

Oral Sensitization to Peanut Is Highly Enhanced by Application of Peanut Extracts to Intact Skin, but Is Prevented when CpG and Cholera Toxin Are Added

Karine Adel-Patient^a Sandrine Ah-Leung^a Hervé Bernard^a
Coralie Durieux-Alexandrenne^b Christophe Créminon^b Jean-Michel Wal^a

^aLaboratoire Inra d'Immuno-Allergie Alimentaire et ^bLaboratoire CEA d'Etude et de Recherche en Immuno-Analyse, CEA de Saclay, Gif-sur-Yvette, France

Key Words

Immunotherapy · Peanut allergy · Intact skin · CpG

Abstract

Background: CpG oligonucleotides might offer an alternative to conventional immunotherapy in preventing and potentially reversing Th2-biased immune deregulation which leads to allergy. However, non-invasive ways of administration, especially in peanut-allergic patients, should be explored. **Methods:** One hundred micrograms of whole peanut protein extract (PE) alone, or mixed with cholera toxin (CT, 50 µg) plus CpG (100 µg) as adjuvant, was applied on intact skin of mice (40 min, twice). Initiation of an immune response was monitored by detection of specific antibodies in sera. The effect of this pretreatment on a further oral sensitization by PE was then evaluated by assaying antibodies and cytokines specific for PE and purified allergens. Cytokine production in liver 40 min after skin application was also assayed. **Results:** Two brief skin applications of PE alone highly potentiated further oral sensitization, as demonstrated by very intense specific IgE, IL-4 and IL-5 productions. Conversely, skin pretreatment with PE and CT + CpG efficiently prevented further sensitization via gastro-intestinal exposure. In both cases, the specificity of the antibodies and cytokines was the same as in control mice. CT + CpG treatment allowed the rapid production of IL-12 and TGFβ in liver

and of specific IgG2a in sera, suggesting the activation of Th1 and/or regulatory T cells. **Conclusions:** Oral sensitization to peanut is highly enhanced by a previous short exposure of allergens to intact skin. Conversely, the use of CT + CpG adjuvant for skin application efficiently prevents further oral sensitization. The potential of such treatment in specific immunotherapy needs to be evaluated.

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Introduction

Classical immunotherapy with allergen extracts, i.e. the induction of a tolerance by injection of increasing concentrations of allergens, is neither effective nor totally safe for the treatment of food allergies, and might present associated risks such as life-threatening anaphylaxis, especially for peanut-allergic patients [1]. Non-invasive administration of peanut allergens in such a way as to induce tolerance and/or Th1 response, then counterbalancing the allergic response, appears to be of great interest. Recent studies suggest that immunostimulatory sequences of oligonucleotides might offer an alternative way to prevent and potentially reverse Th2-biased immune deregulation that leads to allergy. Those sequences comprise unmethylated CpG motifs, which are abundant in bacterial DNA and are recognized by specific endosomal

toll-like receptor 9 (TLR9). This recognition induces an innate immune response via the production of pro-inflammatory cytokines (type I interferons, IL-6 and TNF α) by monocytes, dendritic cells (DC) and macrophages [see 2, for review]. CpG have also been shown to be an effective Th1 adjuvant which triggers Th1 and B cells to produce the Th1-polarizing cytokine IL-12 [3, 4]. Immunization with CpG simultaneously with, or conjugated to, the allergen attenuated allergic sensitization and/or reaction in mice [5]. Although redirection of a pre-established Th2 response failed in some studies [6, 7], others demonstrated 2- to 4-fold reductions in Th2 cytokine production concomitant with increased IFN γ levels [8, 9] or redirection of the immune response in animals with established eosinophilia [10]. Therapeutic administration of the ragweed allergen Amb a 1 conjugated to CpG in mice was demonstrated to be more efficient than traditional immunotherapy as it induced a stronger Th1 response [11]. In humans, subcutaneous administration of the same conjugate leads to a prolonged shift from Th2 to Th1 response and appeared to be safe [12].

The skin-associated lymphoid tissue, including powerful antigen-presenting cells such as immature Langerhans cells (LC) of the epidermis and DC of the dermis, recirculating T cells and regional lymph nodes, ensures efficient presentation of antigens to immune cells, which results in the induction of a specific immune response. TLR9 expression by skin cells is uncertain in mice: epidermal LC but not keratinocytes have been demonstrated to express TLR9 in Balb/c mice [13], whereas TLR9 was demonstrated to be absent in normal Balb/c mouse ear skin, its presence being induced after intradermal injection [14]. The intradermal injection of CpG in Balb/c mice leads to epidermal LC activation which results in phenotypic changes and specific regulation of adhesion molecule surface expression involved in LC/DC migration [15], enhancement of MHC II and expression of costimulatory molecules, and production of IL-12 [16]. Conversely, CpG had no effect on cytokine production or cell surface molecule expression by primary murine keratinocytes [16]. Other cells can be involved in the induction of a specific immune response after skin exposure. Recent publications on contact sensitization suggest that skin application of a hapten induces a rapid IL-4 production by iNKT cells in liver, allowing activation of peritoneal B-1 cells to produce specific IgM within 1 day [17].

The effect of a deposit of allergens on intact skin in the presence or absence of CpG, i.e. non-invasive administration, remains to be explored especially when considering peanut-allergic patients. In this context, cholera toxin

(CT), an ADP-ribosylating exotoxin from *Vibrio cholerae*, is of interest as it allows immunization with various proteins of a molecular weight above 60 kDa, using a brief application on the skin without physical alterations [18]. Systemic toxicity observed after mucosal administration of CT was not observed after application to intact skin. Moreover, CT and CpG have a synergistic effect on both humoral and cell-mediated specific Th1 response in mice immunized by skin application of synthetic peptides from influenza virus [19].

In the present study, we then aimed to evaluate for the first time the effect of the application of whole peanut protein extract (PE) alone, or mixed with CT and CpG as adjuvant, on the intact skin of mice. Initiation of an immune response was monitored by analyzing induced specific antibodies in sera. The effect of this pretreatment on a further sensitization was evaluated using a high IgE responder Balb/c mouse model orally sensitized with peanut [20]. Mechanisms of potentiation/suppression of the immune response were then evaluated by cytokine measurement in liver 40 min after skin application.

Materials and Methods

Apparatus

All enzyme immunometric assays were performed in 96-well microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialized Titertek microtitration equipment from Labsystems (Helsinki, Finland).

Reagents

Unless otherwise stated, all reagents were of analytical grade from Sigma (St. Louis, Mo., USA).

Phosphorothioate-stabilized CpG oligonucleotide (ODN) 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3'; CpG underlined [21]) and control non-CpG-ODN (5'-TCC AAT GAG CTT CCT GAG TCT-3' [22]) were synthesized by MWG Biotech France (Courtaboeuf, France), with the HPSF[®] purification grade (high purity salt free). CT was obtained from List Biological Laboratories (Campbell, Calif., USA).

Peanut and PE

After shelling, kernels of commercially roasted peanuts were ground. Proteins were extracted for 4 h at room temperature in deionized water adjusted to pH 9. After centrifugation (1,000 g, 15°C, 10 min), the supernatant was collected, filtered, and dialyzed against phosphate buffer (pH 7.4) yielding the whole peanut PE. Protein content of PE was 17 mg of protein/ml, as determined with the BCA assay (Pierce). Endotoxin levels were below 0.06 ng/ml (E-Toxate kit, assuming 1 UE = 0.1 ng of endotoxin).

Purified allergens were obtained from PE after ammonium sulphate precipitation, and combination of successive chromatographic separation using concanavalin A affinity, anion exchange and reversed-phase liquid chromatography. Isolated fractions

were characterized by electrophoresis, MALDI-TOF mass spectrometry using a Voyager DE RP apparatus (PE Biosystems) and determination of N-terminal sequence using the Edman method. Ara h 1, Ara h 2, Ara h 6 and polymer of glycinin fragments, containing proteolytically processed Ara h 3/4 polypeptides, were then obtained [23], with >95% purity grade and undetectable endotoxin levels (E-Toxate kit).

Mouse Immunization

Female Balb/c mice were from CERJ (Centre d'Élevage René Janvier, Le Genest-Saint-Isle, France), and were housed under normal husbandry conditions. All experiments were performed according to the European Community rules of animal care, and with permission 91-122 of the French Veterinary Services. Mice were acclimated for 2 weeks before immunizations, and were used when 6 weeks old.

Pretreatment with CpG and PE on Intact Skin

On days 1 and 14, mice were anesthetized by intraperitoneal administration of ketamine (100 mg/kg; Imalgène 500, Merial, Lyon, France) and xylazine (10 mg/kg; Rompun 2%, Bayer Pharma, Puteaux, France). They were anaesthetized for approximately 1 h to prevent self-grooming, i.e. oral immunization. Hair on the abdomen was removed using a depilatory cream (Veet, Reckitt Benckiser, Massy, France), and skin was gently cleaned with water. For each mouse, 30 µl of a solution containing PE alone (100 µg of protein), 100 µg of PE plus 100 µg of CpG and 50 µg of CT, or 100 µg of PE plus 100 µg of non-CpG and 50 µg of CT were applied to the bare skin for 40 min (5 mice/group). Skin was then gently cleaned with water. Control mice received saline as pretreatment and were considered as positive controls for further sensitization.

Mouse Sensitization

On days 31, 37, 43, 49 and 55, pretreated mice received PE (5 mg of protein/mouse) mixed with CT (9 µg/mouse) by means of gavage to induce peanut-specific sensitization [20]. Sera were collected on days 29 (before sensitization), 41, 48 and 62. Faeces were also collected on days 38, 44, 50 and 56.

Semiquantification of Anti-PE IgE, IgG1 and IgG2a

These assays consisted of semiquantitative immunoassays [20, 24]. Specific IgE were assayed on pooled sera, whereas specific IgG1 and IgG2a were determined on individual sera (n = 5/group).

Specificity of the IgE, IgG1 and IgG2a

Ara h 1, Ara h 2, Ara h 6, glycinin polymer (i.e. Ara h 3) and PE were coated on 96-well plates as described [20]. Sera from the same group and the same sampling date were pooled and diluted in EIA buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin, 0.15 M NaCl, 0.01% sodium azide; from 1/50 to 1/10⁶, depending on the isotype). Sera were incubated for 18 h at +4°C on purified allergen-coated plates. After washing, acetylcholinesterase (AChE)-labelled anti-IgE, anti-IgG1 or anti-IgG2a was added, and plates were incubated for 18 h at +4°C. Plates were then extensively washed and solid phase-bound AChE activity was determined by addition of Ellman's medium. Results are reported as absorbance units at 414 nm.

IgA Assay

Specific IgA were determined on sera and faecal samples. Sera (1/50) or faeces (1/15) diluted in EIA buffer plus 0.1% Tween were incubated on plates coated with PE. Specific IgA were detected using goat anti-mouse IgA (Southern Biotechnology Associated, Birmingham, Ala., USA) labelled with AChE, and detected as above. Results are reported as absorbance units at 414 nm.

Cytokine Production

After the last blood sampling, mice were sacrificed by vertebral dislocation. Spleens were harvested under sterile conditions. After lysis of red blood cells (180 mM NH₄Cl, 17 mM Na₂EDTA) and several washes, splenocytes were resuspended in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin. Cells were incubated in 96-well culture plates (10⁶ cells/well) in the presence of Ara h 1, Ara h 2, Ara h 6, glycinin polymer or PE (20 µg protein/ml), or PBS or ovalbumin (20 µg/ml; negative control), or concanavalin A (1 µg/ml, positive control) for 60 h at 37°C (5% CO₂). Supernatants were then removed and stored at -80°C until further assay.

IL-4, IFNγ and TGFβ were assayed using CytoSet™ kits (BioSource International Europe, Nivelles, Belgium) according to the manufacturer's instructions. IL-5 was assayed using an immunometric assay described elsewhere [25], using monoclonal antibodies TRFK4 for capture and AChE-labelled TRFK5 monoclonal antibodies as tracer. IL-10 assay was performed as previously described [26].

Liver Extract

Mice were anaesthetized and treated by skin application of PE, PE + CpG, PE + non-CpG, PE + CT, PE + CT + CpG or PE + CT + non-CpG as described. Forty minutes after skin application, mice were sacrificed and the liver was perfused via the portal vein until opaque with phosphate buffer containing 50 µg/ml bacitracin, 300 µg/ml benzamidine, 80 µg/ml leupeptin, 20 µg/ml chymostatin and 25 µg/ml pepstatin. Individual livers were homogenized in an Ultraturrax and then sonicated and centrifuged (6,000 g, 20 min, +4°C). Supernatant, containing soluble proteins, was aliquoted and kept at -80°C until further assay. Individual samples were assayed for protein content (BCA assay, Pierce) and cytokines IL-4, IL-12, IL-13, TGFβ, TNFα and IFNγ (CytoSet kits), IL-5 [25] and IL-10 [26].

Statistical Analysis

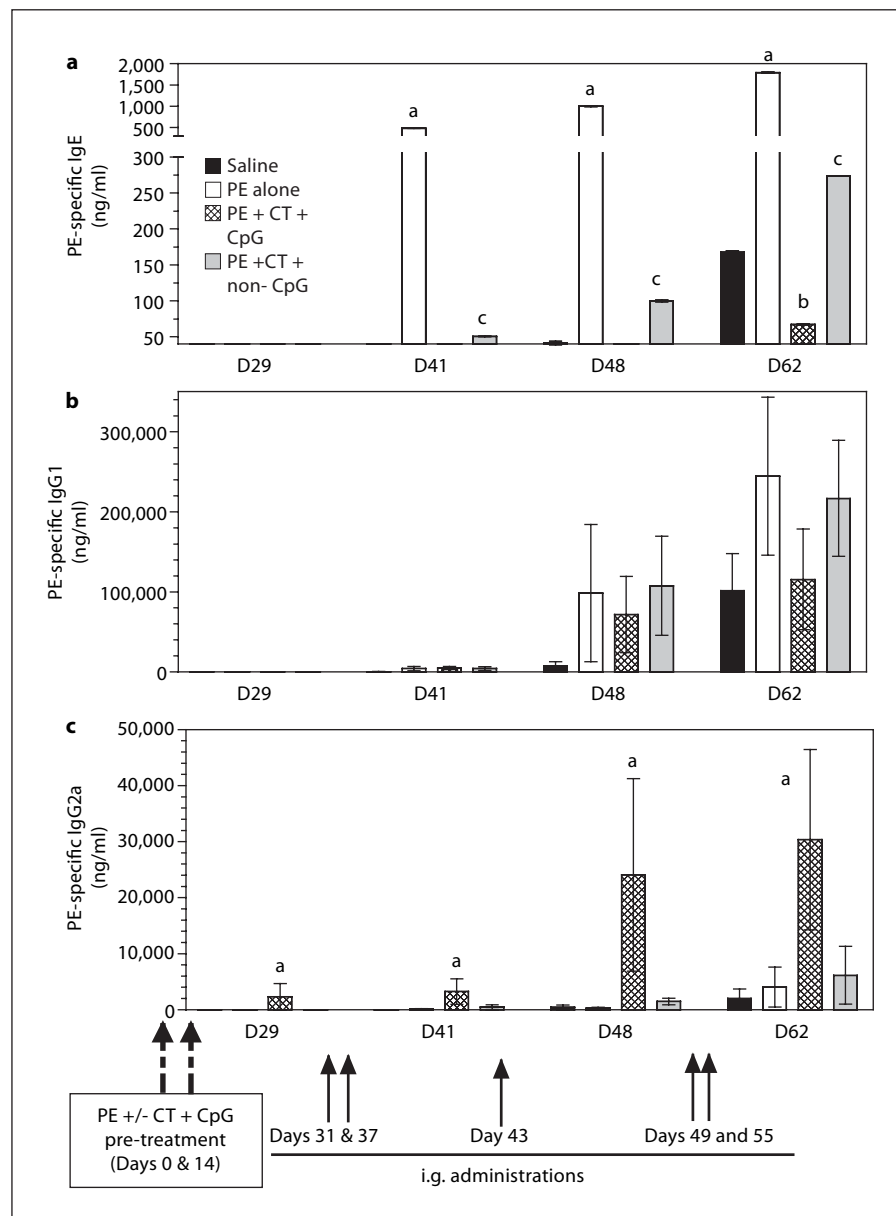
Statistical analyses were conducted with GraphPad software by comparing all the groups with each other using analysis of variance (ANOVA) and Tukey's post-test.

Results

Anti-PE Specific IgE, IgG1 and IgG2a after Skin Treatments

As shown in figure 1, no anti-PE-specific IgE (fig. 1a), IgG1 (fig. 1b) or IgG2a (fig. 1c) was detected in sera of saline, PE or PE + CT + non-CpG-treated mice on day 29, i.e. after two skin applications. A slight specific IgG2a re-

Fig. 1. PE-specific IgE (**a**), IgG1 (**b**) and IgG2a (**c**) in mice pretreated by skin application of saline, PE alone, PE + CT + CpG, or PE + CT + non-CpG. On days 31, 37, 43, 49 and 55, mice were sensitized by gavage with PE, as explained in the Materials and Methods section. Assays were performed as duplicates on sera from 5 individual mice (IgG) or on corresponding pooled sera (IgE). ^a $p < 0.001$ compared to all other groups; ^b $p < 0.01$ compared to control mice; ^c $p < 0.01$ compared to control and PE + CT + CpG mice (ANOVA + Tukey's post-test).



sponse was observed in mice receiving PE + CT + CpG treatment (fig. 1c), whereas neither IgE nor IgG1 was detected. This suggested the induction of a Th1 response in the PE + CT + CpG-treated group, whereas the other pre-treatments failed to induce any detectable immune response.

Effect of CpG Pretreatment on Further Oral Sensitization

After the third gavage (on day 48), specific IgE (fig. 1a) and IgG1 (fig. 1b) were detected in control mice (saline).

These specific responses were further enhanced by two additional gavages, i.e. on day 62, and contrasted with the slight induced IgG2a response (fig. 1c). Mice pretreated with PE alone showed a very intense specific IgE response from the second gavage onwards (fig. 1a). A slight, albeit non-significant increase in IgG1 response was also observed compared to control mice (fig. 1b), whereas IgG2a responses were similar.

Anti-PE IgE was induced weakly and late after oral administration in PE + CT + CpG pretreated mice, and was significantly reduced when compared to control mice

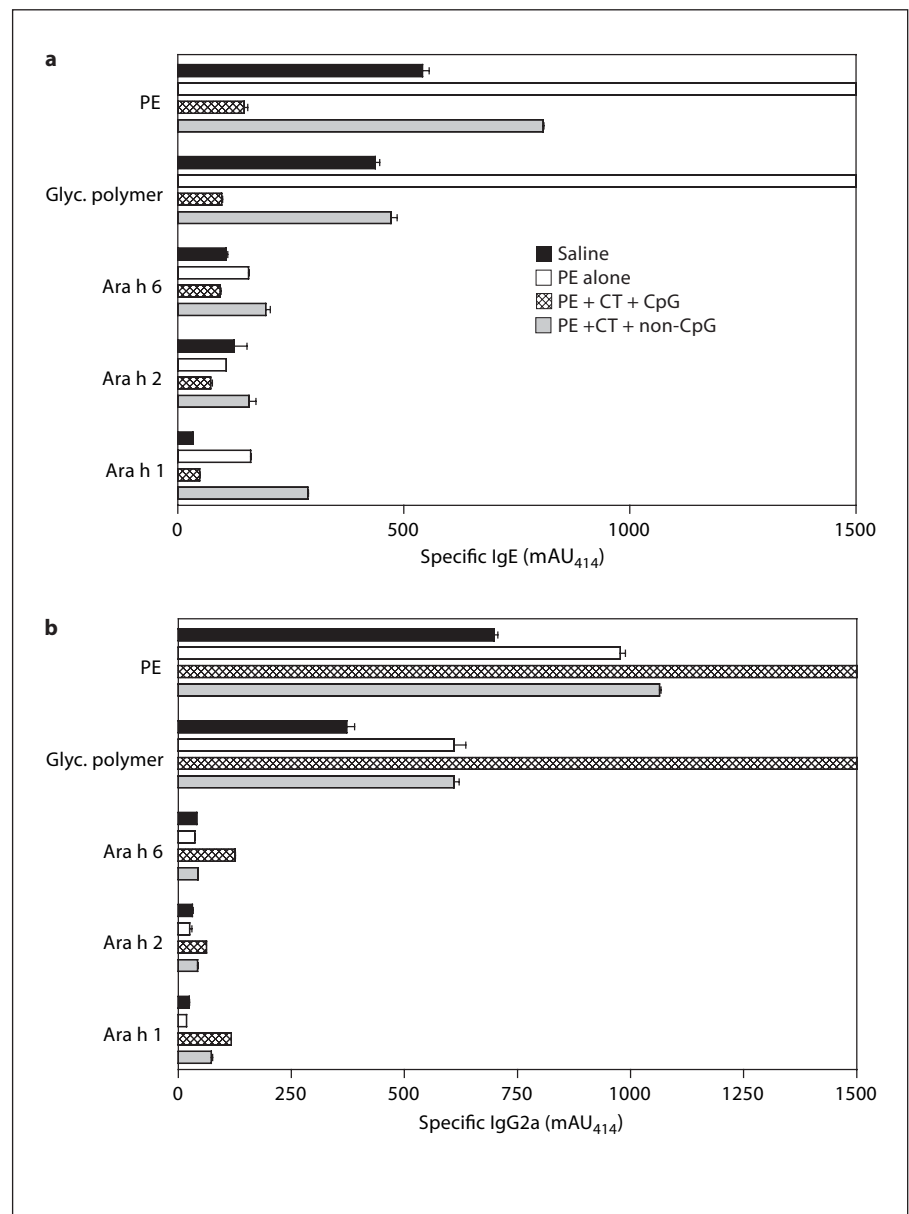


Fig. 2. Specificity of PE-specific IgE (**a**) and IgG2a (**b**) in mice pretreated with saline, PE alone, PE + CT + CpG, or PE + CT + non-CpG, and further sensitized by means of oral gavage. Allergen specificity was performed with pooled serum obtained on day 62 (i.e. after pretreatment and PE sensitization), on immobilized purified allergen or PE, as explained in the Materials and Methods section.

(fig. 1a). IgG1 responses, although delayed, were equivalent when compared to control mice. It is worth noting that anti-PE-specific IgG2a were highly induced in the PE + CT + CpG-treated group (fig. 1c). PE + CT + non-CpG mice showed antibody responses that were intermediate between those of control mice and mice pretreated with PE, i.e. a significant increase in anti-PE-specific IgE (fig. 1a) and a non-significant increase of specific IgG1 (fig. 1b).

IgE, IgG1 and IgG2a Antibody Response to Purified Allergens

We analyzed IgE (fig. 2a) and IgG2a (fig. 2b) specificity on purified allergen-coated plates. Control mice developed IgE and IgG2a mainly directed against glycinin polymer (Ara h 3), as previously described in this mouse model [20]. Ara h 1, Ara h 2 and Ara h 6 were also slightly recognized. Although variable in intensity, the antibody responses showed the same specificity whatever the skin pretreatment, suggesting that pretreatments using a route of administration different from that used for sen-

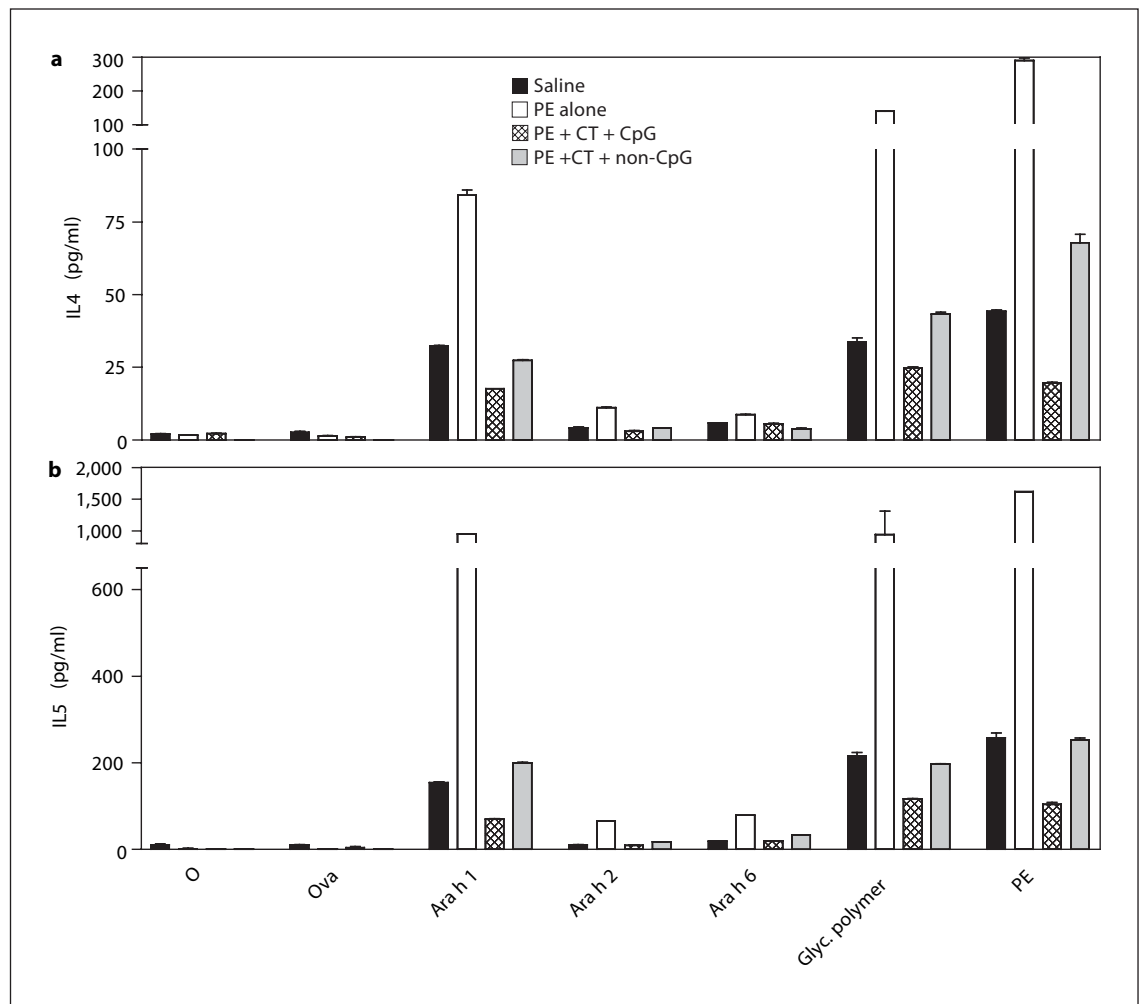


Fig. 3. IL-4 (a) and IL-5 (b) secretions by splenocytes from mice pretreated with saline, PE alone, PE + CT + CpG, or PE + CT + non-CpG. Mice were further sensitized by means of oral gavage. Splenocytes were reactivated by saline (O) or ovalbumin (negative controls), PE and peanut-purified allergens.

sitization do not modify the specificity of the antibody response. IgG1 showed the same specificity as IgE, whatever the pretreatment (data not shown).

Cytokines Secreted by Reactivated Splenocytes

Splenocytes from sensitized mice reactivated with PBS or an irrelevant allergen (ovalbumin) did not secrete IL-4 (fig. 3a) nor IL-5 (fig. 3b), whatever the pretreatment. PE or purified allergens induced significant secretion of IL-4 and IL-5 in control mice, principally Ara h 1 and Ara h 3, whereas Ara h 2 and Ara h 6 induced significant although weaker secretions. Whatever the allergen used for reactivation, PE pretreatment led to intense IL-4 and IL-5 secretions when compared to control mice. Conversely,

PE- and purified allergen-specific IL-4 and IL-5 secretions were reduced in PE + CT + CpG pretreated mice when compared to control mice. This effect was specifically restricted to the peanut allergens, since concanavalin A reactivation produced similar levels of IL-4 and IL-5 in these two groups of mice. An intermediate response was observed for allergen-specific IL-4 production in the PE + CT + non-CpG-pretreated group, while IL-5 secretion was equivalent to that of control mice. The same results as for IL-4 and IL-5 were also observed for IL-10 secretion whatever the pretreatment (data not shown), whereas IFN γ and TGF β secretion levels were comparable in all groups.

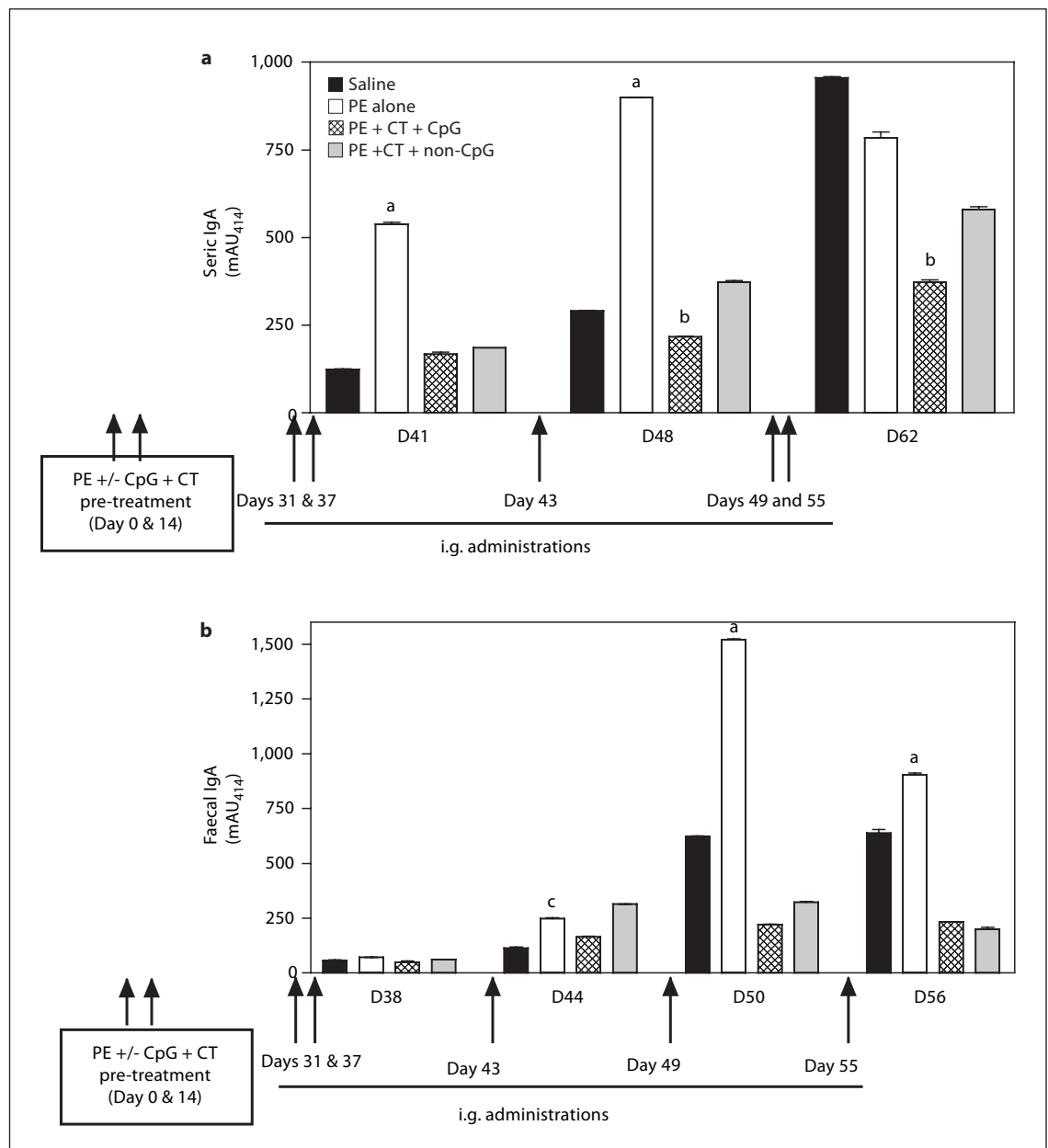


Fig. 4. PE-specific IgA in sera (**a**) and faecal samples (**b**) in mice pretreated with saline, PE alone, PE + CT + CpG, and PE + CT + non-CpG. Sensitization is performed by i.g. administrations of PE and CT, as explained in the Materials and Methods section. ^{a, b} $p < 0.001$ compared to all other groups; ^c $p < 0.001$ compared to control mice (ANOVA + Tukey's post-test).

Serum and Faecal IgA

No specific IgA could be detected in faeces or serum after skin pretreatments and before sensitization (data not shown). Mice pretreated with saline produced IgA antibodies both in sera (fig. 4a) and faeces (fig. 4b), from the second gavage onwards, and this production

was enhanced by further gavages. Mice pretreated with PE alone showed an intense IgA response in serum on days 41 and 48. For this group, faecal IgA levels were also higher than in control mice from day 44 to day 56, and than in other pretreatment groups after day 50. On the other hand, although slightly increased after

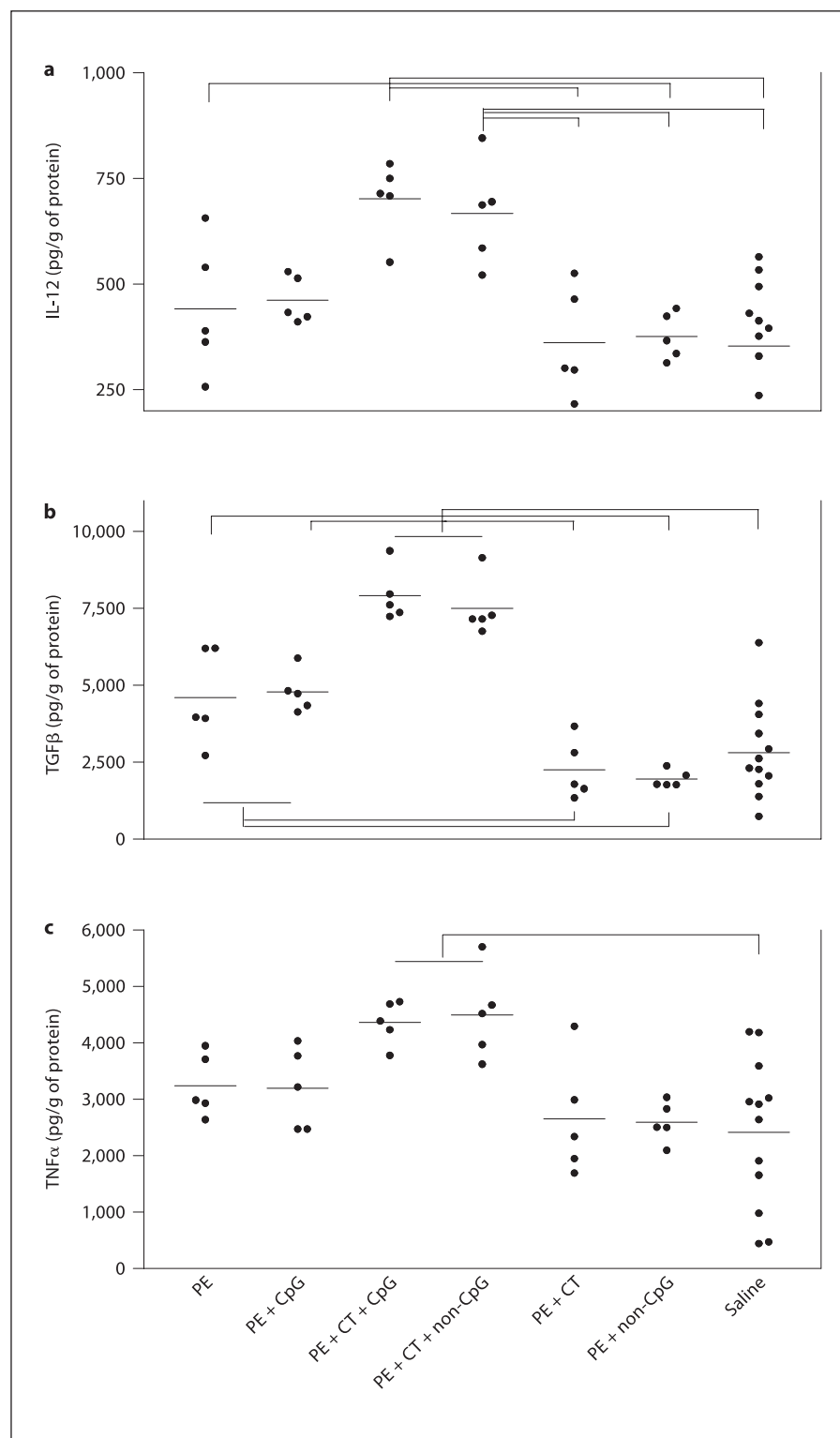


Fig. 5. IL-12 (**a**), TGFβ (**b**) and TNFα (**c**) production in liver 40 min after skin deposit of PE, and PE with or without CpG and/or CT. Bars indicate statistical significance ($p < 0.05$, ANOVA + Tukey's multiple comparison test).

the first gavages, the specific IgA responses in serum and faeces from PE + CT + CpG-pretreated mice were significantly reduced from day 48–50 onwards. PE + CT + non-CpG mice developed an IgA response nearly equivalent to that of PE + CT + CpG-pretreated mice, although slightly more intense for some sampling dates.

Liver Cytokine Production after Skin Application

Skin applications were performed as previously described. Additional control groups were included, consisting of skin application of PE plus CT, PE plus CpG or PE plus non-CpG. Forty minutes later, liver IL-4, IL-10, IL-13 and IFN γ productions were found to be equivalent in the different groups (data not shown). Surprisingly, IL-12 (fig. 5a) and TGF β (fig. 5b) productions were highly enhanced in livers from PE + CT + CpG and PE + CT + non-CpG-treated mice compared to all other groups. PE + CT + CpG- and PE + CT + non-CpG-treated mice also demonstrated an increased liver production of TNF α when compared to PBS control mice (fig. 5c). PE and PE + CpG groups share common cytokine patterns, suggesting inefficient CpG activation if CT is omitted. In parallel, PE + CT or PE + non-CpG groups were equivalent to the PBS control group, for all cytokines assayed.

Discussion

Approximately 80% of peanut-allergic patients react to their first known oral exposure to peanut, implying some prior sensitization, possibly via the skin [27]. In a cohort study, Lack et al. [28] suggested that sensitization to peanut proteins may occur in children through the application of peanut oil to inflamed skin. Skin patch tests have been used to sensitize animals to proteins such as ovalbumin and KLH: occlusive patches were applied for several days and several times on intact skin to allow IgE production [29, 30]. More recently, it was demonstrated that exposure of disrupted skin of mice to peanut proteins can prevent induction of oral tolerance, and can promote sensitization to food proteins upon gastrointestinal exposure, whereas application of antigens to intact skin failed to induce a specific antibody response although a slight specific T cell proliferation was observed [31]. In the present study, we observed that two applications of a crude peanut extract on intact skin without any adjuvant and for a short time led to an intense amplification of further specific IgE and Th2-associated responses (IL-4 and IL-5 secretions). This implies that brief expo-

sure of intact skin to allergens specifically leads to the initiation of a discrete Th2 response, which may then highly enhance sensitization via gastrointestinal exposure. Conversely, two brief applications to intact skin of a crude extract of peanut protein and CT + CpG as adjuvant induced a specific IgG2a response in mice 14 days after the second application. Such a pretreatment efficiently prevented further oral sensitization as suggested by the following criteria: a decrease in specific IgE and IgA responses, a decrease in allergen-specific production of IL-4, IL-5 and IL-10 counterbalanced by an increase in specific IgG2a. Surprisingly, this IgG2a increase was not accompanied by an increased IFN γ production by spleen cells, which has been observed before in preventive protocols [11, 32].

The mechanisms involved in the modulation of the immune response after skin pretreatments remain unclear. Cutaneous sensitization with hapten was demonstrated to stimulate preferential and rapid production of IL-4 by liver iNKT cells. Within 1 h, IL-4 promotes early production of IgM by B-1 cells, which will further initiate antigen-specific T and B cell responses [33, 17]. We thus assayed cytokines in liver 40 min after skin application, to check for a possible increased expression of IL-4 and/or IL-13 in liver after pretreatment with PE alone, which may have explained the enhanced sensitization in this group. However, we could not demonstrate such cytokine production in liver. Moreover, a recent study suggested that collaboration between iNKT cells and B-1 cells in a rapid polyclonal IgM response can depend on TLR4 and TLR2, whereas the TLR9 ligand CpG likely stimulates the B-1 cell response independently of iNKT cells [34]. Altogether, this suggests the nonimplication of iNKT cells in the enhanced/repressed sensitization observed after our skin pretreatment. However, we demonstrated that skin application of PE + CT + CpG rapidly induced IL-12 and TNF α production in liver, while other treatments such as PE + CT or PE + CpG did not result in significant cytokine production. All these data lead to the hypothesis that, in the presence of CT, peanut allergens and CpG rapidly activate immature LC/DC of the skin to migrate into the liver and to produce the Th1-inducing cytokine IL-12 and TNF α . Indeed, CpG have been demonstrated (1) to be rapidly internalized in mouse alveolar macrophages [35], (2) to regulate TNF α and IL-12 production in different macrophage cell lines and in bone-marrow-derived DC [36], (3) to induce LC/DC migration from skin explants after regulation of the required adhesion molecule expression [15], and (4) to enhance expression of MHC II and costimulatory molecules and production of IL-12 [16]. We

also observed TGF β production in the liver of mice pretreated with PE + CT + CpG. This cytokine has been demonstrated to help the differentiation of naïve T cells into Th3, TGF β -secreting cells [37]. Moreover, a CpG prevention of airway remodelling in a murine model of chronic asthma is associated with an increased level of TGF β in bronchoalveolar fluid, suggesting the induction of immunosuppressive regulatory T cells [38]. Then our study suggests that the preventive effect of CT + CpG adjuvant may result from a complex mechanism involving the induction of Th1 cells counterbalancing Th2 response, but also the activation of regulatory T cells that efficiently prevent Th2 cell induction the same as an inappropriate excessive Th1 response. Further studies on CD4+CD25+ depletion/transfer should address this hypothesis.

Surprisingly, the application of PE + CT + CpG or of PE + CT + non-CpG led to identical cytokine profiles in liver (fig. 5), whereas prevention of sensitization was ineffective in this latter group (fig. 1–3). Nevertheless, incorporation of CpG and non-CpG into immune cells is independent of TLR9 intracellular expression [39], and CpG and non-CpG are rapidly endocytosed by macrophage cells suggesting that this uptake is independent of CpG [36, 40]. However, endocytosed non-CpG coupled to antigen failed to activate DC to express co-stimulatory molecules [40]. B cells were also demonstrated to present antigens conjugated to non-CpG to Th1 cells and to induce the secretion of IFN γ , but no increased expression of co-stimulatory molecules such as CD86 was found in these cells [41]. In our experiments, non-CpG may then allow the activation of immature skin DC/LC to produce cytokines, but co-stimulatory molecule expression is not

induced. This would lead to inefficient antigen-specific T cell (Th1 and/or regulatory T cells) activation and then finally resulted in a non-protective effect of the PE + CT + non-CpG pretreatment.

Physical conjugation of allergen to CpG facilitates APC uptake and presentation of antigens [40]. Moreover, it enhances the immunogenicity and reduces the allergenicity of allergens such as Amb a 1 [11, 42] and ovalbumin [5] administered intradermally. However, excessive Th1 cytokine production could potentially have negative effects by exacerbating, rather than reducing, the inflammatory process, as suggested by Peng et al. [7] who observed transient delayed skin reactions in mice after intradermal administration of CpG-conjugated allergen in therapeutic protocols. The application of a peanut PE mixed with CT and CpG as described in the present study, i.e. applied to intact skin, seems to greatly minimize potential adverse effects and could be of great interest in therapy. The use of modified peanut allergens with reduced IgE-binding capacity but retaining T cell activation capacity should also be considered in therapeutic protocol [43].

In conclusion, we have demonstrated that two successive and brief applications of peanut PE alone to intact skin highly potentiate further sensitization via the oral route. In contrast, pretreatment with PE and CT + CpG as adjuvant prevents further oral sensitization, as demonstrated by decreased production of IgE, IL-4 and IL-5. This pretreatment allows the rapid production of IL-12 and TGF β in liver and specific IgG2a in sera, suggesting an activation of skin LC/DC that further induces Th1 and/or regulatory T cells.

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