

Cry1Ab Protein from *Bacillus thuringiensis* and MON810 *cry1Ab*-transgenic Maize Exerts No Adjuvant Effect After Airway Exposure

M. Andreassen*†‡, T. Bøhn*‡, O.-G. Wikmark*, J. Van den Berg§, M. Løvik¶, T. Traavik*‡ & U. C. Nygaard†

*GenØk - Centre for Biosafety, Tromsø, Norway; †Norwegian Institute of Public Health, Oslo, Norway; ‡UiT The Arctic University of Norway, Tromsø, Norway; §North-West University, Potchefstroom, South-Africa; and ¶Norwegian University of Science and Technology, Trondheim, Norway

Received 29 September 2014; Accepted in revised form 30 December 2014

Correspondence to: M. Andreassen, Department of Food, Water and Cosmetics, Norwegian Institute of Public Health, PO Box 4404, 0403 Oslo, Norway. E-mail: monica.andreassen@fhi.no

Abstract

The genetically modified (GM) maize event MON810 has been inserted with a processed version of the transgene, *cry1Ab*, derived from the soil bacterium *Bacillus thuringiensis* (Bt) to express proteins with insecticidal properties. Such proteins may introduce new allergens and also act as adjuvants that promote allergic responses. While focus has been on safe consumption and hence the oral exposure to GM food and feed, little is known regarding inhalation of pollen and desiccated airborne plant material from GM crops. The aim of this study was to investigate whether plant material from the Cry1Ab-expressing maize variety MON810, or trypsin-activated Cry1Ab (trypCry1Ab) protein produced in recombinant bacteria, may act as adjuvants against the allergen ovalbumin (OVA) in a mouse model of airway allergy. A clear proallergic adjuvant effect of the mucosal adjuvant cholera toxin (CT) was demonstrated, determined as increased specific IgE, eosinophils and Th2 cytokines in MLN cell supernates, while no elevation in OVA-specific antibodies or cytokine release from MLN cells after stimulation with OVA were observed in mice receiving Cry1Ab-containing plant materials or the trypCry1Ab protein. Our data suggest that Cry1Ab proteins had no detectable systemic adjuvant effect in mice after airway exposure. Further experiments with purified plant proteins, as well as long-term exposures needs be conducted to further evaluate exposures experienced in real-life situations.

Introduction

Transgenic modifications of crop plants by the insertion of 'foreign' DNA-sequences into the plant genome will often introduce novel proteins into food and feed products intended for human and domestic animal consumption. Concerns have been raised regarding the safety for consumers, including the risk of introducing new allergens [1–4]. Additionally, novel proteins may act as enhancers, the so-called adjuvants, of allergic immune responses without being allergens themselves (possible modes of actions are reviewed by Brunner *et al.* [5]).

In the genome of the genetically modified (GM) maize event MON810, a processed version of the transgene *cry1Ab*, derived from the soil bacterium *Bacillus thuringiensis* (Bt), has been inserted. In the parent Bt bacterium, the *cry1Ab* gene encodes a protoxin, which is transformed into an active toxin by enzymatic cleavage in the insect midgut. The complexity in mode of action(s) and interactions with

other factors is discussed in the literature [6–9], but the main toxic effect of the cleaved polypeptide is ascribed to its binding to receptors in the cell membranes of lepidopteran midgut epithelium, inducing pore formation in the cell membranes. This in turn leads to influx of intestinal bacteria, followed by septicaemia and insect death [10]. The naturally occurring Cry-proteins are known to be non-toxic for human consumption because the human intestines lack the enzymatic prerequisite for activation of the protoxin. The Cry version expressed in GM plants, however, is circumventing this because the *cry1Ab* transgene has been truncated and codon optimized to maize. Consequently, the plant-Cry1Ab molecule lacks the carboxy-terminal part of the protein, and thus resembles the activated version of the protoxin, although they are not identical. Accordingly, it is important to distinguish between the various versions and origins of Cry1Ab proteins when assessing possible adverse immune responses, as the protoxin Cry1Ab and different versions

of toxin Cry1Ab may affect the immune system differently [11].

To date, the plant Cry1Ab has not been extracted and purified in sufficient amounts to enable animal exposure experiments. Feeding of MON810-containing diets to animals indicates Cry1Ab immunogenicity [12], altered proportion of immune cells in the gut [13–15] and increased serum levels of cytokines [13]. Only a few studies have addressed the potential adjuvant effect of Cry1Ab proteins. Guimaraes *et al.* [16] reported a potential adjuvant effect, as oral gavage of the trypsinized version of Cry1Ab plus a major food allergen showed increased Th2 and Th17 cytokine production and eosinophil inflammation in the airways of mice. In contrast, a recent study [17] found no adjuvant effects on the onset or severity of allergic asthma after short-term feeding of mice to a MON810 maize diet.

The dietary intake of Cry1Ab proteins via food and feed is undoubtedly a highly relevant route of exposure. In addition, inhalation of pollen and desiccated airborne plant material from GM crops represent a realistic exposure route for humans, as well as for domestic and wild animals. Exposure may occur in the field, but also during storage and processing of GM plant materials. Nevertheless, to our knowledge, no information exists on the adjuvant capacity of inhaled Cry1Ab proteins. Airway exposure was illustrated in a rat feeding study with *cry1Ab*-transgenic rice [12]. The target groups fed GM and the control groups fed unmodified rice menus were kept in the same room, and both groups developed anti-Cry1Ab antibody reactions. The only reasonable exposure route for the control groups were by airborne plant debris from the feed. Although the inhalation of Cry1Ab-containing feed was unintended, it demonstrates the principle that such exposures may be relevant for immune effects.

Importantly, another Cry1A protein, the structurally related Cry1Ac, has been demonstrated to exert strong adjuvant effects, comparable to the systemic and mucosal effect reported for cholera toxin (CT) [18]. Consequently, the similarity with the mucosal adjuvant Cry1Ac and the gaps in knowledge related to inhalation of plant originating Cry1Ab proteins warrants further investigations. The aim of this study was to investigate whether exposure to pollen and/or leaf material from the *cry1Ab*-transgenic MON810 maize elicited airway immune responses or exerted adjuvant effect on antibody production against an allergen, ovalbumin (OVA), in a mouse model of airway allergy. We also tested a trypsinized, and hence toxicologically activated, version of a Cry1Ab protein produced in recombinant *E. coli*. Effects on immune responses were evaluated by determination of serum levels of OVA-specific antibodies (IgE, IgG1 and IgG2a), cytokines in broncho-alveolar lavage fluid (BALF) and mediastinal lymph nodes (MLNs), and BALF cell differentiation.

Materials and methods

Animals. Female BALB/c mice (Charles River, Sulzfeld, Germany), 5–6 weeks old at arrival, were rested for 1 week before being included in the experiments. The mice were randomly allocated to groups ($n = 8–10$) and housed on Nestpack bedding (Datesand Ltd., Manchester, UK), 3–4 mice together in cages. The Harlan Teklad 2018 rodent OVA-free diet (Madison, WI, USA) and tap water were given *ad libitum*. The mice were exposed to a 12/12 h light/dark cycle, room temperature of 21 ± 2 °C and $55 \pm 10\%$ humidity. The experiments were performed in conformity with the laws and regulations for experimentation with live animals, and approvals were obtained from the North-West University Office of Ethics Committee (approval reference NWU-00025-10-A3) and by the Norwegian Animal Research Authority under the Ministry of Agriculture (approval reference FOTS 4050) for experiments 1 and 2, respectively.

Test materials. The test solutions (Table 1) contained allergen alone or in combination with test materials (pollen, leaf extract or trypCry1Ab protein). A physiological buffer (HBSS or PBS) was used as vehicle providing a total exposure volume of 35 μ l per mouse per day. Endograde ovalbumin, OVA (Hyglos GmbH, Bernried, Germany), was used as allergen. Cholera toxin, CT, (Quadrant Diagnostics Ltd., Surrey, UK) was included as a positive control for adjuvant effect. Purified trypsinized Cry1Ab protein, trypCry1Ab, was purchased from Case Western Reserve University (Dr Marianne Carey, OH, USA). Briefly, the *cry1Ab* gene from the Bt kurstaki HD-1 strain was inserted in recombinant *E. coli*. Inclusion bodies were solubilized at pH 10.5 in the presence of a reducing agent. The precipitated protoxins were digested by commercial bovine trypsin and subsequently purified by ion exchange HPLC. The relevant fractions were analysed by gel filtration, HPLC and SDS-PAGE, desalted and lyophilized (M. Carey, personal communication). For pollen preparation, MON810 (cultivar DKC78-15B) and non-GM (cultivar CRN 3505) maize plants were cultivated under identical conditions in pots in a greenhouse at North-West University. The presence of Cry1Ab in MON810 plants was confirmed with the QuickStix kit for Cry1Ab corn leaf and seed (Envirologix, Portland, ME, USA). Individual plant flowers from the two cultivars were carefully enclosed in plastic bags and shaken to dislodge pollen from flowers 2–3 h before exposure. After sieving twice to remove fragments other than pollen, 5 mg pollen was suspended in 350 μ l sterile HBSS <1 h before exposure. The applied dose of 500 μ g pollen per mice per day was in a pilot study observed to be the highest dose not causing respiratory problems in the mice. Whole leaves were collected from greenhouse-cultivated MON810 (cultivar 6Q-308Bt) and non-GM (cultivar 6Q-121) maize plants. The leaves were enclosed in wet paper and sent by airmail to the Norwegian Institute of Public Health. At arrival, the leaves were cut into 1- to 2-cm-long

Table 1 Exposure schemes for experiment 1 and 2, with doses of test materials and allergen.

Test material Days 0, 1, 2	Allergen Days 0, 1, 2 and 21, 22, 23	Experiment 1	Experiment 2
–	10 µg OVA	x	x
0.5 µg CT	10 µg OVA	x	x
500 µg MON810 pollen	10 µg OVA	x	–
500 µg non-GM pollen	10 µg OVA	x	–
35 µl MON810 leaf extract	10 µg OVA	–	x
35 µl Non-GM leaf extract	10 µg OVA	–	x
0.22, 1.1 or 5.5 µg trypCry1Ab	10 µg OVA	–	x
HBSS or PBS	–	x	x

CT, cholera toxin; OVA, ovalbumin.

pieces before manual grinding with mortar and pestle. Ten millilitre HBSS was added to 1 g of ground leaves, stirred and left overnight at 4 °C. After centrifugation at 877 G for 5 min, the supernates were collected and frozen at –18 °C until the days of exposure. The leaf extracts were not further diluted to give the highest possible concentration of plant Cry1Ab after extraction.

Plant Cry1Ab levels in test suspensions were measured by a semi-quantitative ELISA kit (Agdia, Elkhart, IN, USA) according to the manufacturer's instructions. A twofold dilution standard curve ranging from 0.03–0.96 ng/µl to 3–96 ng/µl trypCry1Ab was included for the quantification of Cry1Ab in pollen and leaf extract, respectively.

Endotoxin levels were measured in the pollen suspension (0.131 EU/ml in both MON810 and non-GM), leaf extracts (0.130 and 0.129 EU/ml for MON810 and non-GM, respectively) and the trypCry1Ab solution (0.046 EU/ml) using ToxinSensor™ Chromogenic LAL endotoxin Assay kit (GenScript, Piscataway, NJ, USA) according to the manufacturers' description. One EU/ml corresponds to 0.1–0.2 ng endotoxin/ml, which correspond to a total dose of <0.003 ng endotoxin per mouse.

Experimental protocol. The mice were intranasally exposed under anaesthesia to 35 µl of test solutions on days 0, 1, 2. On days 21, 22 and 23, all mice, except those in the vehicle control group, were boosted with allergen. A 100-µl blood sample was collected from *vena saphena lateralis* from each animal on day 0 and prior to the challenge on day 21. The mice were terminated by heart puncture under anaesthesia on day 26, and blood and 3 × 0.8 ml BALF were collected as previously described [19] and kept on ice until processing. In experiment 2, also MLNs were collected [20] and MLN single cell suspensions were prepared as previously described [21].

Due to accessibility of fresh pollen from flowering maize plants, experiment 1 was performed at North-West University (Potchefstroom, South Africa). Experiment 2 was performed at the Norwegian Institute of Public Health (Oslo, Norway). Although the experiments were not intended to be replicates as different versions of the Cry1Ab protein were assessed, the same experimental protocol was applied and the experimental

conditions were kept as similar as possible (unless otherwise stated).

Intranasal immunization. For each individual mouse, droplets of the test solution were carefully applied with a pipette onto the left and right nostril repeatedly until the full dose of 35 µl was inhaled. The animals were under anaesthetics and positioned on their backs during the application.

Anaesthetics. In experiment 1, for the intranasal exposures and the exsanguination, an initial dose of 1.4 ml of Halothane (SafeLine Pharmaceuticals (Pty) Ltd., Johannesburg, South Africa) was added to an inhalation chamber and animals were kept in the chamber until sedated. Supplemental doses of 0.4 ml were added when the sedative effect declined. For the intranasal exposures in experiment 2, 3.5% Isoflurane gas (Isoba vet; Intervet/Schering-Plough Animal Health, Lysaker, Norway) were administered in surgical O₂ in an inhalation chamber until sedation. At exsanguination, the mice received ZRF cocktail (18.7 mg Zolazepam, 18.7 mg Tiletamine, 0.45 mg Xylazine and 2.6 mg fentanyl per ml) at 0.1 ml/10 g bodyweight intraperitoneally prior to heart puncture.

OVA-specific antibodies in sera. OVA-specific IgE and IgG1 antibodies in sera were determined in a capture ELISA as previously described [21]. In short, monoclonal rat anti-mouse IgE and IgG1 were used as capture antibodies. Following incubation with optimally diluted test sera (1:10 for IgE and 1:20 000 for IgG1), biotinylated OVA and HRP-conjugated OVA were added in the IgE and IgG1 assays, respectively. In the IgE assay, poly-HRP-streptavidin was added. In both assays, colour development was obtained by the addition of stabilized chromogen TBM. The reaction was stopped with 2N H₂SO₄ solution.

OVA-specific IgG2a antibodies were determined using indirect ELISA as previously described [22]. In short, optimally diluted sera (1:50) were incubated in OVA-coated microtiter plates. Biotinylated rat anti-mouse IgG2a was added. Detection was performed with poly-HRP-streptavidin and stabilized chromogen TBM as described above.

Standard curves were included for all antibody assays on each plate and made from duplicates of diluted IgE

standard (mouse anti-OVA IgE; AbD Serotec, Kidlington, Oxon, UK) or serum pools from mice immunized with OVA and Al(OH)₃ for IgG1 or OVA and CpG oligonucleotides for IgG2a. As the amount of IgG1 or IgG2a in the standards is unknown, their levels are presented as arbitrary units (AU). Absorbance was measured at 450 nm on BioTek Elx808 Absorbance Microplate reader with the Gen5™ Microplate Data Collection & Analysis Software (BioTek® Instruments, Inc., Winooski, VT, USA).

Cytokine analyses. Cytokines in BALF and cell supernates were analysed by Cytometric Bead Array (CBA) flex set kit from BD Biosciences (San Diego, CA, USA). Beads dyed with fluorescence red and covalently coupled to antibodies that capture specific cytokines, allow simultaneous quantification of several cytokines. IL-5, IL-10, IFN γ , TNF α and MCP-1 were measured in BALF from both experiments. IL-10, IFN γ , IL-4, IL-13 and IL-17 were determined in the supernates from mediastinal lymph node cells stimulated with 1 mg/ml OVA and incubated at 37 °C for 120 h, in experiment 2.

Differential counts. Cells (125,000 cells/ml) from a pool of all three BALF collections from each individual animal were spun down on to cytoslides using a cyto centrifuge (Shandon Scientific Ltd., Astmoor, Runcorn, UK) at 70 G for 6 min and stained by the Hemacolor rapid staining according to the manufacturer's protocol (Merc KGaA, Darmstadt, Germany). A total number of 200 cells were counted on each slide in a light microscope (Axioplan2, Zeiss, Göttingen, Germany) at 100 \times magnification, separating macrophages, eosinophils, neutrophils, lymphocytes and epithelial cells based on their morphological characteristics. Cell differential counts for all animals were performed blindly by the same investigator (MA).

Statistical analyses. Statistical analyses were performed with SigmaPlot 12.3 and Minitab 16 Statistical software. When necessary, data were log₁₀-transformed to adjust for non-normal distributions and to obtain comparable vari-

ances. All normally distributed parameters with comparable variances were tested by one-way ANOVA. In the case of significant overall differences, pairwise comparisons between treatment and control were performed with the Dunnett's *post hoc* test. Data that could not be transformed to normal distribution and comparable variances were tested with the nonparametric Kruskal–Wallis test, using Mann–Whitney *post hoc* tests for pairwise comparisons between treatments versus control. Differences between groups were considered significant when *P*-values were < 0.05.

Results

Cry1Ab exposure dose

Based on the measured concentrations (6.92 and 612.51 ng Cry1Ab in 1 mg of pollen and leaf, respectively), the calculated total dose of Cry1Ab given per mice exposed to pollen or leaf extracts was 0.01 μ g and 6.43 μ g, respectively. The total dose of trypCry1Ab was 0.66, 3.3 and 16.5 μ g per mice.

OVA-specific antibodies in serum

OVA-specific antibodies were analysed in sera collected on day 0, before booster on day 21 and at termination on day 26. In experiment 1, the levels of OVA-specific IgE, IgG1 and IgG2a were below or just above the limit of detection for all mice on day 0 (data not shown). On day 21, the levels of OVA-specific antibodies remained low for all mice in all treatment groups except in the positive control group that received OVA together with CT (data not shown). After allergen booster, the levels of OVA-specific IgE and IgG1 in the OVA + CT group were significantly (*P* < 0.05) enhanced compared to the OVA group on day 26 (Fig. 1). A few animals in the OVA + CT group showed elevated levels of OVA-specific IgG2a on day 26,

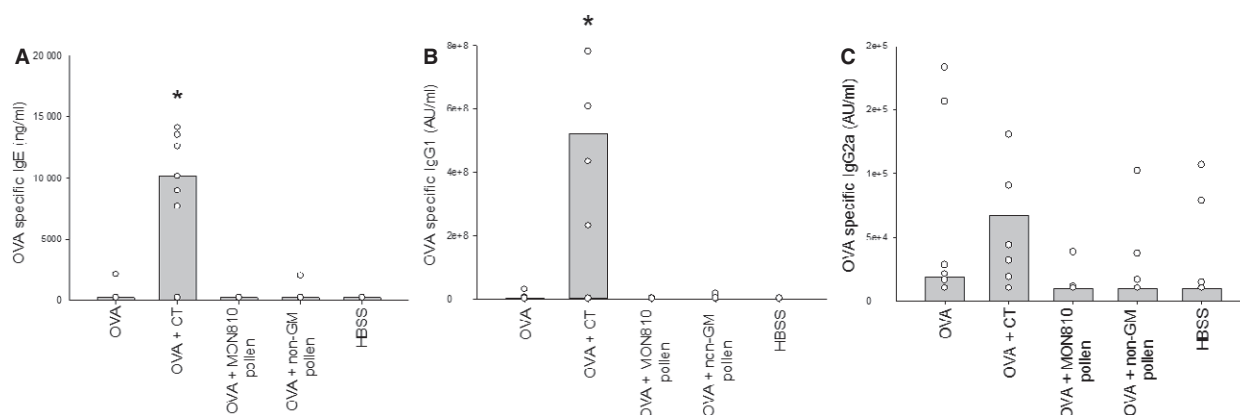


Figure 1 sjiIntranasal exposure of pollen from MON810 and non-GM maize did not promote OVA-specific antibody responses in mice. Allergen (OVA)-specific IgE (A), IgG1 (B) and IgG2a (C) in serum on day 26 after intranasal exposure of mice to allergen and fresh maize pollen (day 0, 1 and 2), followed by OVA boosters (day 21, 22 and 23). Each circle represents one individual animal, and bars represent group medians (*n* = 8). Asterisks (*) denote groups that are significantly different (*P* < 0.05) from the OVA control group.

but no significant elevation was observed on group level. The OVA + CT group also showed elevated levels of OVA-specific IgG2a on day 26 although not statistically significant. Pollen from GM and non-GM plants did not increase antibody production in mice, as none of the two groups receiving pollen demonstrated significant elevated OVA-specific IgE, IgG1 or IgG2a responses. In experiment 2, OVA-specific IgE, IgG1 and IgG2a levels were low, often below the detection limits, for all mice on day 0, and, with the mice exposed to OVA + CT as an exception, also on day 21 (data not shown). At termination on day 26, the levels of OVA-specific IgE, IgG1 and IgG2a antibodies were significantly higher for the mice in the OVA + CT group than for the OVA-exposed mice (Fig. 2). The mice that received OVA together with leaf extract appeared to have a moderate (non-significantly) rise in OVA-specific IgE and IgG1 serum levels; the response was, however, at the same level for the GM and the non-GM leaf extracts. The IgG2a response was somewhat (not significantly) elevated in 2–3 mice that received OVA + non-GM leaf extract.

Cytokines in BALF

In experiment 1, the BALF levels of IL-5, TNF α , IFN γ and MCP-1 were low for all mice in all groups, and not significantly different in mice from the different treatment groups (Fig. 3). The level of IL-10 was below the limit of detection (data not shown). Also, in experiment 2, the levels of IFN γ , TNF α , MCP-1 and IL-10 in BALF were below or just above the detection limit in all mice, irrespective of exposure scheme and no differences were detected between the groups (data not shown).

Cell differential counts

In experiment 2, the relative number of neutrophils, lymphocytes, macrophages and eosinophils in BALF collected day 26, were determined (Fig. 4). Mice exposed to OVA + CT had significantly higher numbers of eosinophils in the BALF than mice exposed to OVA alone. The increased levels of macrophages and decreased numbers of neutrophils seen in animals exposed to OVA + leaf extract cannot be associated with an adjuvant effect of leaf extracts, because a similar pattern was also demonstrated for animals exposed to vehicle alone.

Ex vivo cytokine release from mediastinal lymph node (MLN) cells

In experiment 2, OVA-stimulated MLN cells from the mice in the OVA + CT group demonstrated significantly increased releases of all cytokines measured, that is IL-4, IL-17, IL-13, IL-10 and IFN γ (Fig. 5). The OVA-stimulated MLN cells from mice exposed to OVA + non-GM leaf extract consistently demonstrated elevated secretion of IL-4, IL-13, IL-10 and IFN γ cytokines compared to cells from mice receiving OVA alone, although at lower levels than for the mice exposed to OVA + CT. The exposure of mice to trypsinized Cry1Ab protein or MON810 leaf extract did not affect cytokine release from MLN cells.

Discussion

We have investigated whether Cry1Ab protein after intranasal exposure can act as an adjuvant for allergic sensitization to a protein allergen (OVA) in a mouse

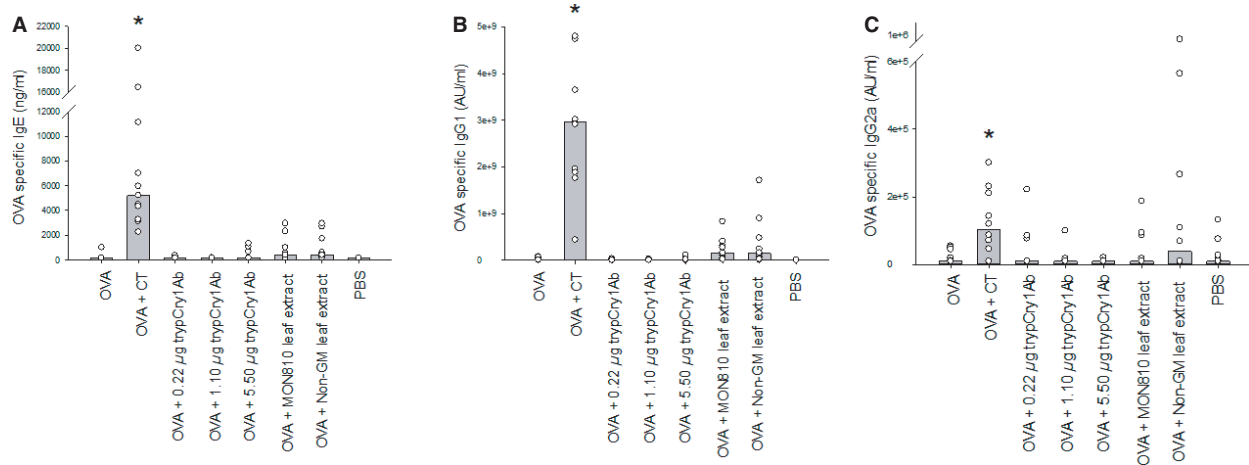


Figure 2 Intranasal exposure of trypsinized Cry1Ab and leaf extracts from MON810 and non-GM maize did not promote OVA-specific antibody responses in mice. Allergen (OVA)-specific IgE (A), IgG1 (B) and IgG2a (C) in serum on day 26 after intranasal exposure of mice to OVA and three doses of trypsinized Cry1Ab (trypCry1Ab), or leaf extracts from MON810 and non-GM maize leaves (day 0, 1 and 2), followed by OVA boosters (day 21, 22 and 23). Each circle represents one individual animal, and bars represent group medians ($n = 10$). Asterisks (*) denote groups that are significantly different ($P < 0.05$) from the OVA control group.

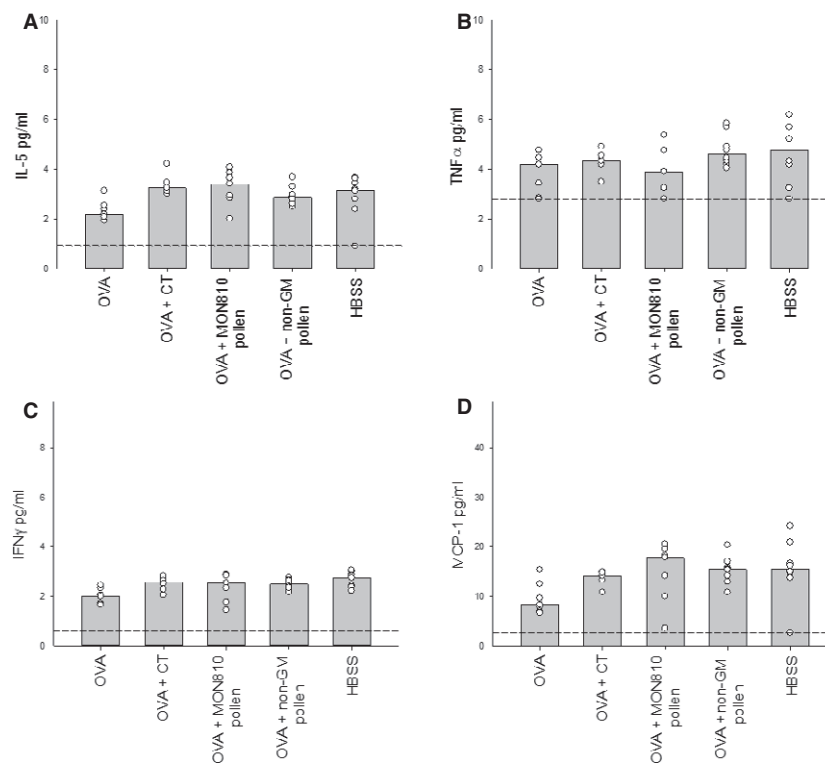


Figure 3 Intranasal exposure of pollen from MON810 and non-GM maize did not affect cytokine levels in BALF. Cytokine IL-5 (A), TNF α (B) IFN γ (C) and MCP-1 (D) levels in broncho-alveolar lavage fluid (BALF) collected on day 26 after intranasal exposure of mice to allergen (OVA) and fresh maize pollen (day 0, 1 and 2), followed by OVA boosters (day 21, 22 and 23). Each circle represents one individual animal, and bars represent group medians ($n = 8$). The dotted lines indicate the lower quantitative detection limit of the assays.

model of airway allergy. Three different sources of Cry1Ab were used: (i) pollen from *cry1Ab*-transgenic maize (event MON810); (ii) leaf extracts from MON810 plants; and (iii) trypsin-activated Cry1Ab produced in recombinant *E. coli*. Homologous materials from an unmodified, near-isogenic maize variety, and the known mucosal Th2 adjuvant, CT, were also included in the test schemes. Immune responses induced by intranasal exposure to OVA in combination with each Cry1Ab protein preparation were compared with those induced by OVA alone or together with CT. Clear proallergic adjuvant effects of CT were demonstrated in the two independent experiments, determined as increased allergen-specific IgE in serum, lung eosinophils and Th2 cytokines in MLN cell supernates after OVA stimulation. Neither the trypCry1Ab protein nor pollen or leaf extract from MON810 maize plants promoted systemic immune responses against OVA, as shown by low levels of all endpoints. These findings, although a different route of exposure was applied, are in line with the recently published study by Reiner *et al* [17]. In that study, feeding of MON810-containing diets for up to 34 days did not affect OVA-induced eosinophilic lung inflammation, or OVA-specific antibody production, using a mouse model of allergic asthma. The authors

concluded on the lack of adjuvant effect upon GM maize consumption on both the onset and severity of allergic responses.

The Cry1Ac protein in its protoxin form has been reported to exert a strong adjuvant effect, as potent as CT [18]. Cry1Ac promoted specific antibody responses towards other proteins, HIV peptides and polysaccharides when administered via the intraperitoneal, intragastric and intranasal route [18, 23]. Cry1Ab can be considered a natural chimera between Cry1Aa (domain III) and Cry1Ac (domains I and II) [24]. Hence, there are reasons to believe that the C-terminally truncated Cry1Ab protein expressed in MON810 maize, mainly composed of domains I and II, may possess adjuvant properties as well. Interestingly, in our study, at the doses employed, neither the plant material from *cry1Ab*-transgenic MON810 maize nor the purified, trypsinized bacterial Cry1Ab protein exhibited an adjuvant effect similar to that observed with CT or reported for Cry1Ac. Cry-proteins produced in recombinant *E. coli* may be contaminated with endotoxin, which have been shown both to promote and inhibit allergic responses dependent on dose and timing [25]. The presence of endotoxins could explain the strong adjuvant effect found for Cry1Ac produced in *E. coli* in the Vazquez *et al.* study [18]; however, the endotoxin levels were never

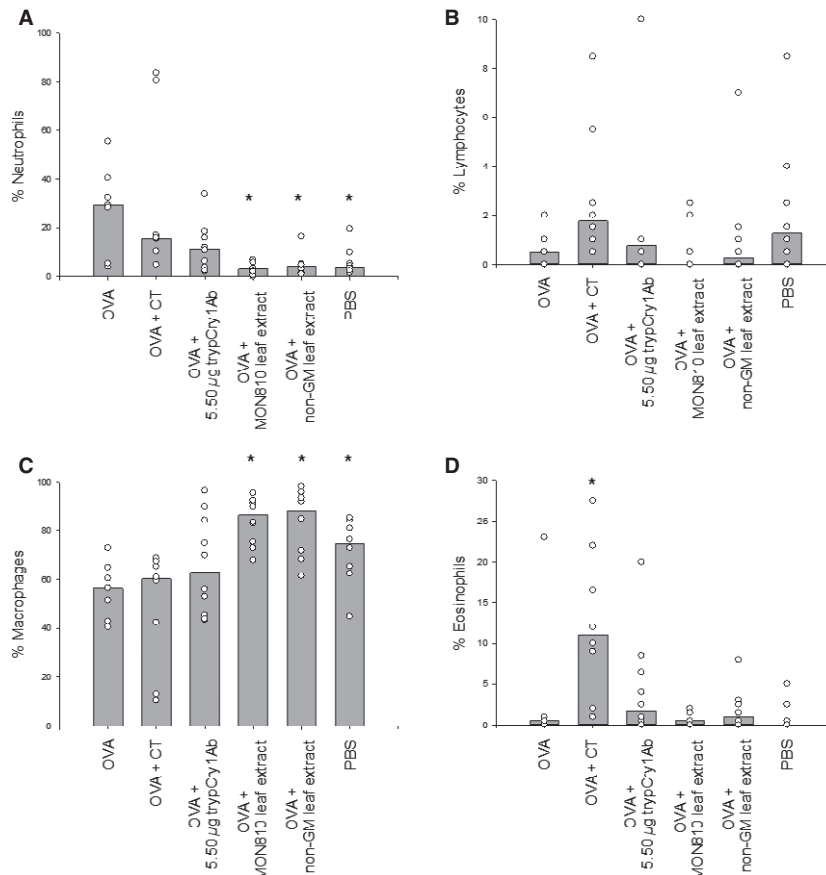


Figure 4 Intranasal exposure of trypsinized Cry1Ab and leaf extracts from MON810 and non-GM maize did not affect the proportion of immune cells in BALF. Percentages (%) of neutrophils (A) lymphocytes (B), macrophages (C) and eosinophils (D) in bronco-alveolar lavage fluid (BALF) collected on day 26 after intranasal exposure of mice to allergen (OVA) and trypsinized Cry1Ab (trypCry1Ab), or leaf extracts from MON810 and non-GM maize leaves (day 0, 1 and 2), followed by OVA boosters (day 21, 22 and 23). Each circle represents one individual animal, and bars represent group medians ($n = 10$). Asterisks (*) denote groups that are significantly different ($P < 0.05$) from the OVA control group.

reported. In our study, the endotoxin levels in the test solution gave a total dose of <0.003 ng per mouse, a dose that is much lower than the dose (100 ng LPS) referred to as an inhibitor of Th2 lung inflammation [26]. We therefore do not expect that the endotoxin level in our samples was interfering with the negative results on Cry1Ab adjuvant effect.

To address the issue that Cry1Ab expressed in transgenic plants has not been extracted and purified in sufficient amounts to enable animal exposure experiments, we included Cry1Ab-containing plant material (pollen and leaf extracts from MON810 maize) as well as Cry1Ab produced in *E. coli*, in our studies. After trypsinization, the latter resembles the truncated, insecticidal protein, that is the expected expression product in the MON810 maize. Using BALB/c mice, it has previously been demonstrated possible adjuvant properties of the trypsinized Cry1Ab on the elicitation, but not the sensitization, of allergic airway reaction after oral exposure of trypCry1Ab and peanut protein. It was evidenced by Th2/Th17 cytokine release

and eosinophil influx in BALF after allergen challenge in the airways [16]. However, our data from intranasal exposure to trypCry1Ab in BALB/c mice do not support an adjuvant effect after allergen airway challenge: While the OVA + CT-exposed mice had a significant eosinophilic infiltration in BALFs, we could not detect local airway effects after the intranasal Cry1Ab exposure, regardless of Cry1Ab source. Compared to mice receiving OVA alone, the mice exposed to OVA + leaf extract demonstrated significantly enhanced macrophage and decreased neutrophil percentages. However, as this was also the case for the control animals that received vehicle only, it illustrated that OVA itself induced some changes to the BALF cell composition in this model. Furthermore, cytokine levels in BALFs were low and no difference between treatments was found. The fact that mice receiving CT + OVA did not have elevated BALF levels of the selected cytokines could reflect that either the choice of cytokines or time point of BALF collection were not optimal. Cytokines after allergen booster have previously been found to have maximum

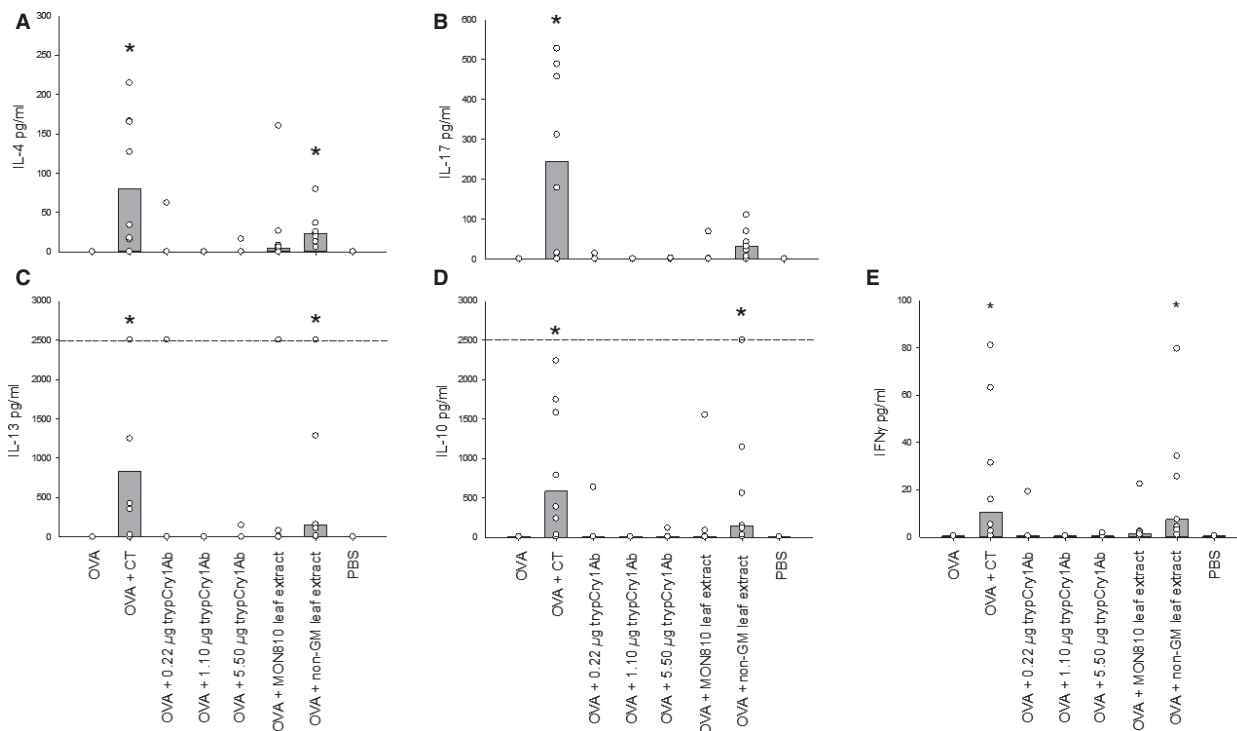


Figure 5 Intranasal exposure of trypsinized Cry1Ab and leaf extracts from MON810 and non-GM maize did not promote cytokine secretion from MLN cells after OVA stimulation. Cytokine IL-4 (A), IL-17 (B), IL-13 (C), IL-10 (D) and IFN γ (E) secretion from mediastinal lymph node (MLN) cells collected on day 26 after intranasal exposure of mice to allergen (OVA) and three doses of trypsinized Cry1Ab (trypCry1Ab), or leaf extracts from MON810 and non-GM maize leaves (day 0, 1 and 2), followed by OVA boosters (day 21, 22 and 23). Each circle represents one individual animal, and bars represent group medians ($n = 10$). The dotted lines indicate the upper quantitative detection limit of the assays. Asterisks (*) denote groups that are significantly different ($P < 0.05$) from the OVA control group.

expression in BALF ≤ 24 h [27]. Furthermore, we gave a maximum total of 16.5 μg trypCry1Ab per mouse intranasally, while the total dose given perorally by Guimaraes and colleagues was 50 μg trypCry1Ab per mouse. The discrepancy in both dose and exposure route may also contribute to the different findings.

Based on an *in vitro* study, Allakhverdi *et al.* [28] suggested that pollen grains themselves may act as adjuvants on allergic immune responses by inducing activation and maturation of dendritic cells. In our *in vivo* study, we did not observe any indications of adjuvant effects exerted by the pollen grains themselves. Dry airborne pollen might, however, have different adjuvant capacity than the vehicle-suspended pollen applied in the present study. The leaf extracts with OVA, and the non-GM extracts in particular, however, appeared to enhance cytokine release from MLN cells. As the extracts were prepared from whole leaves with all constituents present, this cocktail or single element within the cocktail may be responsible for the altered immune responses observed.

Standardized or validated methods to systematically evaluate the effect of adjuvants are not yet available. Animal models are considered robust for hazard identification in the weight-of-evidence approach for GMO issues

recommended by EFSA, and in particular regarded as helpful in addressing the issue of protein immunogenicity or adjuvanticity [29]. Our study design, including a positive control for adjuvant effect and Cry1Ab protein in three different forms, strengthen our ability to conclude on the lack of systemic adjuvant effects.

Taken together, Cry1Ab protein from three different sources did not act as an adjuvant in our mouse model under the experimental conditions used. Although the contents in our MON810 maize tissues may represent 'relevant doses', long-term exposures of plant Cry1Ab as well as purified plant proteins to mimic the total exposures experienced in real-life situations, should be included in future studies.

Acknowledgment

We gratefully acknowledge the technical help and skills of Åse Eikeset, Bodil Hasseltvedt, Berit Stensby, Else-Carin Groeng, Astri Grestad, Trude Olsen, Kari Løken and Henrik Rasmussen at Norwegian Institute of Public Health, and Cor Bester and Antoinette Fick at North-West University. The study was funded by GenØk – Centre for Biosafety and Norwegian Institute of Public Health.

References

- 1 Ladics GS, Cressman RF, Herouet-Guicheney C *et al.* Bioinformatics and the allergy assessment of agricultural biotechnology products: industry practices and recommendations. *Regul Toxicol Pharmacol* 2011;60:46–53.
- 2 Prescott VE, Hogan SP. Genetically modified plants and food hypersensitivity diseases: usage and implications of experimental models for risk assessment. *Pharmacol Ther* 2006;111:374–83.
- 3 Randhawa GJ, Singh M, Grover M. Bioinformatic analysis for allergenicity assessment of *Bacillus thuringiensis* Cry proteins expressed in insect-resistant food crops. *Food Chem Toxicol* 2011;49:356–62.
- 4 Taylor SL, Hefle SL. Will genetically modified foods be allergenic? *J Allergy Clin Immunol* 2001;107:765–71.
- 5 Brunner R, Jensen-Jarolim E, Pali-Scholl I. The ABC of clinical and experimental adjuvants—a brief overview. *Immunol Lett* 2010;128:29–35.
- 6 Hilbeck A, Schmidt JEU. Another view on Bt proteins - how specific are they and what else might they do. *Biopestic Int* 2006;2:1–50.
- 7 Kitami M, Kadotani T, Nakanishi K *et al.* *Bacillus thuringiensis* Cry toxins bound specifically to various proteins via domain III, which had a galactose-binding domain-like fold. *Biosci Biotechnol Biochem* 2011;75:305–12.
- 8 Then C. Risk assessment of toxins derived from *Bacillus thuringiensis*-synergism, efficacy, and selectivity. *Environ Sci Pollut Res Int* 2010;17:791–7.
- 9 Bravo A, Gill SS, Soberon M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 2007;49:423–35.
- 10 Vachon V, Laprade R, Schwartz JL. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. *J Invertebr Pathol* 2012;111:1–12.
- 11 Guerrero GG, Dean DH, Moreno-Fierros L. Structural implication of the induced immune response by *Bacillus thuringiensis* Cry proteins: role of the N-terminal region. *Mol Immunol* 2004;41:1177–83.
- 12 Kroghsbo S, Madsen C, Poulsen M *et al.* Immunotoxicological studies of genetically modified rice expressing PHA-E lectin or Bt toxin in Wistar rats. *Toxicology* 2008;245:24–34.
- 13 Finamore A, Roselli M, Britti S *et al.* Intestinal and peripheral immune response to MON810 maize ingestion in weaning and old mice. *J Agric Food Chem* 2008;56:11533–9.
- 14 Walsh MC, Buzoianu SG, Gardiner GE *et al.* Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. *PLoS ONE* 2011;6:e27177.
- 15 Sagstad A, Sanden M, Haugland O, Hansen AC, Olsvik PA, Hemre GI. Evaluation of stress- and immune-response biomarkers in Atlantic salmon, *Salmo salar* L., fed different levels of genetically modified maize (Bt maize), compared with its near-isogenic parental line and a commercial suprex maize. *J Fish Dis* 2007;30:201–12.
- 16 Guimaraes VD, Drumare MF, Ah-Leung S *et al.* Comparative study of the adjuvanticity of *Bacillus thuringiensis* Cry1Ab protein and cholera toxin on allergic sensitization and elicitation to peanut. *Food Agric Immunol* 2008;19:325–37.
- 17 Reiner D, Lee RY, Dekan G, Epstein MM. No adjuvant effect of *Bacillus thuringiensis*-maize on allergic responses in mice. *PLoS ONE* 2014;9:e103979.
- 18 Vazquez RI, Moreno-Fierros L, Neri-Bazan L, De La Riva GA, Lopez-Revilla R. *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant. *Scand J Immunol* 1999;49: 578–84.
- 19 Samuelsen M, Nygaard UC, Lovik M. Particle size determines activation of the innate immune system in the lung. *Scand J Immunol* 2009;69:421–8.
- 20 Van den Broeck W, Derore A, Simoens P. Anatomy and nomenclature of murine lymph nodes: descriptive study and nomenclatory standardization in BALB/cAnNCrI mice. *J Immunol Methods* 2006;312:12–9.
- 21 Hansen JS, Alberg T, Rasmussen H, Lovik M, Nygaard UC. Determinants of experimental allergic responses: interactions between allergen dose, sex and age. *Scand J Immunol* 2011;73:554–67.
- 22 Nygaard UC, Samuelsen M, Aase A, Lovik M. The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. *Toxicol Sci* 2004;82:515–24.
- 23 Moreno-Fierros L, Ruiz-Medina EJ, Esquivel R, Lopez-Revilla R, Pina-Cruz S. Intranasal Cry1Ac protoxin is an effective mucosal and systemic carrier and adjuvant of *Streptococcus pneumoniae* polysaccharides in mice. *Scand J Immunol* 2003;57:45–55.
- 24 Bravo A. Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. *J Bacteriol* 1997;179:2793–801.
- 25 Zhu Z, Oh SY, Zheng T, Kim YK. Immunomodulating effects of endotoxin in mouse models of allergic asthma. *Clin Exp Allergy* 2010;40:536–46.
- 26 Delayre-Orthez C, de Blay F, Frossard N, Pons F. Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin Exp Allergy* 2004;34:1789–95.
- 27 Busse PJ, Zhang TF, Srivastava K, Schofield B, Li XM. Effect of ageing on pulmonary inflammation, airway hyperresponsiveness and T and B cell responses in antigen-sensitized and -challenged mice. *Clin Exp Allergy* 2007;37:1392–403.
- 28 Allakhverdi Z, Bouguermouh S, Rubio M, Delespesse G. Adjuvant activity of pollen grains. *Allergy* 2005;60:1157–64.
- 29 EFSA. Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. Panel on Genetically Modified Organisms. European Food Safety Authority 2010; rapport.