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Ablation of ovomucoid-induced allergic response by desensitization with recombinant ovomucoid third domain in a murine model

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Summary

Attempts to modulate the allergenic response by hypoallergens aimed at eliminating IgE-binding epitopes have been established recently for allergen immunotherapy. Desensitization offers an alternative approach to mounting a protective immune response. We have shown previously that mutation of the decisive amino acids in the B cell epitope of the ovomucoid third domain suppresses IgE binding reactivity against human patient sera and we hypothesize that this hypoallergenic variant could be a potential candidate molecule for specific immunotherapy against an ovomucoid-induced IgE reaction. The aim of this study was to investigate whether hyposensitization with the ovomucoid-modified isoform could desensitize ovomucoid-sensitized mice. We mapped the immunodominant B cell epitopes of ovomucoid in Balb/c mice. A hypoallergenic ovomucoid mutant isoform, having ablated allergenicity against patient sera, was used to desensitize ovomucoid-sensitized Balb/c mice by intraperitoneal injection. Female Balb/c mice were sensitized with intact ovomucoid molecule (Fovm) and desensitized with the modified isoform of the third domain of ovomucoid (GMFA). Intact ovomucoid-sensitized mice desensitized with phosphate-buffered saline (PBS) served as positive controls to maintain hypersensitivity. To gain insight into the efficacy of the modified ovomucoid variant in desensitization, effects on hypersensitivity reactions and histamine levels, followed by its association with antibody levels and cytokine profiles, were measured. Abrogation of the allergic response with complete suppression of anaphylactic symptoms and lower serum histamine levels was observed in the desensitized group by GMFA, accompanied by significantly reduced ovomucoid-specific IgE and IgG1 levels and enhanced specific IgG and IgG2a levels. The sensitized group showed severe anaphylactic symptoms, enhanced serum histamine concentrations and increased levels of specific IgE and IgG1. The level of interleukin (IL)-4 was decreased dramatically in the desensitized group and higher levels of interferon (IFN)-γ were found, whereas mice sensitized with intact ovomucoid exhibited significantly higher levels of IL-4 favouring a Th2 skewed pathway. We demonstrate clearly that GMFA is able to ablate ovomucoid-induced allergic reactions in sensitized mice. This occurs via a suppression of specific IgE accompanied by an increase in suppressor T cell activity. This approach offers some promise for the development of treatment against ovomucoid-induced allergic response.

Keywords: cytokines, desensitization, egg allergy, IgE, immunotherapy, ovomucoid, Th1/Th2

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Introduction

Food-induced anaphylaxis affects a significant proportion of the population, about 6–8% of children and 4% of adults [1]. In children, peanut, egg and milk allergies are more prominent; in adults, peanuts, tree nuts and shellfish are the main causes of allergic reactions [2]. Eggs are the second major cause of food-induced allergic reactions in the United

States among children, next to milk-induced reactions [3]. Egg is comprised of various proteins, many of which are highly allergenic. Ovomucoid is the major immunodominant allergen in the hen's egg white and accounts for about 11% of total egg white protein. Ovomucoid has 186 amino acids and is stable to digestion and heat [4–6]. Ovomucoid is divided into three domains, each consisting of 60 amino acids, and the third domain of ovomucoid has been shown to be more allergenic than the first and second domains [7]. The carbohydrate content of ovomucoid does not seem to affect allergic reactivity [7–9].

To date, there are no therapeutic options available for the treatment of egg-induced anaphylactic reactions. Avoidance may be an option but is challenging, because egg and egg-derived products are essential ingredients used in a wide variety of food products. Inadvertent consumption of eggs and egg-derived ingredients could be life-threatening, and there is a need for the development of specific immunotherapy against egg-induced allergic reactions. The allergic response is characterized by increased IgE synthesis by B cells and by the cytokine profile of allergen-specific T cells. A major problem for immunotherapy using food allergens is the potentially dangerous risk of severe side effects, including severe anaphylactic shock. Hypoallergenic variants of a number of major recombinant allergens with reduced allergenic activity have been the target for allergen-specific immunotherapy recently [10–12]. To understand of the relationship between protein structure and allergenicity, it is highly important that critical amino acids for IgE binding are identified. Recombinant allergens, mutated to remove decisive B cell epitopes, can prevent allergen cross-linking of IgE on effector cells, including mast cells and basophils, and offer a safer alternative for immunotherapy. Most immunomodulatory approaches aimed at the treatment of food allergy operate on the basis of regulating the Th1/Th2 balance. Down-regulation of the polarized Th2 skewed response or enhancement of production of Th1 favoured cytokines has been shown to be therapeutically effective for immunotherapy. Allergy shots known as immunotherapy or desensitization treatment against specific allergens such as grass, pollen, etc. are normally administered until the body builds specific blocking antibodies that block allergic reactions successfully, and eventually the symptoms disappear.

We have recently reported a hypoallergenic variant of ovomucoid (GMFA) which showed significantly reduced IgE-binding reactivity against human egg-allergic patient sera [13]. This variant could be a target for the development of an ideal vaccine candidate for ovomucoid-induced allergic reaction. The objective of this study was to investigate whether a mutant isoform of ovomucoid third domain (GMFA) could induce desensitization against an ovomucoid-sensitized murine model. We identified the linear IgE binding epitopes for ovomucoid in Balb/c mice and characterized the protection of ovomucoid-induced allergic response *in vitro*. Allergic immune responses with regard to hypersensitivity reactions, plasma histamine, ovomucoid-specific antibody and cytokine levels in splenocytes were measured.

Materials and methods

Mice

Female Balb/c (6-week-old) mice were purchased from Charles River Laboratory (Missisuaga, ON, Canada) and maintained in a pathogen-free environment. All procedures were reviewed and approved by the Animal Care Committee, University of Guelph.

Reagents

Aluminium hydroxide was used as an adjuvant for the intraperitoneal injections (Alhydrogel 2·0%, Superfos Biosector, Vedbæk, Denmark). Cytokine enzyme-linked immunosorbent assays (ELISAs) were performed according to PharMingen standard protocols (PharMingen, San Diego, CA, USA). Opt enzyme immunoassay (OptEIA) mouse interleukin (IL)-10, IL-12, IL-4 and interferon (IFN)-γ kits (PharMingen) were used in this study.

Expression and purification of the antigens

Intact ovomucoid (Fovm) was purified according to the procedure of Fredericq and Deutch [14]. Further purification was carried out using high performance liquid chromatography (HPLC) (Bio-Rad Laboratories, Hercules, CA, USA) using a Bio-Scale Q5 column [7]. Recombinant third-domain (DIII) ovomucoid antigen was cloned as a fusion protein with glutathione S-transferase (GST) in *Escherichia coli* and mutations were made in the DIII of ovomucoid, as reported previously [13]. The recombinant mutant DIII ovomucoid (GMFA) contained two single amino acid substitutions, glycine at position 162 to methionine and phenylalanine at position 167 to alanine. The GMFA ovomucoid construct was transformed into *E. coli* cells. The cell culture containing the fusion protein was induced with 1 mM isopropyl-thio-galactosidase (IPTG) and purified as per the manufacturer's guidelines (Amersham Biosciences, NJ, USA). Briefly, 5 ml of overnight grown culture containing the fusion protein was seeded onto 1 litre of Luria broth (LB) containing ampicillin (50 μg/ml). The culture was grown at 37°C for 4 h until the optical density reached 0·5–0·6. The culture was induced with 1 mM final concentration of IPTG and incubated further in the shaker for 4 h. The cells were harvested by centrifugation (7000 ***g*** for 10 min) and resuspended in 10 ml of lysis buffer (40 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid and 0·5% Triton X-100) and sonicated at 40 W for 10–15 s (Model 250 Sonifier, Branson, Danbury, CT, USA). The mixture was then centrifuged at 7000 ***g*** for 15 min. Glutathione Sepharose 4B (GS4B) resin (Amersham Pharmacia Biotech) was equilibrated with 10

bed-volumes of phosphate-buffered saline (PBS) and the cell extract was loaded onto the column. The column was then washed thoroughly with 10 bed-volumes of PBS to remove unbound material; the fusion protein was cleaved with thrombin (10 U/mg of protein) (Amersham Pharmacia Biotech) overnight at room temperature and eluted with PBS containing 10 mM reduced glutathione. The recombinant protein (GMFA) was passed through a column of polymyxin B-immobilized beads (Sigma, St Louis, MO, USA) to remove contaminating endotoxins according to the manufacturer's instructions (Sigma).

Mapping of IgE binding epitopes of ovomucoid in Balb/c mice

Arrays of synthetic peptides representing the entire ovomucoid gene were synthesized on a nitrocellulose membrane (SPOTs Kit; Genosys Biotechnologies, Woodlands, TX, USA) by Fmoc chemistry (using amino acid active esters N-9-fluorenyl methoxycarbonyl), as described by the manufacturer (Sigma). The membrane-bound peptides were 10 amino acids in length with an offset of five amino acids. Briefly, synthesis of each peptide began by esterifying an Fmoc amino acid to the cellulose membrane derivatized with a dimer of the β-alanine-NH_2 group. Coupling reactions were followed by acetylation with acetic anhydride in *N*,*N*-dimethylformamide to render peptides unreactive during the reactions. Fmoc protective groups were removed by the addition of piperidine to render the nascent peptides reactive. All the remaining amino acids were added by the same process of coupling, blocking and unblocking until the desired peptide was generated. After addition of the last amino acid, the side chains of the peptide were deprotected with a 1 : 1 : 0·05 (v/v/v) mixture of dichloromethane : trifluoroacetic acid : tributylsilane and washed with methanol. Membranes containing the synthetic peptide were blocked with blocking buffer (Genosys Biotechnologies) at room temperature overnight, washed three times with Tris-buffered saline containing 0·05% (v/v) Tween-20 (TBST) and probed with the pooled mice sera (1 : 50 dilution), raised against the intact ovomucoid antigen from five Balb/c mice generated previously in our laboratory for 2 h at room temperature. The membrane was washed further three times with TBST. For detection of IgE binding, biotinylated anti-mouse IgE (1 : 1000) (Caltag) was added and incubated for 2 h at room temperature. The membrane was washed a further three times with TBST and extravidin conjugated to alkaline phosphatase was added (1 : 3000) (Sigma) and incubated for 1 h at room temperature. The membrane was washed six times with TBST and developed with chemiluminescent substrate, CDP-Star (Boehringer Ingelheim, Mannheim, Germany) and enhancer Nitro-Block II (Tropix, Bedford, MA, USA) (both diluted 1 : 100 with 0·1 M Tris–HCl, 0·1 M sodium chloride, pH 9·5) solution. The membrane was visualized under a light imager (EG&G Berthold, Bad Wildbad, Germany). Image processing was performed using Win Light software (EG&G Berthold) as per the manufacturer's instructions.

Antigen sensitization, desensitization and challenge

Balb/c mice ($n = 5$ per group) were sensitized by intraperitoneal (i.p.) injection with 50 μg of ovomucoid in 100 μl of 2% of aluminium hydroxide (Alum) (Superfos Biosector) on day 1 followed by a second injection on day 7. Subsequently, some mice were desensitized by receiving three i.p. injections with 50 μg of the GMFA on days 21, 28 and 35. On day 42, mice were challenged i.p. with a higher dose (1 mg/ml) of intact ovomucoid to induce systemic anaphylaxis (ASA). The group of sensitized mice which received placebo treatments with 100 μl of phosphate-buffered saline (PBS) was subjected to ASA. Mice injected at days 1, 7, 21, 28 and 35 with PBS served as negative controls throughout and were subjected to ASA at day 42 in the manner described.

Assessment of hypersensitivity symptoms

The clinical severity of ovomucoid-induced ASA was assessed by scoring symptoms every 10 min for 40 min after the i.p. injection on day 42. The symptoms were recorded as follows: 0, no symptoms; 1, scratching and grooming of face, mild shock including slow movement; 2, diarrhoea and puffiness of eyes and mouth; 3, wheezing and breathing problems; 4, tremor and convulsions; 5, death.

Measurement of serum histamine levels

Blood was collected from mice 40 min after challenge and the levels of histamine were determined by an inhibition assay using the enzyme immunoassay kit (Neogen Corporation, Lexington, KY, USA), following the manufacturer's protocol. The concentration of histamine was calculated by comparison with a standard histamine curve which was run together with the assay for quantification.

Determination of specific and total serum IgE and IgG levels

Levels of specific and total IgG and IgE in both the sensitized and the desensitized groups of mice were determined by ELISA. In short, 96-well microtitre plates were coated with 100 μl of capture anti-IgE monoclonal antibody (Caltag, Burlingame, CA, USA) or anti-mouse IgG (EMD Biosciences, Madison, WI, USA) at 1 μg/ml final concentration. After overnight incubation at 4°C, the plates were washed with phosphate-buffered saline with 0·05% Tween-20 (PBST) three times and blocked with 200 μl of 2% bovine serum albumin (BSA) in PBS for 2 h at 37°C and the plates were washed with PBST three times. Dilutions of murine IgE and IgG standards (31·250–2000 ng/ml)

(PharMingen) or serum samples (1 : 20 for IgE and 1 : 2000 for IgG) in 1% BSA in PBS were added (100 µl) to the wells in duplicate and incubated for 1 h at 37°C. The plates were washed further with PBST three times and 100 µl of biotinylated anti-IgE monoclonal antibody (1 : 1000) (Caltag) or alkaline phosphate-conjugated rabbit anti-mouse IgG (1 : 15000 dilutions) (Sigma) were added to the wells and incubated for 2 h at 37°C. For IgE reaction the plate was incubated further with 100 µl of extravidin conjugated to alkaline–phosphatase (1 : 3000 dilutions) (Sigma) and incubated for 1 h at 37°C. The reaction product was visualized with 100 µl *p*-nitrophenol phosphatase (1 mg/ml) (Sigma), the reaction was incubated for 20 min followed by the addition of 25 µl of stop solution (3 N sodium hydroxide). The absorbance readings (405 nm) were read using a microplate reader (model 550, Bio-Rad Laboratories) and readings obtained from the individual serum samples were converted to concentrations of IgG and IgE per millilitre for each assay from the values obtained from the standard curve. Specific IgG and IgE levels were measured by coating the plates with 2 µg/ml of the intact ovomucoid antigen and a similar procedure to that described above was followed.

Measurement of specific IgG1 and IgG2a levels

Measurement of specific IgG1 and IgG2a in sera samples was performed by an indirect ELISA. In brief, 100 µl of intact ovomucoid (5 µg/ml) was coated onto 96-well microplates overnight at 4°C and then blocked with 200 µl of 2% BSA. Diluted serum samples (1 : 1000 dilutions for specific IgG1 levels and 1 : 500 for specific IgG2a levels) were added to each well and incubated. After washing with PBST, biotinylated monoclonal antibodies were added for IgG1 and IgG2a (100 µl/well; 1 µg/ml) (BD Biosciences, Mississauga, ON, Canada). The plates were washed and incubated further with 100 µl of peroxidase-conjugated mouse anti-rat Ig avidin–peroxidase for 1 h before detection and the reaction was visualized using 50 µl of 3,3′, 5, 5′-tetra methyl benzidine

Overlapping amino acid residues of Domain I and Domain II (a) and Domain III (b) of ovomucoid synthesized on a nitrocellulose membrane

1. AEVDCSRFPN (1-10) (a)
2. SRFPNATDKE (15)
3. ATDKEGKDVL (20)
4. GKDVLVCNKD (25)
5. VCNKDLRPIC (30)
6. LRPICGTDGV (35)
7. GTDGVTYNNE (40)
8. TYNNECLLCA (45)
9. CLLCAYSIEF (50)
10. YSIEFGTNIS (55)
11. GTNISKEHDG (60)
12. KEHDGECKET (65)
13. ECKETVPMNC (70)
14. VPMNCSSYAN (75)
15. SSYANTTSED (80)
16. TTSEDGKVMV (85)
17. GKVMVLCNRA (90)
18. LCNRAFNPVC (95)
19. FNPVCGTDGV (100)
20. GTDGVTYDNE (105)
21. TYDNECLLCA (110)
22. CLLCAHKVEQ (115)
23. HKVEQGASVD (120)
24. GASVDKRHDG (125)
25. KRHDGECRKE (130)

1. LAAVSVDCSE (131-140) (b)
2. VDCSEYPKPD (145)
3. YPKPDCTAED (150)
4. CTAEDRPLCG (155)
5. RPLCGSKNKT (160)
6. SKNKTYGNKC (165)
7. YGNKCNFCNA (170)
8. NFCNAVVESN (175)
9. VVESNGTLTLSHFG (184)
10. TLTLSHFGKC (186)

Fig. 1. Sequential IgE epitope mapping of intact ovomucoid containing the first and second (a) and third (b) domains probed with anti-ovomucoid Balb/c mice sera. The Spots membrane is shown and the positive spots on the figure indicate the position of the synthetic peptide recognized by the antibody. Each spot represents a separate 10 mer peptide and the sequence of each peptide synthesized is represented below. Peptides in bold represent spots that were positive.

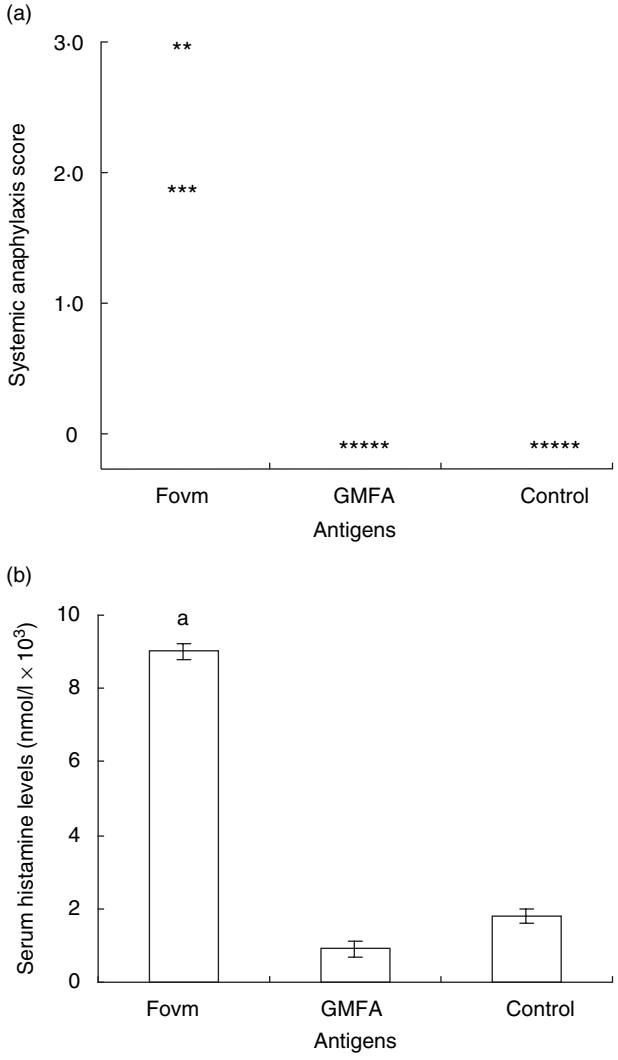

Fig. 2. Characterization of hypersensitivity symptoms (a) and serum histamine levels (b). Blood was obtained from all immunized mice at the end of the experiment, and hypersensitivity symptoms were scored and serum histamine levels were analysed compared to a standard histamine curve as described in Methods.

(TMB) substrate and incubated for 20 min. The reaction was terminated with 50 µl of 1 N H_2SO_4 and absorbance was read at 450 nm.

Immunological analysis of cytokines

Individual spleens were removed at the end of the experiment and single-cell suspensions were prepared from splenocytes. Depletion of erythrocytes by lysis using red blood cells (RBC) lysing buffer (Sigma) was performed. Cells were cultured in RPMI-1640 (Invitrogen Life Technologies, Gaithersburg, MD, USA) supplemented with 8% (v/v) heat-inactivated FCS (Hyclone, Fisher Scientific, Pittsburg, PA, USA). Cells were either unstimulated (cells alone) or stimulated with intact ovomucoid (50 µg/well) in a 96-well plate at 37°C for 72 h. Supernatants were harvested after 72 h, and cytokine levels (IL-4, IFN-, IL-10 and IL-12) were measured by ELISA. Sandwich ELISAs were performed to quantify levels of specific cytokines in the supernatants from cell cultures. Commercial kits (OptEIA mouse cytokine ELISA sets) (Pharmingen) were used to measure IL-4, IFN-γ, IL-10 and IL-12 according to the manufacturer's instructions. Standard cytokines were run together with the samples to quantify the levels of the individual cytokines present.

Statistical analysis

The statistical significance of the data obtained was determined using a software package from INSTAT version 3·0 (Graphpad, San Diego, CA, USA; http://www.graphpad.com) using the Tukey–Kramer one-way analysis of variance (ANOVA) test. Values for all measurements are expressed as mean ± s.d. Differences between groups were considered significant at $P < 0{\cdot}05$.

Results

Identification of IgE epitopes of ovomucoid in mice

Sera from Balb/c mice recognized two IgE epitope regions in the second (II) and third (III) domains of ovomucoid (GKVMVLCNRAFNPVC and YGNKCNFCNAVVESN); the third-domain peptides exhibited stronger IgE binding. This agrees with previous data, that the third domain is more allergenic than the first and the second domains [7]. One epitope in each domain is 15 amino acids in length, thus defining major B cell-restricted antigenic spots on the ovomucoid sequence (Fig. 1). Interestingly, the sequence motifs recognized by the Balb/c mice in the third domain of ovomucoid were also recognized by the sera from egg-allergic human patients, indicating that the immune-dominant IgE epitope is common to mouse and man [15]. Substitution of these critical amino acids in the third domain of ovomucoid at position 162, where glycine was substituted for methionine (G to M) and phenylalanine at position 167, was substituted for alanine (F to A), which led to complete loss of activity of IgE binding using sera from egg-allergic patients when analysed with ELISA and Western blot [13]. Incubation with normal control mice serum alone revealed no non-specific binding of the murine immunoglobulin or of the secondary antibodies to the peptide sequences (data not shown).

Desensitization with recombinant ovomucoid DIII prevents anaphylaxis and lowered histamine levels

GMFA was able to desensitize ovomucoid-sensitized mice, as assessed by symptom scores recorded for various groups after i.p. challenge. Acute symptoms scoring levels 2 or 3 were observed in all mice sensitized with the intact ovomu-

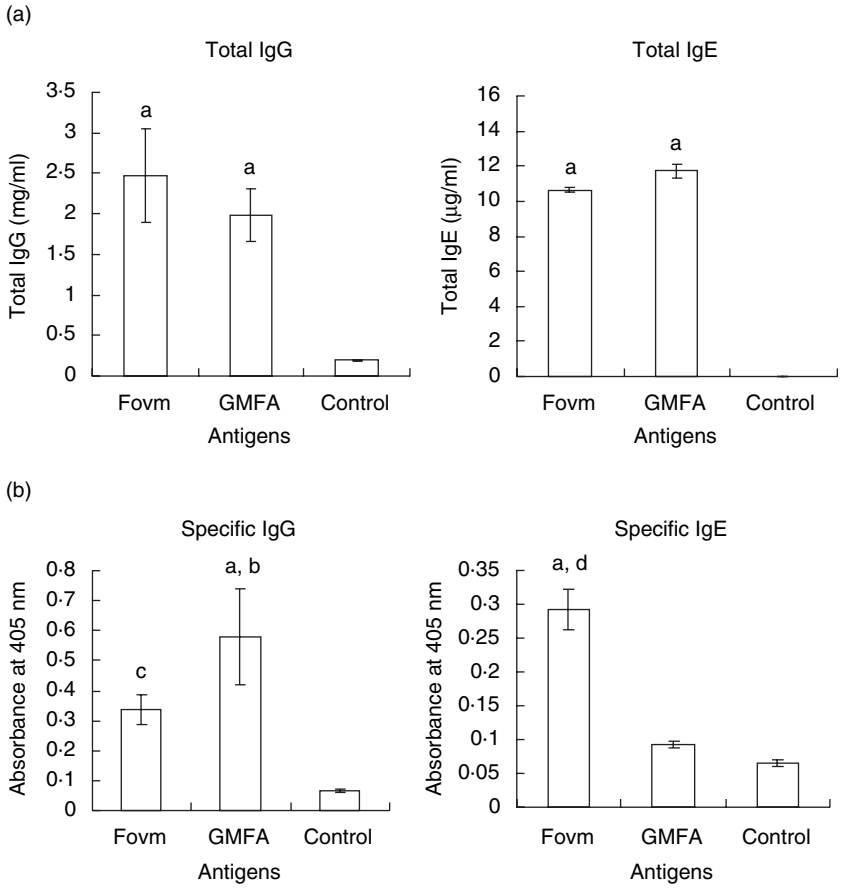

Fig. 3. Serum levels of total IgG and IgE (a) and specific IgG and IgE (b) as determined by enzyme-linked immunosorbent assay (ELISA). Mice were sensitized, desensitized and challenged and sera was collected and analysed as described in Methods. Levels of serum total IgG and IgE were determined at the end-point (day 42) in each individual mouse sera. The total levels of antibodies were quantified using standards. Values are expressed as mean ± s.e.m. ($n = 5$). (a) Significantly higher ($P < 0{\cdot}001$) than the control group; (b) significantly higher than ($P < 0{\cdot}05$) than the intact ovomucoid (Fovm) group; (c) significantly higher than ($P < 0{\cdot}05$) than the control group; (d) significantly higher than ($P < 0{\cdot}001$) than the modified ovamucoid group.

coid antigen, whereas the desensitized group barely showed any sign of hypersensitivity reaction except for slow movement (Fig. 2a). We conclude that the desensitized mice were protected by treatment with GMFA against an otherwise severe anaphylactic challenge. Because histamine is one of the major mediators for anaphylactic reaction, we analysed serum histamine levels in the samples. Histamine levels were much lower in GMFA-treated mice, indicating a blockage of histamine release in desensitized mice. In contrast, sensitized mice showed significantly higher serum histamine levels (Fig. 2b). The control group showed low levels of serum histamine.

Suppression of specific IgE and elevation of specific IgG levels

The group sensitized by intact ovomucoid showed moderate levels of specific IgG, while the group of GMFA mutant desensitized induced strong ovomucoid-specific IgG responses (Fig. 3). This indicates that the modified mutated ovomucoid (GMFA) allergen is able to induce antibodies cross-reactive with non-mutated ovomucoid antigen. Also, ovomucoid-specific IgE levels were reduced significantly in desensitized mice ($P < 0{\cdot}001$) when compared to the dramatic increase in the sensitized group. Specific IgE levels are a direct indication of an antigen-induced allergic response and it was significant from the data obtained that GMFA desensitization caused significant suppression of the ovomucoid-specific IgE level. No significant difference was observed in total IgG and IgE ($P > 0{\cdot}05$) levels (Fig. 3). The PBS control sera elicited a minimal or only very low antibody response.

Isotyping of IgG antibodies in mice sera

In order to evaluate whether GMFA modulates IgG subclass antibody levels involved in allergic responses, the levels of ovomucoid-specific IgG1 and IgG2a were measured. Subtyping of the IgG class revealed that the mice immunized with the intact ovomucoid (Fovm) showed detectable levels of specific IgG1 and low levels of specific IgG2a (Fig. 4). In contrast, in the sera sample collected from the desensitized mice group treated with GMFA protein, the specific levels of IgG1 were lower when compared to the Fovm group, but a significant increase in the level of specific IgG2a was observed ($P < 0{\cdot}001$). Desensitization with GMFA is thus shown to inhibit ovomucoid-specific IgG1 formation and up-regulate ovomucoid-specific IgG2a production, reflecting the development of a Th1-orientated response in the desensitized group (Fig. 4).

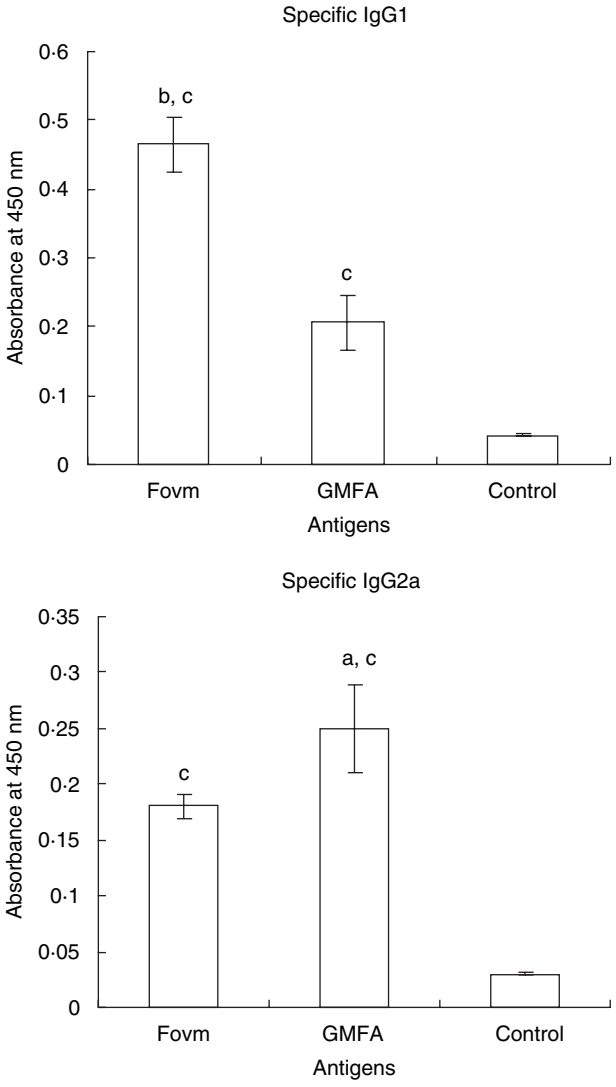

Fig. 4. Determination of specific IgG1 and IgG2a levels by enzyme-linked immunosorbent assay in the intact ovomucoid (Fovm) (sensitized) or modified ovamucoid (GMFA) (desensitized) or control [phosphate-buffered saline (PBS)]-treated groups. Mice were sensitized, desensitized and challenged and sera was collected and analysed as described in Methods. Values are expressed as means ± s.e.m. ($n = 5$ per group). (a) Significantly higher ($P < 0{\cdot}05$) than the Fovm group; (b) significantly higher ($P < 0{\cdot}05$) than the control group; (c) significantly higher ($P < 0{\cdot}001$) than the control group; (d) significantly higher ($P < 0{\cdot}001$) than the GMFA group.

Modulation of cytokine response by GMFA

We hypothesized that desensitization with GMFA may promote an antigen-specific Th1 response and consequently inhibit Th2 cytokine production. In order to test this hypothesis, we analysed the levels of cytokines in the culture supernatant of splenocyte samples from the sensitized and desensitized groups of mice. The spleen was isolated from individual mice, stimulated with intact ovomucoid antigen and incubated for 72 h, and cytokine levels were analysed with reference to standard cytokines. It was observed that the desensitized group showed significantly lower levels of IL-4 ($P < 0{\cdot}001$) and increased levels of IFN-γ ($P < 0{\cdot}05$) secretion in the culture supernatant samples. This suggests the occurrence of a discrete and prominent Th1 response, whereas significantly higher levels of IL-4 and lower levels of IFN-γ were seen in the intact ovomucoid sensitized group (Fig. 5). Control group supernatant samples showed low levels of IL-4 and IFN-γ production. The levels of IL-10 and IL-12 in culture supernatants were also quantified in comparison to standard cytokine curves (Fig. 5), to clarify whether GMFA desensitization induced these cytokines *in vitro*. It was found that significantly higher levels of IL-10 ($P < 0{\cdot}01$) and IL-12 ($P < 0{\cdot}001$) were secreted in the desensitized group in comparison to the sensitized group.

Discussion

It was of interest to note that the immunodominant IgE epitope binding sequence against Balb/c mice sera was homologuous to the human IgE binding sequence, and this prompted us to follow the murine model system as a model for ovomucoid-induced allergy to validate the efficacy of the mutant modified ovomucoid antigen-GMFA, and to characterize its potential role of suppression of allergic reactions in mice. Therefore, this experimental rodent model may be more predictive of human responses.

Desensitization (specific immunotherapy) is the only disease-modifying treatment or therapeutic option currently available for cure against allergic diseases, other than avoidance of a particular food. Earlier immunotherapy with natural allergen extracts led to serious problems with severe systemic symptoms, and rush immunotherapy with peanut extracts showed adverse systemic reactions [16]. Recombinant immunotherapy is an alternative option for promising therapeutic modalities for the treatment and prevention of food allergy [17]. In this study we have reported desensitization of the allergic response to ovomucoid-sensitized mice by a modified isoform of ovomucoid having reduced allergenicity (GMFA), where the decisive B cell epitopes for ovomucoid have been mutated. Desensitization was confirmed by the lack of hypersensitivity symptoms, low level of serum histamine and suppression of the ovomucoid-specific IgE response.

In earlier mouse studies, a rise in allergen-specific IgG was noted to compete with IgE antibodies for allergen binding, thereby blocking IgE-mediated events [18]. In our experiment a significant increase in the level of specific IgG was observed in the desensitized sera samples, so specific IgE inhibition could be associated with the production of blocking antibodies. An increase in specific IgG2a and a decrease in specific IgG1 in desensitized mice demonstrates the augmentation of a Th1 response. IL-4 is critical for the synthesis of IgE by B lymphocytes and IL-4 stimulates growth of T and B cells *in vitro*, and seems to deactivate macrophages and

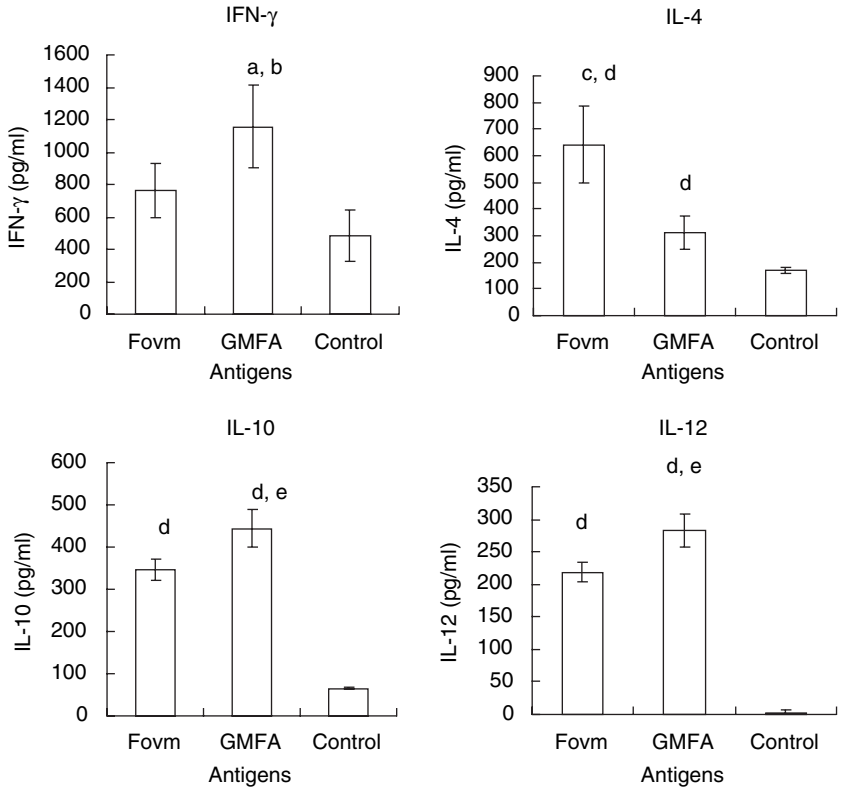

Fig. 5. Effects on cytokine production from antigen-stimulated splenocytes from actively sensitized and desensitized mice. After the final challenge, splenocytes were isolated from each mouse and cultured in the presence or absence of intact ovomucoid (50 μg/ml). The supernatants were harvested after 72 h of culture. Non-Ag-stimulated cultures showed very low cytokine production. Intact ovomucoid-activated splenocyte supernatants were collected from all the three groups and analysed for levels of interleukin (IL)-4, interferon (IFN)-γ, IL-10 and IL-12 as described in Methods. Values are expressed as mean ± s.e.m. of five mice. (a) Significantly higher ($P < 0{\cdot}05$) than the intact ovomucoid (Fovm) group; (b) significantly higher ($P < 0{\cdot}01$) than the control group; (c) significantly higher ($P < 0{\cdot}001$) than the modified ovamucoid (GMFA) group; (d) significantly higher ($P < 0{\cdot}001$) than the control group; (e) significantly higher ($P < 0{\cdot}01$) than the Fovm group.

regulate induction of Th2 cells, thereby inhibiting the development of Th1 cells [19]. Low levels of IL-4 found in the desensitized mice culture supernatant could be an effect of class-switching, attributed to the inhibition of specific IgE levels and bias towards a Th1 pathway. This mechanism is confirmed further by higher levels of IFN-γ in culture supernatants of the desensitized group.

IL-12 has been shown to be an endogenous regulator of Th1 cell development and is reported to be a cytokine that determines the balance between Th1 and Th2 cells [20,21]. In mice administration of an IL-12-allergen fusion protein results in the development of a Th1-specific response to the allergen, with increased production of an allergen-specific IgG2, rather than the normal Th2 response with IgE formation [22]. IL-12p70 is the most potent signal directing force for T cells towards an IFN-γ-producing Th1 phenotype. Our study supports this hypothesis, and we observed an increase in IL-12 which could possibly be driving the production of IFN-γ towards a Th1 bias in the desensitized group. Also, the enhanced levels of IL-10 in the desensitized group observed in our studies could be associated with successful immunotherapy, as shown earlier, resulting in an anergic state [23]. Human studies with various allergens have shown that allergen immunotherapy is associated with increased production of IL-10 [24,25], which agrees with our results. We thereby conclude that successful desensitization is possible with the modified isoform of ovomucoid in ovomucoid-sensitized mice as a therapeutic modality, and with further characterization this strategy could also be expanded to human subjects. To use the GMFA variant for the purposes of clinical study, the optimal dosage and provocation of IgE senstization when administered to humans should be considered in future work.

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