incubated for 2 h at RT and then for 1 h with a conjugate of antigen and digoxigenin (DIG) (OVA-DIG and OM-DIG, 200 ng/ml; KSTI-DIG, 65 ng/ml). The antigen-DIG conjugation was performed using a DIG protein-labeling kit (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The maximal response was obtained using an antigen/DIG molar ratio of 1:10 for OVA-DIG and OM-DIG and 1:3 for KSTI-DIG. After washing, the plates were incubated for 1 h at RT with peroxidase-labeled sheep anti-DIG antibodies (0.05 U/ml; Roche). The plates were developed for 30 min as described above. Positive controls were obtained from mice immunized intraperitoneally with 10 µg/200 µl/mouse of antigen adsorbed on 200 µg Al(OH)₃ at day 1 and with 5 µg/200 µl/mouse of antigen at day 14 and 28. Blood from untreated mice was used as negative control. Moreover, to test for the occurrence of increased unspecific background in dosed animals, samples from animals administered a high dose of CT together with KSTI were analyzed after 7 weeks for anti-OM and anti-OVA antibodies and samples from animals administered high-dose CT and OVA were tested for anti-KSTI antibodies. No increased background was found (data not shown). Titers were determined as the log₂ to the sample dilution giving an absorbance of 0.2.

Statistics

The Proc Mixed procedure of the SAS software 6.12 (SAS Institute, Cary, N.C.) for two-way analysis of variance with repeated measurements on one factor was used to determine the effect of group, week and the interaction between them. If significant, a least squares means test was performed to determine effects of groups at individual times. Likewise, for data having three parameters (week, CT dose and antigen type or dose), a three-way analysis of variance was performed. $p \leq 0.05$ was considered significant.

Results

Effect of CT Dose

To assess the effect of CT dose on the antigen-specific antibody response, a preliminary study involving oral immunization of mice with KSTI combined with none, low, medium or high dose (0, 0.1, 1 or 10 µg, respectively) of CT was undertaken. In previous studies in our laboratory, KSTI was found to induce a strong antibody response in the CT model based on a high dose of CT [unpubl. data]. A clear dose-dependent response was observed for the CT dose on the KSTI-specific antibody response, with the strongest response at the highest CT dose for any of the tested antibody isotypes IgG1, IgG2a and IgE (fig. 1) and also IgA (data not shown). As expected, the most prominent response occurred for the Th2-associated IgG1 response whereas the Th1-associated IgG2a response remained absent at a low CT dose, very weak at a medium CT dose and only modest at the

high CT dose. In comparison with the response of the high CT dose group, a medium CT dose resulted in a more slowly induced IgG1 response, which leveled out at a lower level. Moreover, as evident from the scatter plot depiction, the medium CT dose led to a far less consistent response among animals with the majority of the animals grouped relatively homogeneously at a certain response level while the remaining animals showed no detectable response at any time. This response pattern not only indicates a dose-response effect of the CT dose on the response kinetics and intensity but also the presence of a threshold value for the lowest CT dose needed for response initiation, which embraces a greater biological variation than evident from the high CT dose response. Since the low CT dose group did not statistically differ from the control group, this CT threshold dose ranges between 0.1 and 1 µg at least for the anti-KSTI IgG1 response. However, when looking at the specific IgE response, much fewer animals responded at a medium CT dose than at a high CT dose, indicating that a higher dose of CT was necessary for tipping the response toward IgE production, than for initiation of IgG1 production.

Importantly, great care must be taken when comparing titers obtained from different ELISA formats due to differences in detectability. However, independent of assay detectability, the IgE response was induced at a slower rate than the IgG1 response, as the IgE response steadily increased until immunizations were terminated (week 7) and thus apparently never reached an upper level whereas the IgG1 response had already stabilized by week 5 as indicated by no additional statistically significant increase.

The response of the control group given 5 mg of KSTI weekly administered through the drinking water over 1 day, closely resembled the group dosed by gavage the same amount of KSTI with no CT, confirming that the procedure of prolonged gavage had no effect per se. The results described above, obtained in two independent experiments (fig. 1, 2), clearly demonstrate that CT dose affects the kinetics, as well as the intensity of the KSTI-specific antibody response.

As the antibody response, especially IgE, was found to be a useful and feasible output for thorough evaluation of the parameters of the CT-based food allergy model, evaluation of clinical symptoms upon challenge of the sensitized animals by re-exposure to antigen was not performed in the present study. During the sensitization course, no clinical symptoms were observed in any of the animals.

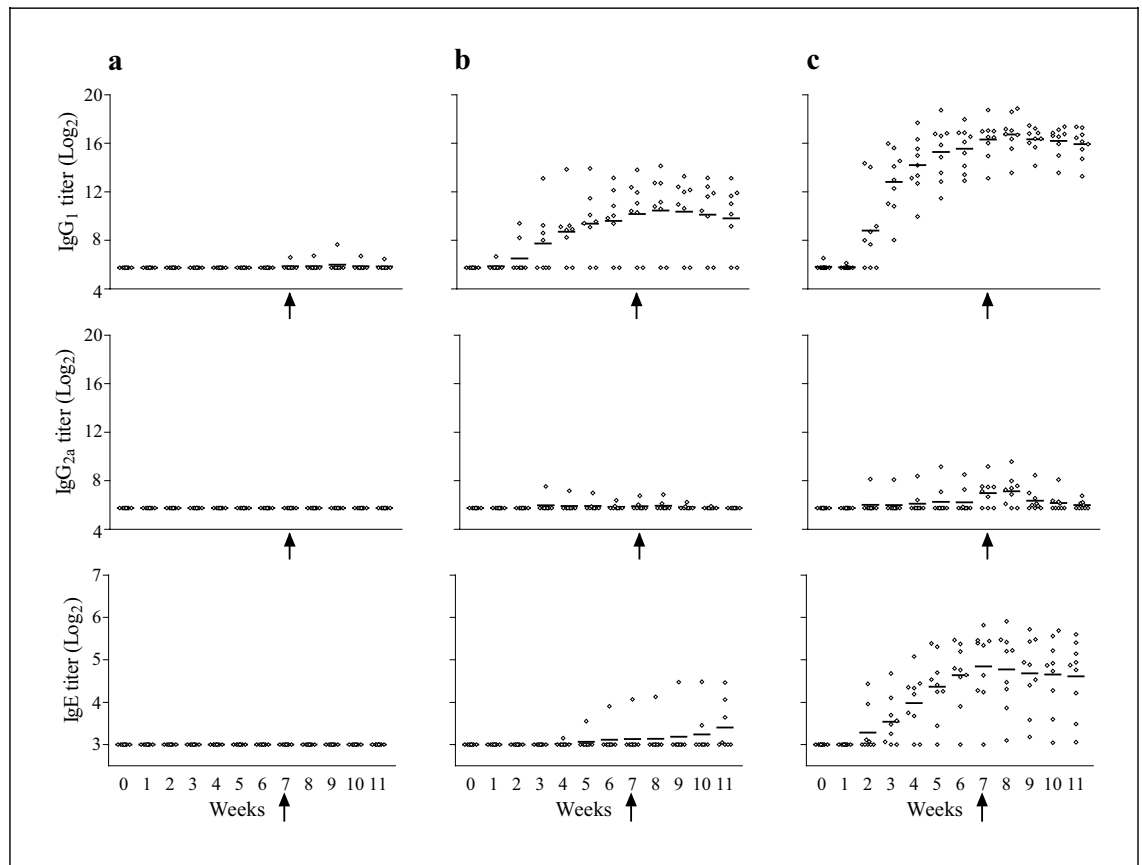


Fig. 1. Effect of varying doses of CT on the KSTI-specific IgG1, IgG2a and IgE response monitored by ELISA. Groups of mice ($n = 8-9$) were immunized weekly by gavage for 7 weeks with 5 mg/mouse of KSTI together with 0 (not shown), 0.1 (low) (**a**) 1 (medium) (**b**) or 10 (high) (**c**) $\mu\text{g}/\text{mouse}$ of CT. Blood samples were obtained weekly the day before each oral immunization until 5 weeks after the last immunization. KSTI-specific IgG1, IgG2a and IgE for the non-CT-treated group were the same as for the low-CT-treated group (data not shown). Bars indicate means. Arrows indicate 1 week after the last immunization.

Effect of CT Dose for Different Antigens

To examine whether the effect of CT dose on the antibody response varies with different antigens, a new study was performed employing KSTI, OVA or OM orally administered to mice, together with none (control), low, medium or high dose of CT. Antigen given alone (control) showed no significant response (data not shown). Again, the study showed a clear effect of the CT dose on the specific antibody response for all three antigens (fig. 2).

However, the response pattern towards the different CT doses was found to vary significantly between the antigens. For example, at a low CT dose, KSTI, as opposed to OVA and OM, showed no detectable IgG1 response, whereas at a medium and high CT dose, the response resembles that of OVA and OM. Moreover, at a low CT dose, a detectable IgE response was elicited only against

OM but not against OVA or KSTI. Altogether, this indicates that the threshold value of CT necessary for response initiation differs among antigens. The kinetics of the responses also differ among the antigens, e.g. the anti-OM IgG2a response developed later than the anti-OVA while the anti-OM IgE response developed earlier. These differences were statistically significant and indicate that different antigens exhibit different types of antibody iso-type patterns of the CT-mediated response.

Effect Antigen Dose and Number of Immunizations

In order to assess the effect of the antigen dose on the antigen-specific antibody response, groups of mice were orally immunized with a low or high dose of OVA combined with a low, medium or high dose of CT. In addition, to study the effect of the number of immunizations, one

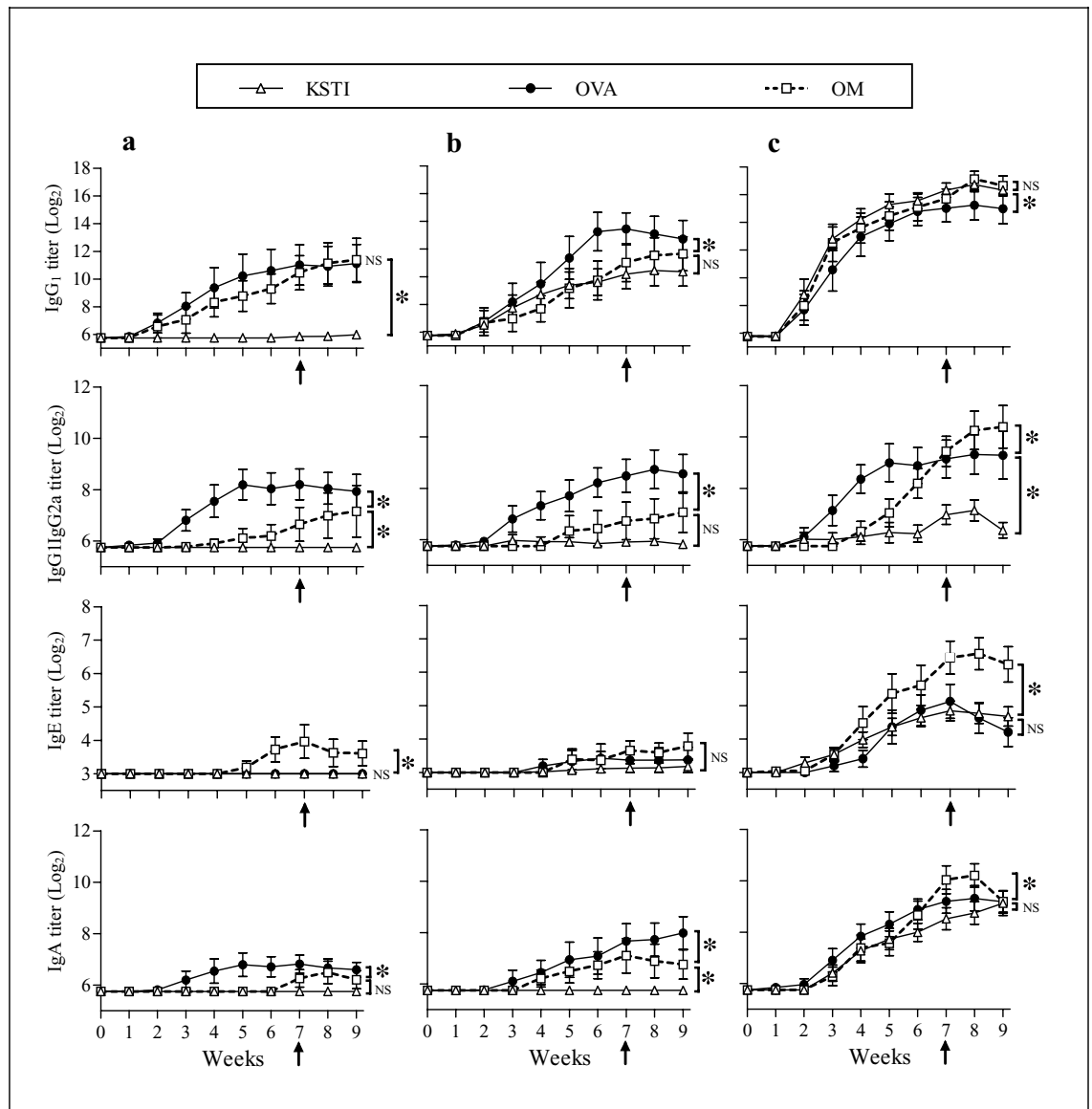


Fig. 2. Antigen-specific IgG1, IgG2a, IgE and IgA responses against different antigens (KSTI, OVA and OM) administered with various doses of CT. Groups of mice ($n = 8-9$) were immunized weekly by gavage for 7 weeks with 5 mg/mouse of antigen together with 0.1 (low) (a) 1 (medium) (b) or 10 (high) (c) μg CT/mouse. Blood samples obtained weekly the day before the oral immunization and until 3 weeks after the last immunization were analyzed by ELISA. Arrows indicate 1 week after the last immunization. Error bars represent $\pm$ SEM. * $p \leq 0.05$, only indicated for the least significant groups. NS = Not significant.

group received only three oral immunizations of a high dose of OVA with a high dose of CT. As in the previous study for OVA (fig. 2), the results showed an effect of CT dose for all antibody isotypes (fig. 3). In contrast, a statistically significant effect of OVA dose was found only for the IgG2a (low CT-dosed groups) and in particular for the IgE response (high CT-dosed groups), but surprisingly not

for the IgG1 or IgA response. Nevertheless, most notably for groups given a low CT dose, a low OVA dose tended to delay the onset of IgG1, IgG2a and IgA antibody production as compared to a high OVA dose, while the strength of the final response tended to be independent of OVA dose. The IgE response for the high CT dose was affected likewise as the onset of response was substantially delayed

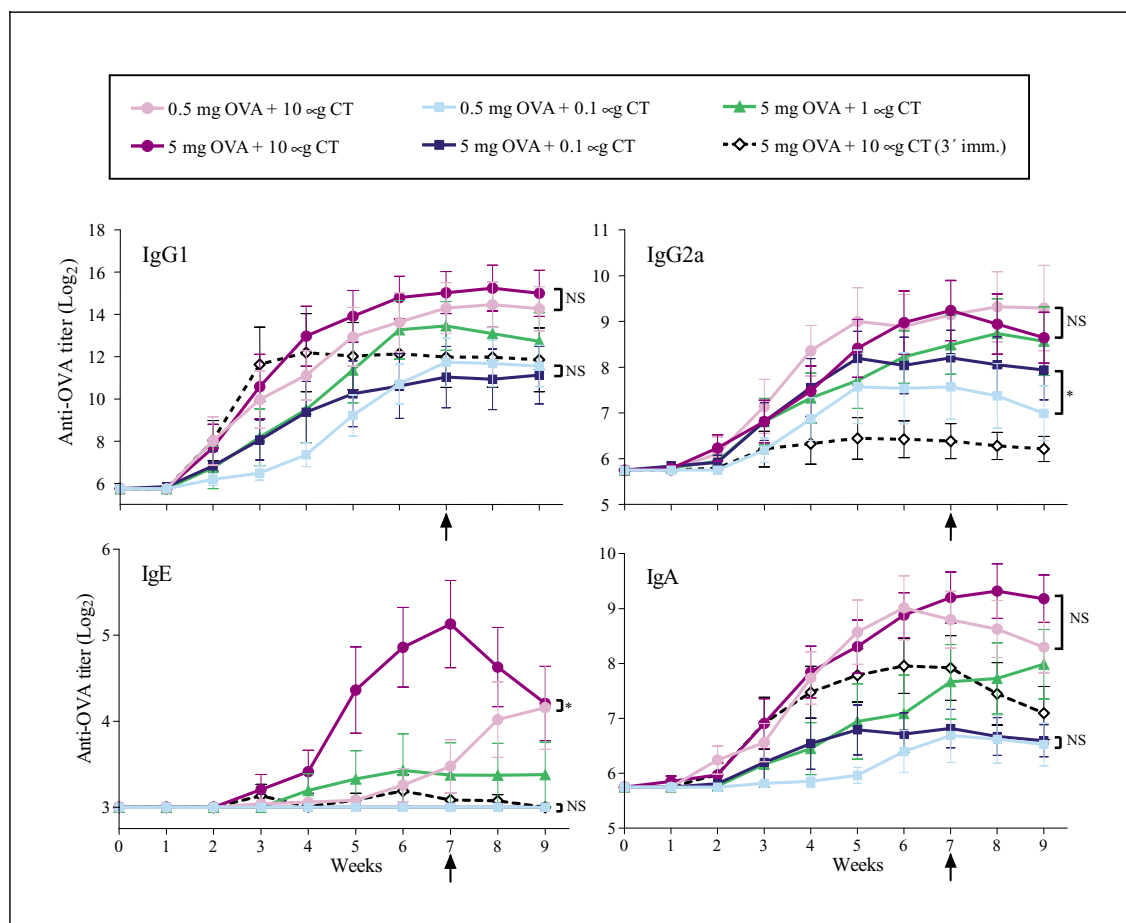


Fig. 3. Effect of OVA dose, CT dose and number of oral immunizations on the development of the OVA-specific antibody response. Groups of mice ($n = 8$) were immunized weekly by gavage for 7 weeks with 0.5 or 5 mg of OVA combined with either a low or a high dose of CT (0.1 and 10 $\mu\text{g}/\text{mouse}$, respectively) or 5 mg of OVA together with a medium dose of CT (1 $\mu\text{g}/\text{mouse}$). One group received only 3 oral immunizations of 5 mg/mouse of OVA with the high dose of CT. Blood samples obtained weekly the day before the oral immunization and until 2 weeks after the last immunization were analyzed by ELISA. Arrows indicate blood samples taken 1 week after the last immunization; solid lines: immunized for 7 weeks; dotted lines: immunized for 3 weeks. Error bars represent $\pm$ SEM. * $p \leq 0.05$ between curves. NS = Not significant. Only statistics for OVA dose are depicted.

for a low OVA dose in comparison with a high OVA dose. Since the IgE response did not level off within the immunization period, information about maximal antibody level was not obtained. Finally, the study showed an important effect of the number of immunizations for the antibody response in the CT model. Fewer immunizations gave rise to a more moderate but stable IgG1 response while only a very weak IgG2a and IgE response developed. Thus, the number of immunizations also represents a potential parameter for manipulating the antibody response raised in the murine CT model.

Discussion

Although the CT-based murine model of food allergy is being increasingly used, only very few studies have focused on evaluating the effect of the individual parameters forming the experimental protocol. In the present study, we were particularly interested in characterizing these parameters primarily in an attempt to provide a basis for tailoring the CT allergy model towards a sub-maximal response level potentially more susceptible to influence by possible immunomodulating factors investigated in our laboratory.

The CT dose represents one obvious key factor in the CT model. In the initial studies on the mucosal adjuvanticity of CT, Lycke and Holmgren [7] showed a dose-dependent effect of CT on the antigen-specific antibody-producing cells in the lamina propria with a minimum dose of CT in the range of 1–10 µg required for response (doses larger than 10 µg cannot be used due to incipient toxicity). Later studies have almost consistently used 10 µg CT in order to generate a maximal response. In agreement with the work of Lycke and Holmgren, the present data demonstrate a clear dose-dependent effect of CT adjuvanticity on the antigen-specific antibody response (IgG1, IgG2a, IgE and IgA) with a CT dose as low as 0.1 µg still efficient in inducing a significant specific response, only for some antigens however.

Importantly, substantial differences were observed among the tested antigens, KSTI, OVA and OM. These antigens, though being only minor allergens, were chosen to represent antigens with various physicochemical properties, as the outcome of the CT-adjuvantized response conceivably depends on the nature of the antigen, e.g. through differences in mode of processing of the particular antigen by the gastrointestinal tract [10]. The anti-KSTI response was in general considerably lower than the anti-OVA and OM response, which was most likely due to the presence of oral tolerance in the animals, since KSTI was present in the diet. Although CT has been known to abrogate oral tolerance, recent studies [11, 12] including studies in our laboratory [unpubl. data] have shown that the presence of oral tolerance reduces the strength of the CT-induced antigen-specific antibody response including IgE. OVA and OM, representing antigens to which the animals had not been exposed pre-experimentally through the diet, also caused significantly different response patterns to different CT doses; that is, OM showed a much greater IgE response than OVA. These results clearly indicate that the antibody response in the CT model differs between antigens, which, especially when deciding upon a CT dose for the purpose of establishing a suboptimal response, necessitates the type of antigen used to be taken into consideration.

The dose of antigen represents another key factor in the CT model. Several studies have reported a dose-dependent effect of antigen on the specific antibody response [3, 4, 7, 13]. Our studies, involving OVA, showed that the antigen dose in the tested amounts (0.5 vs. 5 mg/mouse) only weakly influenced the final IgG1, IgG2a and IgA response level after repeated oral immunizations whereas the IgE response was significantly affected by the antigen dose. A tendency for the antigen

dose to particularly affect the initial stage of the development of the response was observed since a reduced dose caused development of a delayed response. Studies by Li et al. [3, 4] demonstrated a maximal antigen dose for IgE production, where both lower and higher doses generated a weaker IgE response. In our studies, such a maximal dose was not found within the dose range tested. It should be noted that the studies of Li et al. employed complex protein matrixes (cow's milk and peanut), and therefore cannot be directly compared to the present data. Studies involving higher OVA doses, as well as dose-response studies for other pure antigens, and simple well-defined antigen mixtures thereof, are thus of interest.

Although generally recognized as being a Th2-driving adjuvant, it has been contentious whether CT induces a clear Th2 response characterized by IgG1 [11, 14] and IgE [3–5], or also induces a weak Th1 antibody response featuring IgG2a [15–17]. In this study, repeated oral immunizations with CT and antigen induced an antigen-specific IgG1 and IgE response along with an IgG2a response. However, the level of IgG2a in comparison with the other antibody isotypes was strongly influenced by the type of antigen, CT dose and number of immunizations. KSTI induced the lowest level of IgG2a, which, again, could relate to an already established oral tolerance. OVA and OM exhibited very different kinetics for the developing IgG2a response. The OM response emerged much later but reached a higher final response level than that of OVA for a high CT dose. When immunizing only three times, OVA elicited a substantial IgG1 response while the IgG2a response remained low, a pattern from which a different conclusion may be drawn than from the results obtained using more immunizations resulting in a higher IgG2a response. Since the experimental protocol varies greatly between studies reported in the literature, this could explain, at least in part, the discrepancy between the reported results on the Th1/Th2-driving adjuvanticity of CT and also other diverging results.

In summary, the present study showed that in particular the CT dose, but also the type and dose of antigen, in addition to total number of oral immunizations, are all important parameters for the pattern of the antibody response obtained in the CT-based murine model of food allergy. Other factors such as immunization interval and particularly mouse strain and age may be of importance. Adjustment of these individual parameters represents a promising line of approach for controlling the resulting immune response of the CT model rendering the model potentially more useful for reinvestigation of immunomodulating factors affecting food allergy. Different exper-

imental approaches of the CT model in accordance with the data reported herein are currently being used in our laboratory for studying the anti-allergic effect of probiotic bacteria found to have opposite Th1/Th2-driving capacities in vitro [18].

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