

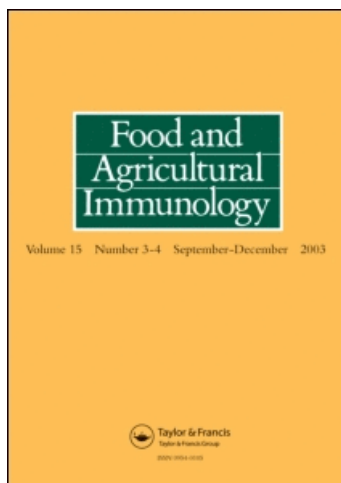
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Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713422299>

Comparative study of the adjuvanticity of *Bacillus thuringiensis* Cry1Ab protein and cholera toxin on allergic sensitisation and elicitation to peanut

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Online Publication Date: 01 December 2008

To cite this Article Guimaraes, V. D., Drumare, M. -F., Ah-Leung, S., Lereclus, D., Bernard, H., Créminon, C., Wal, J. -M. and Adel-Patient, K. (2008) 'Comparative study of the adjuvanticity of *Bacillus thuringiensis* Cry1Ab protein and cholera toxin on allergic sensitisation and elicitation to peanut', *Food and Agricultural Immunology*, 19:4, 325 — 337

To link to this Article: DOI: 10.1080/09540100802495651

URL: <http://dx.doi.org/10.1080/09540100802495651>

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Comparative study of the adjuvanticity of *Bacillus thuringiensis* Cry1Ab protein and cholera toxin on allergic sensitisation and elicitation to peanut

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(Received 21 August 2008; final version received 22 September 2008)

We aimed to investigate the adjuvanticity of Cry1Ab on allergic sensitisation and elicitation to peanut in mice, in comparison with cholera toxin (CT), a known mucosal Th2 adjuvant. Balb/c mice were gavaged with phosphate buffer saline (PBS) (control), peanut protein extract (PE) alone or PE co-administered either with CT or Cry1Ab. Peanut-specific IgE, IgG1 and IgG2a and Th1/Th2/Th17 cytokine secretion by splenocytes were assayed. Gavaged mice were further challenged with PE. Markers of the immediate and late phases of the allergic reaction were assayed in bronchoalveolar lavages (BAL) fluids collected 10 minutes or 36 hours after airway challenge, respectively. Sensitisation to peanut was only observed in mice receiving PE plus CT, as demonstrated by specific IgE and IgG1 production in sera and secretion of Th2 (and Th17) cytokines by splenocytes. Following challenge, mice sensitised with PE plus CT produced leukotrienes (LT) E4 and C4, then Th2/Th17-cytokines and showed eosinophil/neutrophil influx in BAL. Interestingly, LT production, Th2/Th17 cytokine release and eosinophil influx were also significant in mice gavaged with PE plus Cry1Ab, but not with PBS or PE alone. Cry1Ab did not demonstrate adjuvant effects on oral sensitisation to peanut. However, this study shows possible adjuvant properties of Cry1Ab on the elicitation of the allergic reaction, at least in the Balb/c mouse model and in the experimental conditions used.

Keywords: Cry1Ab; *Bacillus thuringiensis*; adjuvanticity; Balb/c mouse model; allergic sensitisation; allergic reaction

Introduction

The prevalence and severity of food allergy, mainly involving IgE-mediate reactions, have increased during the past few decades. The major foods responsible for food-allergic reactions are milk, eggs, peanuts, soy, fish and shellfish (Sampson, 1999) and the prevalence of allergy to individual foods varies geographically due to genotypes and different dietary habits. Environmental conditions may also be involved in particular the exposure to novel proteins including those that are expressed in genetically modified (GM) crops and that may have immunological properties such as immunogenicity or adjuvanticity. Adjuvanticity, i.e. the capacity to facilitate and/or enhance an immune response and particularly a sensitisation

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to a (food) protein is not currently assessed using the weight-of-evidence approach as recommended by different guidance documents (Codex Alimentarius Commission, 2003; EFSA, 2006).

Bacillus thuringiensis is a gram-positive bacterium that produces insecticidal crystal ('Cry') proteins (Höfte & Whiteley, 1989). After ingestion by susceptible insect, protein inclusions, or protoxins, are cleaved by insect midgut protease, leading to the production of N-terminal active fragment (Choma & Kaplan, 1990) which binds to specific receptors leading to increased gut permeability and finally to insect larva death (Van Rie, Jansens, Höfte, Degheele, & Van Mellaert, 1990).

We recently demonstrated that Cry1Ab protein is immunogenic in Balb/c mice, leading to the induction of Th1- and Th2-immune responses after intra-peritoneal (i.p.) administration without adjuvant (Adel-Patient et al., in press). This is in line with studies demonstrating that another Cry1A protein, namely Cry1Ac which shares 86% sequence homology with Cry1Ab, is highly immunogenic in mice (Vazquez-Padron et al., 2000). In addition, the adjuvant effect of Cry1Ac on systemic and mucosal specific antibody responses and on cellular immunity after intra-nasal (i.n.) or i.p. administration of microbial antigens was evidenced (Moreno-Fierros, Ruiz-Medina, Esquivel, López-Revilla, & Piña-Cruz, 2003; Rojas-Hernández, Rodríguez-Monroy, López-Revilla, Reséndiz-Albor, & Moreno-Fierros, 2004). The adjuvant effects of Cry1Ac and cholera toxin (CT), a major oral adjuvant that induces a Th2 type immune response, were also compared after i.g. administration (Vazquez-Padron, Moreno-Fierros, Neri-Bazan, de-la-Riva, & Lopez-Revilla, 1999). This latter study showed that Cry1Ac was a mucosal and systemic adjuvant as potent as CT for the hepatitis B surface antigen (HBsAg) and bovine serum albumin (BSA), and that its effect depended on the antigen and the route of administration used. However, no study evidenced the induction of an IgE response and, to date, no data is available on the adjuvant potential of the Cry proteins for sensitisation to food allergens. The aim of this study is to assess the potential adjuvanticity of Cry1Ab on the sensitisation to peanut, a highly allergenic food that may cause severe IgE mediated allergic reactions in sensitised children and adults (Rona et al., 2007; Sampson & Ho, 1997; Sampson & McCaskill, 1985). Several major peanut allergens have been identified, namely Ara h 1, Ara h 2, Ara h 6, Ara h 3 (Bernard et al., 2007; Burks et al., 1991; Burks et al., 1992; Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004; Shin et al., 1998). We previously reported that intra-gastric co-administration of whole peanut protein extracts (PE) and CT lead to sensitisation of Balb/c mice, i.e. to production of peanut-specific IgE and Th2 cytokines (Adel-Patient, Bernard, Ah-Leung, Creminon, & Wal, 2005; Adel-Patient et al., 2007). The present study thus aims to assess the potential adjuvant effect of Cry1Ab on allergic sensitisation in comparison to CT, using PE as a model food allergen.

Materials and methods

Apparatus and reagents

Enzyme immunometric assays were performed in 96-well microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialised Titertek microtitration equipment from Labsystems (Helsinki, Finland). Unless otherwise stated, all reagents were of analytical grade from Sigma (St Louis, MO).

Immunogens

CT (97kDa MW) was purchased from Sigma. Whole peanut PE and purified peanut allergens were prepared from commercial roasted peanuts as previously described (Bernard et al., 2007).

The *Bacillus thuringiensis* 407 strain harbouring the pHT315Ωcry1Ab plasmid (Vincent Sanchis, unpublished results) overproducing the Cry1Ab protein was grown in 100 ml HCT medium (Lecadet & Martouret, 1967) in 1l flasks at 37°C, 175 rpm, until sporulation was completed. The culture pellet was washed twice with water and loaded on a 31–80% two-phases discontinuous sucrose gradient and spun down at 37,000 *g* for 90 minutes at room temperature. Parasporal bodies were recovered at the 31–80% sucrose interface and extracted during 60 minutes at 37°C with NaOH 0.1N, 10mM EDTA, 1mM PMSF. The solubilised Cry1Ab protoxin (i.e. full length protein of 130 KDa MW) was then precipitated by decreasing the pH to 6.0 using small volumes of Bis Tris 1.5M, 20mM EDTA, 1mM PMSF, pH 2.0. Protoxin was cleaved using trypsin, and toxin fragment, corresponding to the δ-endotoxin Cry1Ab (65 KDa MW) was further purified. SDS-PAGE analysis demonstrated >95% purity. No endotoxin was detected in the Cry1Ab preparations, as determined by the Limulus Amebocyte Lysate test from Sigma. Purity was confirmed by HPLC using Akta purifier and/or SDS-PAGE electrophoresis systems from Pharmacia Biotech (data not shown).

Sensitisation of mice

Intra-gastric administrations

Five week-old female Balb/c mice (Centre d'Élevage René Janvier, France) were housed under normal husbandry conditions and were acclimated for 2 weeks before immunisations. All experiments were performed according to the European Community rules of animal care and with permission 91–122 of the French Veterinary Services. Balb/c mice were intra-gastrically administered 200 µl of PE alone (0.5 mg/g of mouse b.w.) or mixed with either CT or Cry1Ab (0.6 µg/g of mouse b.w., i.e. ca. 10 µg of Cry 1Ab or CT /mouse) at days 1, 7, 13, 19 and 25 (*n*=16 per group). Control mice (*n*=9) received 200 µl of phosphate buffer saline (PBS).

Determination of specific IgE, IgG1 and IgG2a

Serum samples were obtained by puncturing the retro-orbital venous plexus of mice at day 29. Peanut-specific antibodies, i.e. IgE, IgG1 and IgG2a were assayed as previously described (Adel-Patient et al., 2000; Adel-Patient et al., 2005). Briefly, sera were incubated at convenient dilutions on PE coated plates (5 µg/ml, diluted in 50 mM phosphate buffer pH 7.4) for 18h at 4°C. After washing, anti-IgE, anti-IgG1 or anti-IgG2a labelled with acetylcholinesterase (AChE) were added for 3 hours at 20°C, and AChE activity measured. Results were expressed as absorbance unit at 414 nm (mAU).

Determination of mediators released during the immediate phase of the allergic reaction

Mice sensitised by gavage, including control mice which received PBS, were challenged with PE by intra-tracheal (i.t.) route on day 33 (*n*=5–8 per group). Mice were deeply anaesthetised by i.p. injection of 200 µl/mice of Ketamine (15 mg/ml)/Xylazine (2 mg/ml) cocktail (Imalgène 500, Merial, Lyon France; Rompum 2% Bayer Pharma, Puteaux,

France). Mice were then i.t. challenged with 20 µg of PE in 50 µl PBS. Bronchoalveolar fluids (BAL) were taken 10 minutes later, according to Adel-Patient et al. (2003). Leukotrienes (LTs) E4 and C4 (LTE4 and LTC4) were analysed according to Pradelles, Grassi, and Maclouf (1985) using specific enzyme immunoassays provided by SPI-Bio Bertin, France.

After BAL collections, mice were sacrificed by vertebral dislocation and spleens were harvested for specific cytokine secretions assays. After lysis of red blood cells (180 mM NH₄Cl, 17 mM Na₂EDTA) and several washes, splenocytes were resuspended in RPMI-10 (RPMI supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin, all from Gibco). Cells were incubated in 96-well culture plates (10⁶ cells/well) in presence of PE (2, 10 or 50 µg/ml), purified peanut allergens (Ara h 1, Ara h 2, Ara h 3 or Ara h 6; 20 µg/ml), RPMI-10 or irrelevant protein (negative controls) or concanavalin A (1 µg/ml, positive control) for 60 h at 37°C and 5% CO₂. Supernatants were then removed and stored at -80°C until further assay. Th1-, Th2- and Th17-cytokines (TNFα, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17, GM-CSF and IFNγ) were assayed in duplicates using x-MAP kit from Biorad and BioPlex technology, following manufacturer's recommendations. Coefficients of variation between duplicates were all inferior to 10%.

Determination of mediators released during the late phase of the allergic reaction

On day 32, all the mice sensitised by gavage were challenged with 20 µg of PE (in 50 µl of PBS) by i.n. route under light anaesthesia (Isoflurane, Baxter) (*n* = 4–8 per group). After 36 hours, the mice were deeply anaesthetised by i.p. injection of 200 µl/mice of Ketamine/Xylazine cocktail. The trachea was cannulated for bronchoalveolar cells collection in sterile saline and kept on ice. Total cells counts were performed after Trypan Blue exclusion on Malassez cells. Differential cell counts were performed after cytocentrifugation and staining with Diff-Quick stain (LaboModerne, France). Aliquots of the remaining BAL were centrifuged and supernatants were collected and stored at -80°C until cytokine analysis. Th1-, Th2- and Th17-cytokines were assayed as above described. All BAL samples were analysed individually.

Statistical analysis

Non-parametric Kruskal–Wallis test and Dunn's multiple comparison post-test were used to compare all groups together, and *t*-test was performed to compare PE plus Cry1Ab or PE treated groups to control group. *P* values of less than 0.05 were considered statistically significant.

Results

Peanut specific IgE, IgG1 and IgG2a

The concentrations of peanut-specific antibodies induced in Balb/c mice after i.g. administration of PE alone or plus CT or Cry1Ab were compared (Figure 1). No specific antibodies were detected in mice gavaged with PBS (*n* = 9). As already observed, co-administration of PE plus CT induced a high production of specific IgE and IgG1, and to a lesser extent of IgG2a (not shown), in all mice, thus demonstrating the efficiency of CT as adjuvant to induce an intense specific Th2 immune response.

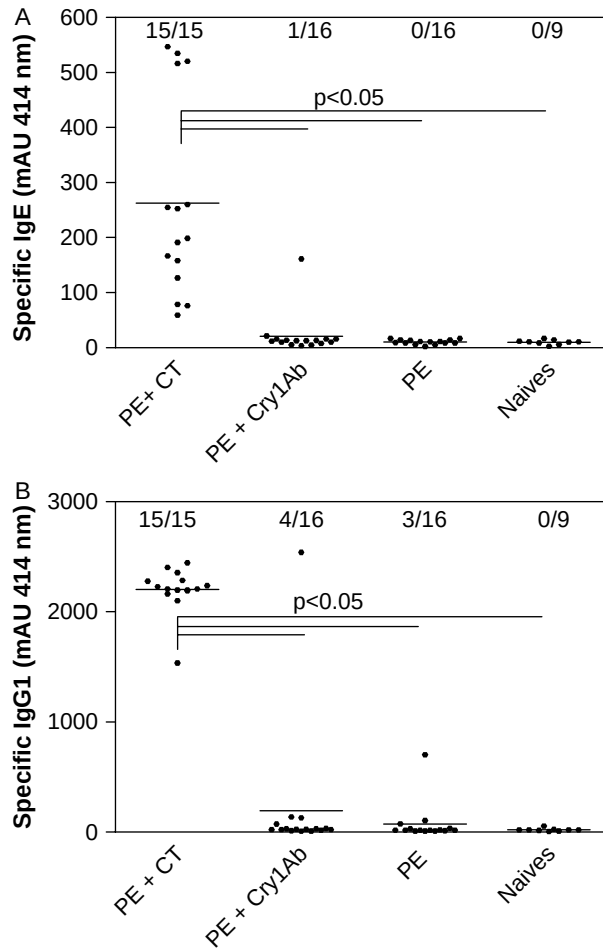


Figure 1. Specific IgG1 and IgE induction after intra-gastric administration of PE alone or co-administered with CT or Cry1Ab. Balb/c mice received PE plus CT, PE plus Cry1Ab, PE alone or PBS by gavage. On day 29, sera were collected and specific IgE (A) and IgG1 (B) were assayed as described in Materials and methods section. Serum dilutions were 1/500 for IgG1 and 1/20 for IgE determinations. Bars represent the mean of 15–16 mice per group, except for the control group (PBS – ‘naïve’, $n = 9$). $p < 0.05$ indicates statistical significance using non-parametric Kruskal–Wallis test and Dunn’s multiple comparison post-test.

Out the 16 mice administered PE plus Cry1Ab, only four produced peanut-specific IgG1, and one produced specific IgE (and IgG2a, not shown). The specific immune response induced in mice which received PE plus Cry1Ab was similar to that induced by PE alone.

Cytokine productions by splenocytes after antigen reactivation

The Th1-, Th2- and Th17-cytokines secreted by the splenocytes reactivated *ex-vivo* by PE or by the purified peanut allergens were analysed. We observed important productions of IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF and IL-17 after PE reactivation of splenocytes from PE plus CT mice (Figure 2). Conversely, low levels of TNF α and no IFN γ or IL-12

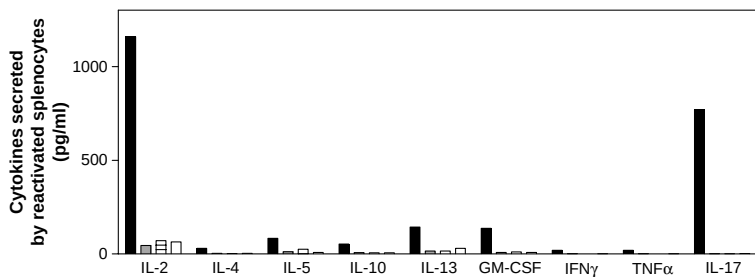


Figure 2. Cytokines secreted by splenocytes reactivated with PE. Balb/c mice received PE plus CT (black bars), PE plus Cry1Ab (grey bars), PE alone (hatched bars) or PBS (empty bars) by gavage. On day 33, spleens were removed and pooled within the same treatment group, and splenocytes were reactivated *ex vivo* with PE (50 μ g/ml). Expected results were obtained for positive (ConA) and negative controls (medium alone) (data not shown).

were detected which demonstrates that intra-gastric administrations of PE plus CT induced both Th2 and Th17 cells. As previously reported (Adel-Patient et al., 2007), Ara h 1 is the most potent Th2 (and Th17) inducer of cytokines production (data not shown). Conversely, we did not observe cytokine productions after reactivation of splenocytes from mice administered PBS, PE alone or PE plus Cry1Ab (Figure 2).

Analysis of the immediate phase of the allergic reaction after challenge of mice

The immediate phase of the allergic reaction was analysed by assaying LTs (LTE4 and LTC4) released in bronchoalveolar lavage (BAL) fluids collected 10 minutes after i.t. challenge with PE as previously described by Adel-Patient et al. (2003). An important production of LTE4 and LTC4 was observed after challenge with PE in mice sensitised with PE plus CT, but also in mice administered PE plus Cry1Ab (Figure 3). Conversely, neither LTC4 nor LTE4 were detected in BAL after PE challenge of mice gavaged with PE alone or with PBS.

Analysis of the late phase of the allergic reaction after challenge of mice

Mice gavaged with PE and CT showed high levels of both IL-4, IL-5 and IL-17 in BAL collected 36 hours after a single i.n. challenge with PE (Figure 4), thus demonstrating a Th2/Th17 local response. Interestingly, mice gavaged with PE plus Cry1Ab also demonstrated a weak but significant release of IL-5 and IL-17 in BAL when compared to PBS (control) mice (Figure 4). Release of IL-17, but not of IL-5, was also significant in mice gavaged with PE alone as compared to PBS mice (Figure 4). In no group Th1 cytokine IFN γ and IL-12 were detected (not shown).

The analysis of cellular influxes showed that mice gavaged with PE plus CT and challenged with PE presented a significant increase in total cell counts in BAL, as a consequence of an important influx of eosinophils and neutrophils (Figure 5). Although less intense, a significant eosinophil influx was also observed in mice sensitised with PE plus Cry1Ab when compared to control mice, whereas neutrophil influx was not significant in those mice. It is worth noting that mice demonstrating the highest eosinophil influx were also characterised by the highest levels of IL-5, IL-17 and IL-4 in BAL. Conversely, no significant eosinophil influx was observed in mice gavaged with PE alone.

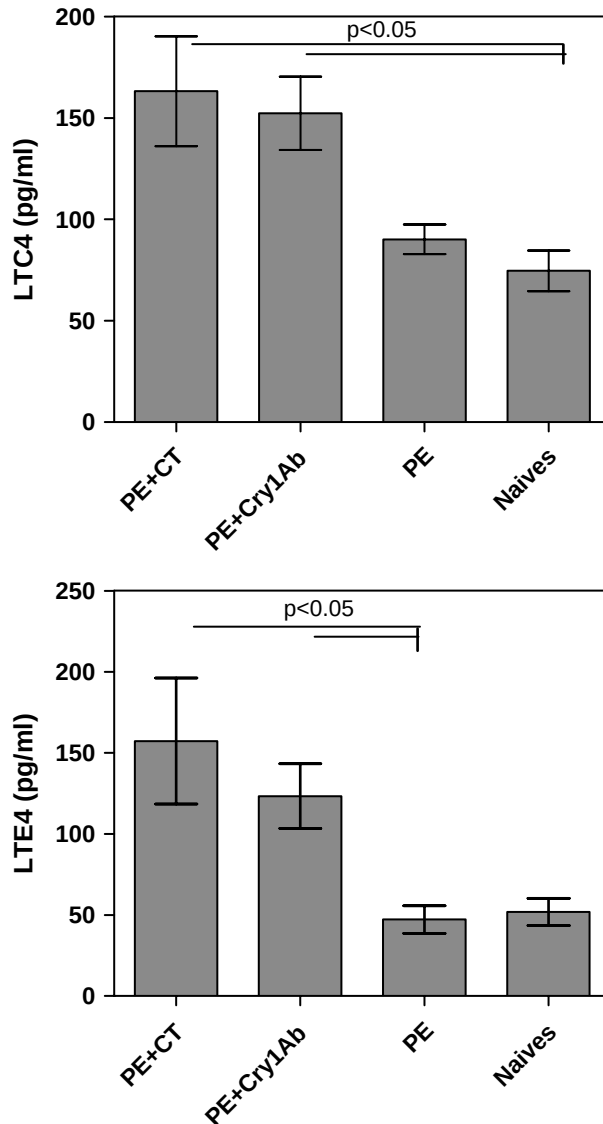


Figure 3. Leukotrienes released in bronchoalveolar fluid (BAL) after intra-tracheal challenge: Balb/c mice received PE plus CT, PE plus Cry1Ab, PE alone or PBS by mean of gavage on days 1, 7, 13, 19 and 25. On day 33, all mice were challenged with 20 μ g of PE by intra-tracheal route and BAL were collected 10 minutes later for LTC4 (A) and LTE4 (B) assays. $p < 0.05$ indicates statistical significance using non-parametric Kruskal–Wallis test and Dunn’s multiple comparison post-test.

Discussion

In this study we investigated the adjuvant properties of Cry1Ab on allergic sensitisation and elicitation to a major food allergen, i.e. peanut. We compared the immune response induced by i.g. administration of PE plus Cry1Ab to that observed after similar administration of PE but in presence of CT, a strong mucosal Th2 adjuvant. Mice were thereafter challenged with PE to compare the intensities of the allergic reactions thus elicited.

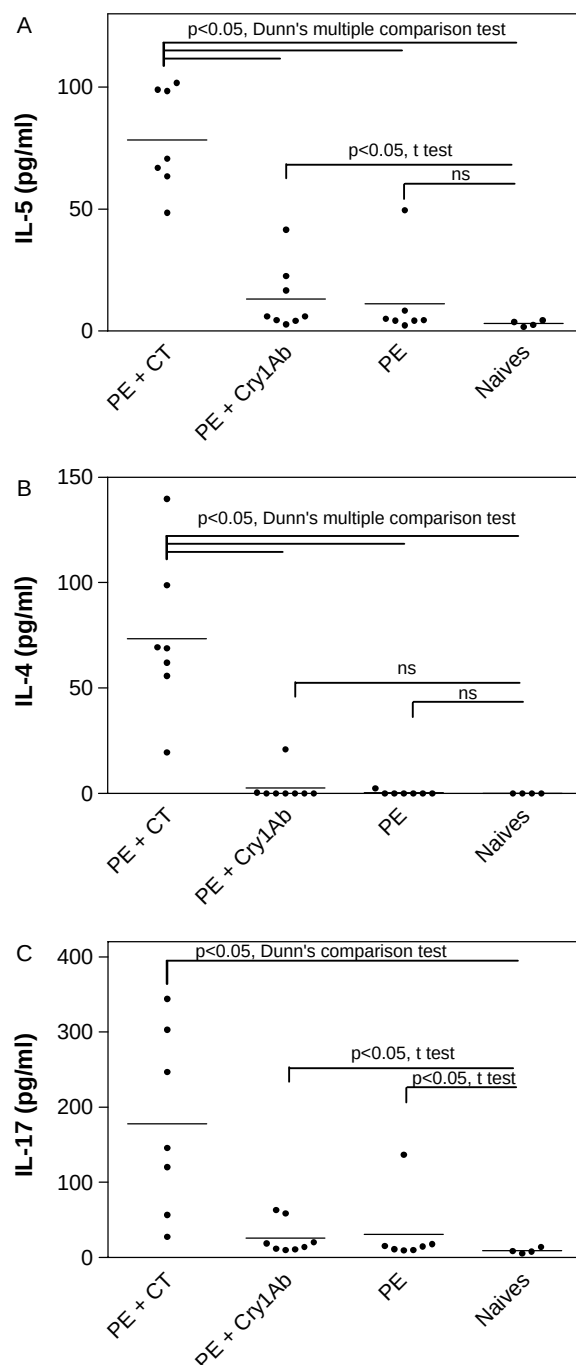


Figure 4. Cytokines secretion in bronchoalveolar lavage fluids (BAL) after intra-nasal challenge: Balb/c mice received PE plus CT, PE plus Cry1Ab, PE alone or PBS by mean of gavage on days 1, 7, 13, 19 and 25. On day 33, all mice were challenged with 20 μ g of PE by intra-nasal route. IL-5 (A), IL-4 (B) and IL-17 (C) cytokines were assayed in BAL collected 36 hours after challenge. $p < 0.05$ indicates statistical significance using non-parametric Kruskal–Wallis test and Dunn's multiple comparison post-test or t -test. ns: not statistically significant.

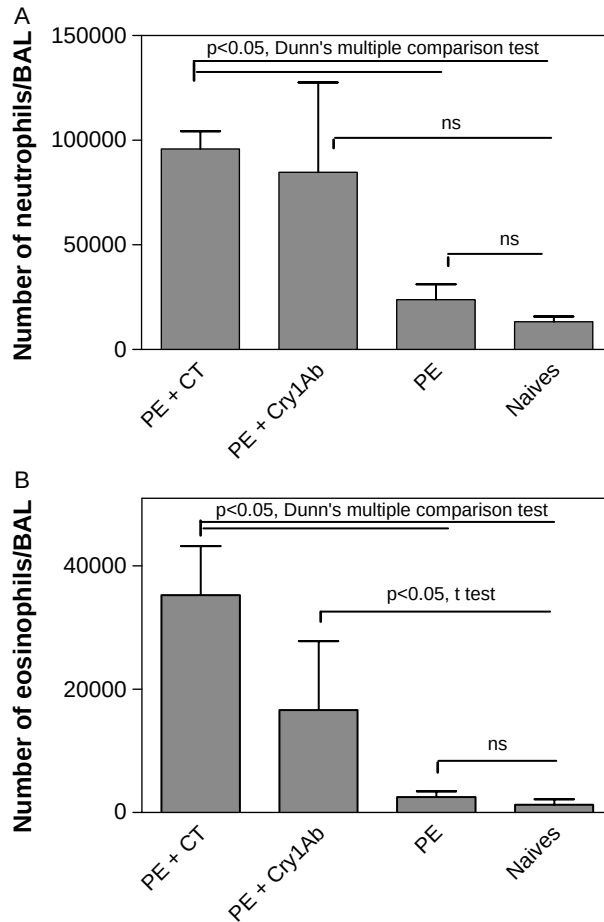


Figure 5. Cellular influx in bronchoalveolar lavage fluids (BAL) after intra-nasal challenge: Balb/c mice received PE plus CT, PE plus Cry1Ab, PE alone or PBS by mean of gavage on days 1, 7, 13, 19 and 25. On day 33, all mice were challenged with 20 μ g of PE by intra-nasal route. BAL were collected 36 hours after challenge. Neutrophils (A) and eosinophils (B) were counted after cytocentrifugation and staining with Diff-Quick stain. At least 300 cells per samples were considered. Mean of five (control) to eight (others groups) mice/group $\pm$ SEM are represented. $p < 0.05$ indicates statistical significance using non-parametric Kruskal–Wallis test and Dunn's multiple comparison post-test or *t*-test. ns: not statistically significant.

As previously reported, a high sensitisation was observed in mice gavaged with PE plus CT, as evidenced by the production of specific IgE and secretion of Th2 cytokines by reactivated splenocytes (Adel-Patient et al., 2005, 2007). We now demonstrate that the allergic reaction is highly elicited after airway challenge of sensitised mice with PE. In fact, both early (e.g. LTC₄ and LTE₄) and late (e.g. Th2 cytokines and eosinophil influx) allergic reaction mediators are evidenced in BAL of challenged mice. Although it has been demonstrated that IL-17 may inhibit the activation of Th2 cells, and IL-4, IL-5 and IL-13 productions (Schnyder-Candrian et al., 2006), we evidenced the induction of both a Th2 and Th17 response in mice i.g. sensitised with PE plus CT. This is in agreement with a

previous report demonstrating that the activity of Th17 cells within the draining lymph nodes interfered with induction of oral tolerance in CD11b^{-/-} mice (Ehrichtiou et al., 2007). We also evidenced high IL-17 secretion and neutrophil influx in BAL after i.n. challenge which confirms the important role of IL-17 in allergen-specific T cell-mediated immune responses and in neutrophil recruitment (Schnyder-Candrian et al., 2006). It is then noteworthy that our study is the first report demonstrating the induction of both a Th2 and a Th17 response in mice i.g. sensitised with PE plus CT, although this was recently suggested (Blázquez & Berin, 2008).

Conversely, we observed low levels of peanut specific antibodies in mice after i.g. administration of PE in presence of Cry1Ab at the same dose as that used for CT. In addition, no Th2-, Th1- or Th17 specific cytokines were detected in supernatants from reactivated splenocytes of those mice. These results were similar to those observed in mice sensitised by PE alone. CT and Cry1Ab were used at the same dose (i.e. 0.6 µg/g) which is the classical effective dose used to get a strong adjuvant effect with CT in experimental allergic sensitisations (Adel-Patient et al., 2005). Molecular weights of Cry1Ab and CT are similar (65KDa versus 97Kda, respectively) which means that both toxins were used at a similar molarity and that even some more molecules of Cry1Ab than CT were administered to mice. The lack of mucosal adjuvant effect of Cry1Ab when compared to CT can thus not be explained by differences in the dosage of the two toxins. It is likely due to the low intrinsic capacity of Cry 1Ab to enhance an allergic sensitisation to peanut in mice when used in the same conditions as CT and therefore that the mode of action would be different.

These results are not in agreement with previous studies performed on different models and/or in different conditions. Vazquez-Padron et al. (1999) showed that Balb/c mice immunised by i.g. route with BSA or HBsAg in presence of 10 or 100 µg of a recombinant Cry1Ac protein showed increased antigen-specific IgG in sera when compared to mice receiving the antigen alone. The use of magnesium-aluminum hydroxide for i.g. administrations can partially explain the discrepancies between the latter study and our results since neutralisation of acidic gastric conditions may promote sensitisation via the i.g. route (Untersmayr et al., 2003). Moreover, it has been proposed that the N-terminal region of Cry proteins is mainly responsible for the specific antibody responses induced by those proteins (Guerrero & Moreno-Fierros, 2007) and that orally administered Cry1Ac may turn on extra-intestinal but not gut associated lymphoid tissues antibody-secreting cells (Moreno-Fierros et al., 2003; Vázquez-Padron et al., 1999). The processing of Cry1Ac and Cry1Ab may be different in the gastrointestinal tract and associated lymphoid tissue, thus explaining variations in the adjuvant effects observed.

Surprisingly, although no peanut-specific humoral and cellular responses were detected in mice gavaged with PE plus Cry1Ab, we evidenced the elicitation of an allergic reaction in those mice after challenge with PE. This was demonstrated by the early production of LTs in BAL fluids, and the late Th2- and Th17-cytokine productions and eosinophil/neutrophil influx. Conversely, mice gavaged with PE alone did not elicit an allergic reaction. This clearly demonstrates that Cry1Ab has an adjuvant effect on the active phase of the allergic reaction and particularly in its early phase, which suggests an IgE-mediated mechanism. Further studies are then needed to clarify the mechanisms of action and identify the cells locally involved.

Mucosal adjuvant properties of recombinant Cry1Ac protein were also demonstrated for some proteins, such as amoebal lysates from *Naegleria fowleri*, *Streptococcus pyogenes* lysates, or polysaccharides, such as *Streptococcus pneumoniae* capsular polysaccharides after administration via the intra nasal route (Moreno-Fierros et al., 2003; Rojas-

Hernández et al., 2004; Vazquez-Padron et al., 1999). Intra-nasal administration of Cry1Ac with polysaccharides from *S. pneumoniae* increased both IgA and IgM systemic and local responses, but also a mucosal response at distant sites (i.e. in the gastro-intestinal tract) (Moreno-Fierros et al., 2003). Increased specific IgG production in sera and BAL were also observed after i.n. administration of Cry1Ab with a polyvalent pneumococcal vaccine (Moreno-Fierros et al., 2003). The fact that adjuvant effects were evidenced after administration by the i.n. route may raise an important issue since Cry1Ab is also expressed in pollen of genetically modified crops. Exposure of humans by inhalation may also occur and the absence of adjuvant effect of Cry1Ab on IgE production against allergens that might be present in the pollen should be clarified.

In conclusion, Cry1Ab did not demonstrate adjuvant effects on oral sensitisation to peanut when compared to the effects of CT in similar conditions. However, this study shows possible adjuvant properties of Cry1Ab on the elicitation of the allergic reaction, at least in the Balb/c mouse model and in the experimental conditions used.

Acknowledgements

The authors thank the French Ministry of Research and Technology for financial support (ANR 'Impact des OGM'). V. Guimaraes was supported by a postdoctoral grant from the AlimH department of INRA. We also thank Jean-Charles Robillard and Sylvain Thessier for providing excellent care for the animals.

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