



Engineered recombinant ovomucoid third domain can modulate allergenic response in Balb/c mice model

Prithy Rupa, Yoshinori Mine *

Department of Food Science, University of Guelph, Guelph, Ont., Canada N1G 2W1

Received 24 January 2006

Abstract

IgE-mediated allergic reactions to egg white are a serious health problem and ovomucoid being the dominant egg white allergen has been on focus in the past decade. Engineered hypoallergens with reduced reactivity for IgE antibodies are being examined to modulate the allergic response and develop prophylactic allergen vaccines. In this study, we evaluated the immunomodulatory effect of a genetic variant of the third domain of ovomucoid (GMFA) which showed reduced IgE binding with egg allergic patient's sera in comparison to the native form of the third domain of ovomucoid (DIII) in a murine model system. Balb/c mice were injected intraperitoneally with DIII and GMFA antigens. Allergen-specific serum IgG, IgG1, IgG2a, and IgE responses were evaluated using enzyme-linked immunosorbent assay. Splenocyte cytokine levels in the medium of the cultured cells were examined by ELISA and levels of IL-4, INF- γ , and IL-12 (p70) cytokines were quantified. Neutralization with anti-IL-12 monoclonal antibody was assayed and cytokine levels with respect to GMFA mutant antigen stimulation were measured. GMFA mutant form was found to have significantly reduced levels of specific IgE when compared to the DIII suggesting a mutation-induced abrogation of the IgE binding epitope in mice. The increase in IgG2a levels in GMFA together with the decline of IgE and IgG1 points to a shift from a Th2 response to a Th1 dominated response. The cytokine profile showed a modulation of anti-allergic Th1 phenotype in GMFA from a proallergic Th2 response observed with DIII. Low levels of IL-4 and increased levels of INF- γ and IL-12 were observed and anti-IL-12 monoclonal antibody restored the levels of IL-4 and suppressed levels of INF- γ and IL-12 in the GMFA sensitized group. These results indicate that GMFA has a marked suppressive effect on the allergic response of ovomucoid and caused a shift towards a Th1 pathway, thereby modulating the Th1/Th2 cytokine balance and could be used as a potential hypoallergenic candidate for allergen-immunotherapy in the treatment of egg white allergy.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Food allergy; Ovomucoid; IgE; Balb/c mice; Site-directed mutagenesis; Th1/Th2; Immunotherapy

Food allergy is normally the result of IgE-mediated hypersensitivity reaction (Type I allergy) and incidence of food induced allergic reactions is shown to be increasing lately [1]. Food allergies are more prevalent in children due to their immature gastrointestinal epithelial membrane barrier, allowing more proteins through the barrier and thereby into the circulatory system. Ovomucoid is a major food allergen in the hen's egg white and accounts for about 11% of the total protein fraction of egg white proteins and is comprised of 186 amino acids.

The molecular weight of ovomucoid is 28 kDa and contains about 20–25% of carbohydrates [2]. The molecule consists of three structurally independent tandem domains (DI, DII, and DIII) that are interconnected with peptide bonds and each domain has three intradomain disulfide bonds. IgG and IgE binding reactivity to the third domain (DIII) is significantly more higher when compared to the first (DI) and the second (DII) domains [3]. We have cloned and characterized the third domain of ovomucoid earlier and reported its allergenic properties [4]. Detailed epitope mapping of the whole ovomucoid primary sequence has been reported and amino acids that are critical for IgG and IgE immunoglobulin binding have

* Corresponding author. Fax +1 519 824 6631.

E-mail address: ymine@uoguelph.ca (Y. Mine).

been identified [5]. Genetic attachment of an undecane peptide into the third domain of ovomucoid (DIII) was found to be effective in reducing the production of specific IgE and IgG antibodies in mice [6].

Allergen-specific IgE levels are directly associated with the pathogenesis of allergic response and hence there is an immediate need to suppress specific IgE levels and to dissect the mechanism of allergic reactions in a given system. Cytokines present at the initiation of CD4⁺ T cell responses predominantly determine the pathway of a particular Th subtype. The cytokines secreted by the T helper cells are known to modulate the IgE response. IL-4 induces the IgM to IgE switch and allows IgE secretion in vitro and INF- γ inhibits this process [7]. The production of allergen-specific IgE requires the help of T lymphocytes which are specific to the allergen and stimulated by linear peptide fragments of antigen. In murine systems, it has been reported that IL-4 has a major role in IgE production and INF- γ suppresses the response [8]. The same has been documented with human cells as well [9]. Specific immunotherapy represents the only curative approach toward type I allergy. The use of hypoallergenic (low IgE binding activity) forms of allergens is shown to modulate the allergic immune response at the level of the T helper cell [10]. Modulation of the immune response at the level of Th cells is a key target for successful immunotherapy [11]. Li et al. [12] have demonstrated immunomodulatory effect of heat-killed *Escherichia coli* producing engineered peanut allergens using mice, resulting in downregulation of Th2-associated cytokines and upregulation of Th1 and T-regulatory cytokines.

In the present study, we have established the effect of the allergic response of a variant of the third domain of ovomucoid in comparison to the native third domain of ovomucoid in a murine model of egg allergy. It was shown earlier that the engineered mutant (GMFA) had low IgE binding reactivity upon replacement of the two critical IgE binding amino acids (G³² and F³⁷) on Western blot and ELISA using egg allergic patient's sera [13]. The epitope sequence where the mutation was done was also predominantly recognized with Balb/c mice sera against the recombinant wild type of the third domain of ovomucoid [6]. In order to study the effects of this mutant isoform (GMFA) in accordance with the native form of the allergen (DIII) in mice, we investigated in detail the allergen-specific immune response in Balb/c mice and found that GMFA seems to inhibit and suppress allergic response inducing a dominant Th1 phenotype mediated by IL-12 and INF- γ . These findings draw attention to a potential use of this hypoallergenic variant of ovomucoid as an agent for specific-immunotherapy to prevent egg allergy.

Materials and methods

Animals and reagents. Female Balb/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Mississauga, ON, Canada) and maintained in accordance with standard animal housing conditions at the

Central Animal Facility of University of Guelph in a pathogen-free environment. The mice were maintained under an egg-free diet (Teklad Global 14% protein and 3.5% fat) and a 12/12 h lighting cycle for the duration of the experiment. Food and water were available ad libitum. All animal studies were conducted under guidelines and permission from University of Guelph, Animal Utilization Committee. All antibodies used in the experiments were purchased from either BD Biosciences (Pharmingen, San Diego, CA), Sigma (Sigma-Aldrich, ON, Canada) or Cedarlane (Cedarlane Laboratories, Hornby, ON, Canada).

Expression and purification of the antigens. Recombinant DIII ovomucoid antigen was cloned as a fusion protein with glutathione *S*-transferase (GST) in *Escherichia coli* and mutations were made in the third domain of ovomucoid as reported earlier [4,13]. The recombinant mutant ovomucoid was designed to substitute two single amino acids, glycine at position 32 to methionine and phenylalanine at position 37 to alanine (GMFA). The GMFA ovomucoid construct was transformed into *E. coli* cells. The cell culture containing the fusion protein was induced with 1 mM IPTG (isopropyl-thio-galactosidase) 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 L of Luria broth (LB) containing ampicillin (50 μ g/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 further incubated in the shaker for 4 h. The cells were harvested by centrifugation (7000g 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 7000g for 15 min. Glutathione Sepharose 4B (GS4B) resin (Amersham Pharmacia Biotech) was equilibrated with 10 bed-volumes of 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 and 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 of reduced glutathione. The recombinant protein (GMFA) was passed through a column of polymyxin B-immobilized beads (Sigma) to remove contaminating endotoxins according to the manufacturer's instructions (Sigma). The native ovomucoid DIII without carbohydrate was prepared by hydrolysis of ovomucoid with *Staphylococcus aureus* V₈ (Spase V₈) protease (Sigma), as described earlier [2].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, to analyze the purity of the antigens, according to the earlier described method by Laemmli [14] in a Mini-Protein II Electrophoresis Cell (Bio-Rad). Samples were run on 15% homogeneous gel. Proteins were dissolved in sample buffer in the presence of 5% (w/v) β -mercaptoethanol, heated for 5 min at 95 °C in an Eppendorf Thermomixer 5436 (Fisher Scientific, Unionville, CA, USA), and loaded onto the gel. Gels were run at a constant current (18 mA/slab gel) and visualized by silver staining for homogeneity.

Mice immunization and collection of samples. Balb/c mice (four per group) (6–8 weeks old) were rested for a week after arrival at the facility and were prebled and sensitized with the native third domain ovomucoid antigen (DIII) and mutant ovomucoid antigen (GMFA). Hundred micrograms of the antigens was emulsified with 100 μ l of complete Freund's adjuvant (CFA) (Sigma) and injected through the subcutaneous route. Animals were monitored for stress, pain, and lesions. Four weeks later, a booster was given with the same amount of antigens using Incomplete Freund's adjuvant (IFA) (Sigma). Two weeks later all the animals were euthanized and blood samples and spleen were collected. The control animals were immunized with phosphate-buffered saline (PBS) (adjuvant control). Sera collected from mice at the end point were used to determine antigen-specific and total antibody levels.

Measurement of ovomucoid-specific and total IgE by ELISA. The total and specific IgE levels in the mice sera were measured according to a previously described procedure [15]. The total IgE level was determined using 100 μ l of rat anti-mouse IgE at 1 μ g/ml (BD Bioscience) in 50 mM sodium carbonate buffer (pH 8.5) as a capture antibody and incubated

overnight at 4 °C. The wells were washed with PBS containing 0.05% Tween (PBST) three times and were blocked with 200 µl of blocking buffer [2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2], for 1 h at 37 °C; subsequently, the wells were washed three times with PBST. Serum samples were diluted in blocking buffer (1:20) and incubated with the bound anti-IgE monoclonal antibody overnight at 4 °C. The plate was further washed with PBST three times and 100 µl of biotinylated rat anti-mouse IgE (Caltag, CA, USA) was added to each well (diluted with blocking buffer, final concentration 2 µg/ml), and the plate was incubated at 37 °C for 1 h. The plate was further washed with PBST and incubated with 100 µl of avidin-conjugated alkaline phosphatase (1:3000) (Sigma) for 1 h and developed with *p*-nitrophenol phosphate (1 mg/ml) (Sigma) in 0.1 M di-ethanolamine buffer (pH 9.8) for 60 min at 37 °C. The plate was read after 60 min of development after terminating the reaction with 3 M NaOH (sodium hydroxide). Absorbance at 405 nm was read using a microplate reader (Model 550, Bio-Rad Laboratories). Purified mouse anti-IgE antibody (Pharmingen) was used as a standard. Specific IgE levels were detected in a similar way except for coating the plate with 2 µg/well of the ovomucoid antigen (DIII or GMFA).

Measurement of total and specific IgG. Total and specific IgG levels were obtained according to the above-mentioned ELISA procedure. Goat anti-mouse IgG (EMD Biosciences, CA, USA) was used as a capture antibody at a dilution of 1 µg/ml in coating buffer (PBS), pH 7.2. Alkaline phosphatase-conjugated to rabbit anti mouse IgG (Sigma) (1:2000 dilutions) was used as a detection antibody. Specific IgG levels were detected by coating the plates with 2 µg/well of the antigen and all other steps being the same as mentioned above.

Measurement of specific IgG1 and IgG2a levels. For specific IgG1 and IgG2a determination, indirect ELISA was performed. Native (DIII) and mutant ovomucoid (GMFA) antigens were coated (2 µg/well) and subsequent steps were the same as above. Samples were diluted at 1:1000 dilutions for IgG1 and 1:500 dilutions for IgG2a. Rat anti-mouse IgG2a and IgG1 (final concentration of 1 µg/ml) (100 µl) (BD Bioscience) were added to check the bound antibodies and peroxidase-conjugated mouse anti-rat Ig (1:2000) (BD Bioscience) was added to detect the bound antibodies and the reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate developing solution (Sigma) and stopped with 1 N H₂SO₄. Absorbance was measured at 450 nm.

Isolation and culturing of splenocytes. Balb/c mice (*n* = 5 per group) were sensitized intraperitoneally with 100 µg of DIII or GMFA antigen. Both the antigens were adsorbed onto equal volumes of aluminum hydroxide (2%) (Superfos Biosector, Frederikssund, Denmark). Control mice received phosphate-buffered saline. Seven days later, the animals were euthanized and the spleen was removed aseptically in ice-cold RPMI 1640 media (Life technologies, Gaithersburg, MD) and minced, and cell suspensions were prepared. The splenocytes were isolated after the removal of the red blood cells using red blood cell lysing buffer (RBC) (Sigma) and viable counts were performed by exclusion of trypan blue dye. More than 95% of cells were determined viable by trypan blue dye exclusion. Single cell suspensions of splenocytes were seeded in triplicate into 96-well flat-bottomed tissue culture plates (Corning Costar, Fischer Scientific, Pittsburg, USA) (1×10^6 cells/well) and cultured in the presence of 50 µg of native (DIII) or mutant antigen (GMFA) of ovomucoid for 72 h at 37 °C in a humidified atmosphere of 5% CO₂.

Cytokine ELISA. Culture supernatants from the incubated plates were separated after 72 h and analyzed for levels of IL-4, INF-γ, and IL-12 (p70). The protocol for cytokine ELISA was followed as per the BD Bioscience capture ELISA protocol instructions with slight modifications. Paired monoclonal antibodies which are specific for cytokines IL-4, INF-γ, and IL-12 (p70) were used. The purified anti-cytokine capture antibodies were coated at a concentration of 2 µg/ml in binding solution (0.1 M Na HPO₄, adjusted to pH 9.0 with 0.1 M NaH₂PO₄). The 96-well ELISA plates (Corning, NY, USA) were incubated overnight at 4 °C. The plates were washed three times with Tris buffered saline containing 0.05% Tween 20 (TBST) and incubated with 2% BSA in TBST (blocking buffer) to block the nonspecific binding for 1 h at 37 °C. The plates were washed three times with TBST and further incubated with the culture supernatant samples and recombinant cytokine standards diluted in blocking buffer

containing TBST for 2 h at 37 °C. After incubation, the plates were washed three times with TBST and biotinylated anti-cytokine detection antibodies for IL-4, INF-γ, and IL-12 (2 µg/ml) were added at 37 °C for 1 h. The plates were washed thrice with TBST and avidin-horseradish peroxidase (HRP) (Pharmingen) was added (1:2000) and incubated for 1 h at 37 °C. The plates were subsequently washed six times with PBST and developed with 50 µl of TMB substrate (tetramethylbenzidine) (Sigma) in dark and the reaction was stopped with 25 µl of 0.5 M H₂SO₄ and absorbance was determined at 450 nm.

IL-12 neutralization assay. For neutralization study, the levels of cytokines secreted were measured by adding monoclonal anti-IL-12 antibody (Pharmingen) (various concentrations of 0.02, 0.2, and 2 µg/ml) along with the mutant ovomucoid GMFA antigen for stimulation. Control wells were stimulated with GMFA alone. The plates were incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO₂ and levels of IL-4, INF-γ, and IL-12 were measured using the above-mentioned cytokine capture ELISA protocol.

Statistical analysis. The results were expressed as means ± SEM, with the number of mice/group indicated in each figure. Differences between groups were analyzed using 1-way analysis of variance (ANOVA—from GraphPad Instat Version 3.05 software, San Diego, CA, USA) followed by Tukey–Kramer multiple intergroup comparison test and the level of significance was defined at *P* < 0.05.

Results

Purification of ovomucoid third domain antigens

Recombinant DIII ovomucoid mutant antigen (GMFA) was expressed as a fusion protein and purified to homogeneity using glutathione–Sepharose 4B resin and was found that ovomucoid gene was highly expressed as a soluble fusion protein with GST in *E. coli*. Nearly 1 mg of purified protein was obtained per liter of induced culture. SDS–PAGE

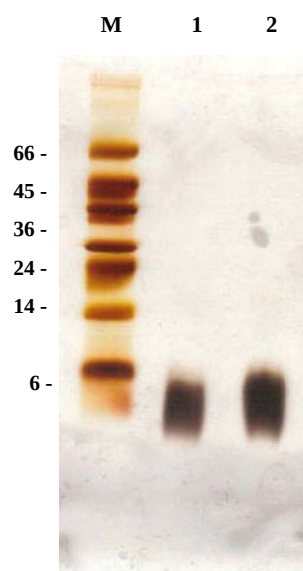


Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis profile of ovomucoid third domain antigens. The antigens of ovomucoid were purified and subjected to electrophoresis and analyzed on a 15% SDS–PAGE gel. The protein bands were visualized with silver stain. M represents the low range molecular markers (Sigma). Lane 1 represents the mutant form (GMFA) of ovomucoid and lane 2 represents the native third domain of ovomucoid (DIII).

analysis showed the presence of a predominant homogeneous band. The fusion protein from lysates was purified by glutathione–Sephadex 4B affinity chromatography, a single band as shown by SDS–PAGE analysis in Fig. 1. The protein has an apparent molecular size of 6 kDa after cleavage of the GST–protein, which corresponds well with the predicted molecular weight of the third domain ovomucoid antigen. Adsorption of GMFA to polymyxin B, an inhibitor of lipopolysaccharides, confirms negligible amounts of endotoxins in the sample. The native DIII antigen was also purified to homogeneity and silver staining was performed to analyze the purity of the antigens and the results showed that there were no contaminating cellular proteins in the purified fraction.

Suppression of specific IgE levels of ovomucoid in mice

Allergen-specific and total IgG and IgE responses were analyzed by direct and indirect ELISA using mouse sera obtained at the endpoint. Sensitization of ovomucoid antigen DIII in BALB/c mice resulted in high levels of specific IgG and IgE antibodies (Fig. 2). On the contrary, GMFA

treated mice maintained significantly low serum specific IgE levels ($P < 0.001$), although no significant difference was observed in the total IgE and IgG levels (Fig. 2). Up regulation of serum specific IgE and IgG levels was suppressed with the GMFA treated sample. The discrete difference in specific IgE levels was more pronounced than in specific IgG.

GMFA skews isotyping profile of IgG subclass antibodies in mice sera

In order to evaluate if GMFA modulates additional immunoglobulins involved in allergic responses, the levels of allergen (ovomucoid DIII)-specific serum IgG1 and IgG2a levels were measured. Subtyping of the IgG class revealed that the mice immunized with the native third domain of ovomucoid (DIII) showed detectable levels of specific IgG1 and low levels of specific IgG2a (Fig. 3). On the contrary, in the sera sample collected from the mice immunized with GMFA protein, the specific levels of IgG1 were considerably low when compared to the native third domain (DIII), but a significant increase in the level

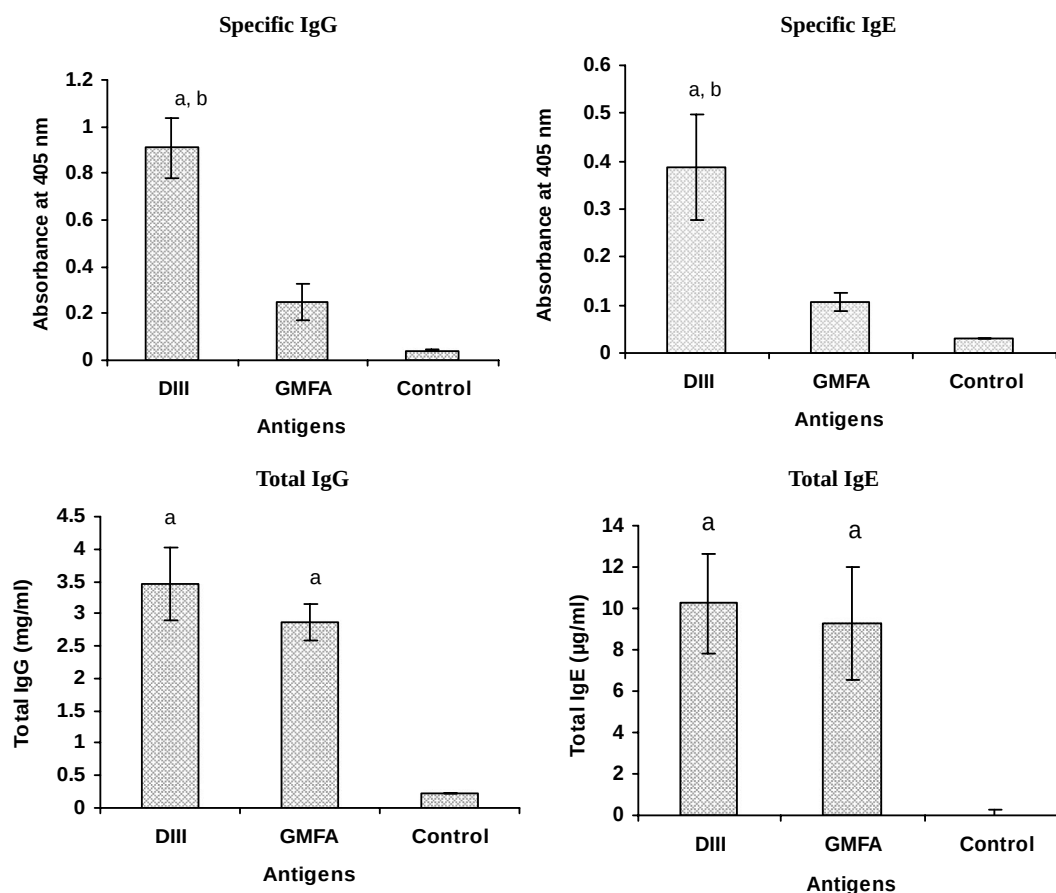


Fig. 2. Determination of total and specific serum IgE and IgG antibody levels in immunized mice sera. BALB/c mice were sensitized with the native (DIII) antigen and GMFA antigen as described in the Materials and methods. Control mice received PBS. Sera were collected at the end point (week 6), and total and specific IgE and IgG levels were detected by ELISA. Results are expressed as means of individual sera $\pm$ SEM ($n = 5$ mice/group). Total IgG and IgE levels were quantified using standards. Statistical differences between designated groups are indicated by ^a $P < 0.001$ vs control group and ^b $P < 0.001$ vs GMFA group.

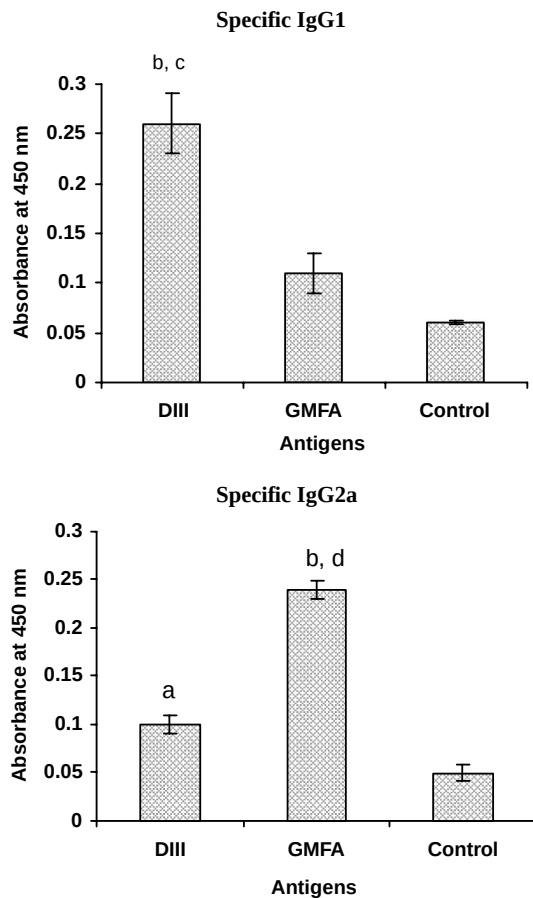


Fig. 3. Serum ovomucoid-specific levels of IgG1 and IgG2a. Balb/c mice were sensitized with the native (DIII) and the mutant (GMFA) antigens, and the serum specific IgG1 and IgG2a levels of antibodies were detected using indirect ELISA. The levels are represented as absorbance (OD) at 450 nm. Results are expressed as means of individual sera $\pm$ SEM ($n = 4$ mice/group) (^a $P < 0.01$ vs control group; ^b $P < 0.001$ vs control group; ^c $P < 0.001$ vs GMFA group; ^d $P < 0.001$ vs DIII group).

of specific IgG2a was observed ($P < 0.001$). GMFA is thus shown to inhibit IgG1 levels and up-regulate IgG2a production.

Cytokine profile in mice skews T cell response

Th1 immune mediators include IL-12 and IFN- γ while Th2 cytokines include IL-4. These immune responses can balance one another, as Th2 mediators suppress Th1 responses and Th1 mediators similarly inhibit Th2 responses. Th1 and Th2 cytokines are antagonistic in activity [16]. The balance between Th1 and Th2 activity may steer the immune response in the direction of cell-mediated or humoral immunity [17]. In order to determine if the IgE suppression effect of GMFA had any influence on the alteration of the Th1/Th2 cytokine profile, we analyzed the levels of Th1 and Th2 cytokines, secreted by the culture supernatants from splenocytes of mice sensitized and stimulated with DIII, GMFA, and PBS, respectively. We detected elevated levels of IL-4 in mice sensitized with DIII

antigen and significantly reduced levels of IL-4 in mice sensitized with GMFA ($P < 0.001$). Considerable increase in the levels of INF- γ and IL-12 was observed with GMFA treated sample when compared to DIII antigen ($P < 0.001$) (Fig. 4). The inhibition of IL-4 by GMFA showed an inclination towards the Th1 pathway. Th1 cytokine IFN- γ inhibits proliferation of Th2 cells and stimulates B cells to secrete IgG2a and inhibits secretion of IgG₁ and IgE. Thus, the results obtained suggest that the suppression effect of GMFA is due to the modulation of the cytokine profile from an existing allergic (Th2) to an

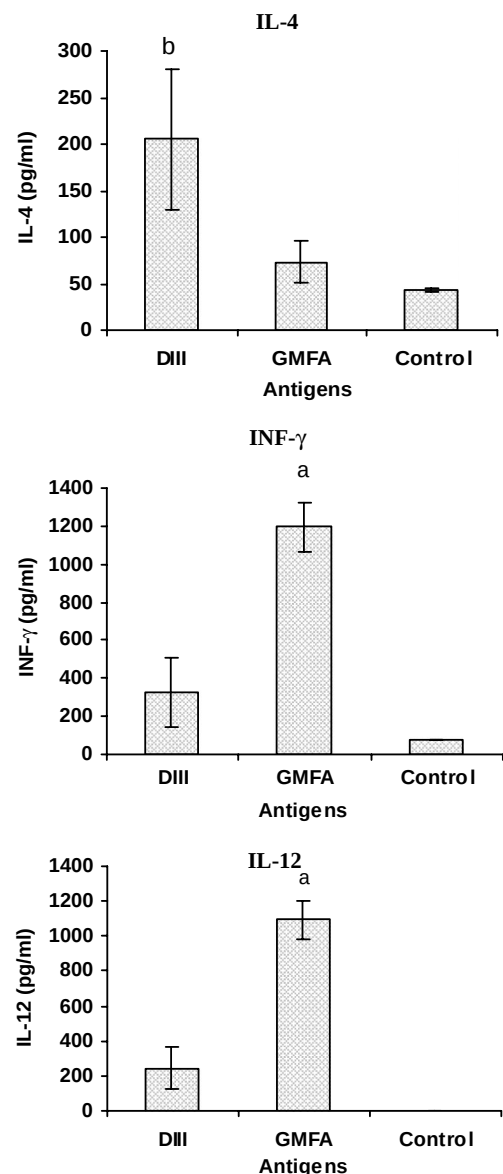


Fig. 4. Cytokine measurements in the culture supernatants of the splenocytes obtained from the immunized mice. The levels of cytokines IL-4, INF- γ , and IL-12 (p70) were analyzed after 72 h of culturing the splenocytes in the presence of the respective antigens (DIII and GMFA) and were compared to standard recombinant cytokines and quantified using ELISA. The absorbance was measured at 450 nm. Results are expressed as means $\pm$ SEM ($n = 5$ mice/group) (^a $p \leq 0.001$ DIII and control group; ^b $p \leq 0.001$ vs GMFA and control group).

anti-allergic (Th1) skewed pathway. IL-12 enhances IFN- γ production by natural-killer T cells [18]. Increase in both IL-12 and IFN- γ cytokine levels in the GMFA supernatant sample indicates clearly that it was biased

towards an anti-allergic Th1 skewed response, whereas the DIII antigen was biased towards the allergenic Th2 pathway.

Addition of anti-IL-12 antibody inhibits enhancement of IL-12 and INF- γ , and restores IL-4 levels

IL-12 can induce a dominant Th1-type cytokine profile [19,20]. To test whether IL-12 is involved in the IL-4-inhibitory activity of supernatants in GMFA samples, we examined the effect of neutralization of GMFA on the production of IL-4, IFN- γ , and IL-12 using different concentrations of monoclonal anti-IL-12 antibodies along with the control GMFA antigen to stimulate the culture supernatants. Addition of anti-IL-12 antibody abrogated the suppression of IL-4 in case of the GMFA treated samples (Fig. 5). The suppressed IL-4 levels were restored ($P < 0.05$), and IFN- γ and IL-12 levels were suppressed with monoclonal anti-IL-12 antibodies tested at diminishing concentrations ($P < 0.001$) (Fig. 5). The IL-12 augmentation by GMFA to induce Th1 development preferentially was clearly evident from this neutralization experiment.

Discussion

Cytokine stimulation controls immune responses. Th2-type cytokines (i.e., interleukin [IL]-4, IL-5, IL-9, and IL-13) influence a wide range of events associated with allergic reactions [21]. Modulation of the Th1/Th2 balance, namely, shifting the balance from Th2 to Th1 dominance, should be a rational strategy for treating allergic diseases involving Th2 cells. The balance between Th1 and Th2 is critical for IgE production. Identification and modification of amino acid residues on sequential and conformational epitopes of allergens that interact with IgE are a rationale target for curing allergic reactions. Inhibition of IgE production through promoting the Th1-type immune response thereby enhancing cellular immunity is an approach for allergen-immunotherapy [22]. A molecule having highly reduced ability to bind IgE should provide a valuable candidate molecule for immunotherapy. Recombinant allergen derivatives are now being used to produce engineered molecules with reduced IgE binding activity for allergy vaccinations [23]. Point mutations of the critical amino acids within the IgE epitope have been shown to abolish the IgE binding activity [24]. Our earlier data indicate that the mutant form of ovomucoid (GMFA) is effective in inhibiting the IgE binding in human patient's sera and reduces an established IgE response [13].

In this report, we demonstrated the effect of the mutant form of ovomucoid (GMFA) in inhibiting the allergic response in a murine model system, since mice model system is being commonly used by different research groups to study various mechanisms involved with molecular pathogenesis of allergic diseases. Although mice immune system does not reflect humans, it is noteworthy

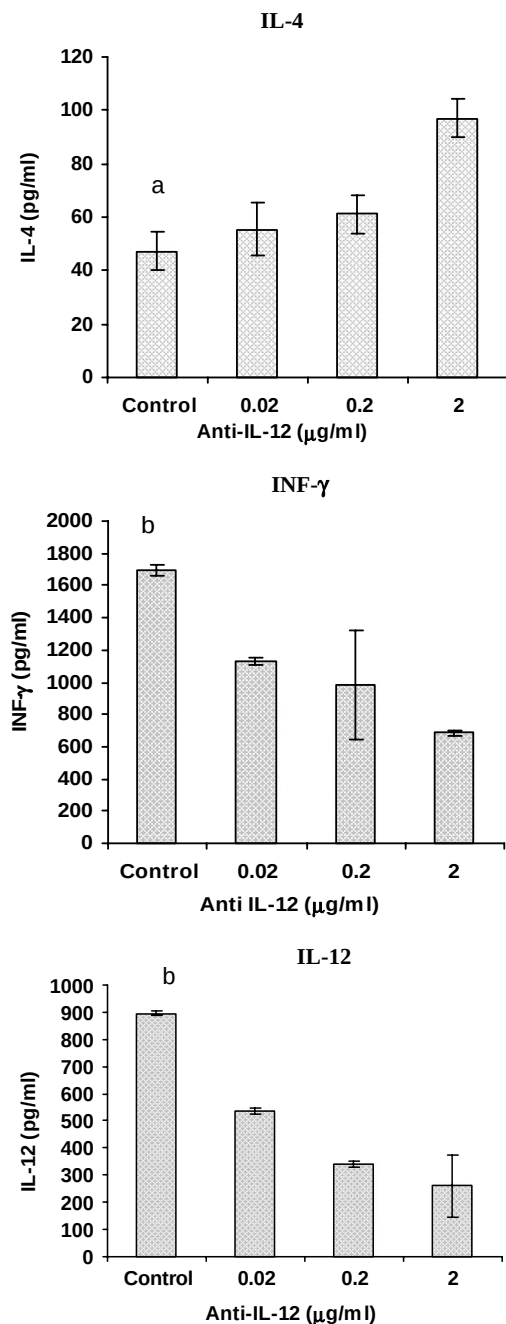


Fig. 5. Effect of anti-IL-12 antibody on the levels of IL-4, INF- γ , and IL-12 (p70) cytokines in the stimulated splenocyte cultures. Splenocytes from the immunized mice were primed with mutant GMFA antigen and with monoclonal anti-IL-12 antibody at various concentrations and the levels of cytokines IL-4, INF- γ , and IL-12 (p70) were measured. Control cultures contained GMFA mutant only. The results were read by measuring the absorbance at 450 nm. Results are expressed as means $\pm$ SEM ($n = 5$ mice/group) (^a $P < 0.05$ vs groups treated with anti-IL-12 antibody; ^b $P < 0.001$ vs groups treated with anti-IL-12 antibody).

to trust the information obtained from murine models, before attempting with human trials. Genetically fused affinity fusion partners are widely used to express recombinant proteins in *E. coli*, because fusion partners with high affinity for a specific ligand make it much more easy to purify fusion protein from hundreds of proteins. GMFA antigen has thus been purified to homogeneity which yielded a single band on a silver-stained SDS–PAGE gel.

Deletions of two amino acids specific for IgE binding could have a major impact on the whole immune response of the molecule which is clearly illustrated by the decreased levels of serum specific IgE in GMFA sera. The cause of the decreased levels of IgG subclasses is mostly unknown. Disturbances in the regulation of IgG subclass protein synthesis probably often underlie the decreased levels. The levels of allergen-specific IgG do not consistently correlate with allergic reactions unlike specific IgE, although it has been stated that increase in IgG1 exerts a blocking effect on circulating allergens by binding and preventing mast cell degranulation [25]. It was shown that levels of specific IgG1 and IgE in GMFA sera are suppressed, but there are probably various other immunological factors involved, that combine to effect suppression of allergic mechanisms.

It has been shown that INF- γ secreting Th1 cells can stimulate B cells to synthesize IgG2a antibodies and inhibit IgE production [26]. The observed isotypic differences indicate that GMFA antigen favors IgG2a production which is more inclined towards the Th1 response. The inhibitory effect of GMFA on IgE production by promoting the Th1-type of immune response is clearly defined from the levels of cytokine measured. The regulatory effect of GMFA on inhibition of allergen-induced IgE production and modulation of cytokine levels which is dependent on the IL-12 driven pathway is clearly demonstrated. In the neutralization experiment, the INF- γ levels are associated or dependent on the IL-12 driven pathway. Our results show that the combination of the hypoallergen concept with the Th1-inducing approach which is cytokine-driven would pave a way for therapeutic treatment of egg allergy. GMFA mutant ovomucoid antigen may represent an ideal target to be used as a hypoallergenic derivative of ovomucoid in the design of a vaccine, against egg allergy for use in immunotherapy. GMFA offers a ray of hope for cure of egg-induced allergy. Although the precise mechanism of the suppression effect of GMFA is unknown, it is clear from the above experiment that GMFA suppresses specific IgE levels which is associated with modulation of cytokines from the Th2 to Th1 pathway. Thus, the findings obtained from this in vitro study suggest that GMFA has the potential to suppress symptoms of allergy towards ovomucoid. In future, for therapeutic studies the modified mutant ovomucoid (GMFA) would prove to be an ideal candidate for desensitizing egg allergic mice and thereby extended to human subjects as well.

Acknowledgment

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC).

References

- [1] D.B. Corry, F. Kheradmand, Induction and regulation of the IgE response, *Nature* 402 (1999) B18–B23.
- [2] I. Kato, J. Schrodde, W.J. Kohr, M. Laskowski, Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site and preparation of all three of its domains, *Biochemistry* 26 (1987) 193–201.
- [3] J.W. Zhang, Y. Mine, Characterization of IgE and IgG epitopes on ovomucoid using egg-white-allergic patients' sera, *Biochem. Biophys. Res. Commun.* 253 (1998) 124–127.
- [4] E. Sasaki, Y. Mine, IgE binding properties of the recombinant ovomucoid third domain expressed in *Escherichia coli*, *Biochem. Biophys. Res. Commun.* 282 (2001) 947–951.
- [5] Y. Mine, J.W. Zhang, Identification and fine mapping of IgG and IgE epitopes in ovomucoid, *Biochem. Biophys. Res. Commun.* 292 (2002) 1070–1074.
- [6] Y. Mine, P. Rupa, Genetic attachment of undecane peptides to ovomucoid third domain can suppress the production of specific IgG and IgE antibodies, *Biochem. Biophys. Res. Commun.* 311 (2003) 223–228.
- [7] H.L. Spiegelberg, L. Beck, H.P. Kocher, W.C. Fanslow, A.H. Lucas, Role of interleukin in human immunoglobulin E formation in hu-PBL-SCID mice, *J. Clin. Invest.* 93 (1994) 711–717.
- [8] C.M. Snapper, W.E. Paul, Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production, *Science* 236 (1987) 944–947.
- [9] J. Pene, F. Rousset, F. Briere, I. Chretien, J.Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K. Arai, J. Banchereau, J.E. Vries, IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2, *Proc. Natl. Acad. Sci. USA* 85 (1988) 6880–6884.
- [10] F. Ferreira, C. Ebner, B. Kramer, G. Casari, P. Briza, A.J. Kungl, R. Grimm, B. Jahn-Schmid, H. Breiteneder, D. Kraft, M. Breitenbach, H.J. Rheinberger, O. Scheiner, Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy, *FASEB J.* 12 (1998) 231–242.
- [11] C. Ebner, U. Siemann, B. Bohle, M. Willheim, U. Wiedermann, S. Schenk, F. Klotz, H. Ebner, D. Kraft, O. Scheiner, Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from Th2 to Th1 in T-cell clones specific for Phl p1, a major grass pollen allergen, *Clin. Exp. Allergy* 27 (1997) 1007–1015.
- [12] X.M. Li, K. Srivatsava, A. Grishin, C.K. Huang, B. Schofield, W. Burks, H.A. Sampson, Persistent protective effect of heat-killed *Escherichia coli* producing engineered recombinant peanut proteins in a murine model of peanut allergy, *J. Allergy Clin. Immunol.* 112 (2003) 159–167.
- [13] Y. Mine, E. Sasaki, J.W. Zhang, Reduction of antigenicity and allergenicity of genetically modified egg white allergen, ovomucoid third domain, *Biochem. Biophys. Res. Commun.* 302 (2003) 133–137.
- [14] U.K. Laemmli, Cleavage of structural proteins during assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [15] P. Rupa, Y. Mine, Immunological comparison of native and recombinant egg allergen, ovalbumin, expressed in *Escherichia coli*, *Biotechnol. Lett.* 25 (2003) 1917–1924.
- [16] E. Maggi, The TH1/TH2 paradigm in allergy, *Immunotechnology* 3 (1998) 233–244.

- [17] T.R. Mosmann, R.L. Coffman, TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties, *Ann. Rev. Immunol.* 7 (1989) 145–173.
- [18] M. Taniguchi, M. Harada, S. Kojo, T. Nakayama, H. Wakao, The regulatory role of Valpha14 NKT cells in innate and acquired immune response, *Ann. Rev. Immunol.* 21 (2003) 483–513.
- [19] T. Germann, E. Rude, Interleukin-12, *Int. Arch. Allergy Immunol.* 108 (1995) 103–112.
- [20] G. Trinchieri, Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes, *Blood* 84 (1994) 4008–4027.
- [21] A.B. Kay, Allergy and allergic diseases. First of two parts, *N. Engl. J. Med.* 344 (2001) 30–37.
- [22] E. Raz, H. Tighe, Y. Sato, M. Corr, J.A. Dudler, M. Roman, S.L. Swain, H.L. Spiegelberg, D.A. Carson, Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization, *Proc. Natl. Acad. Sci. USA* 93 (1996) 5141–5145.
- [23] S. Vrtala, M. Focke-Tejkl, I. Swoboda, D. Kraft, R. Valenta, Strategies for converting allergens into hypoallergenic vaccine candidates, *Methods* 32 (2004) 313–320.
- [24] I. Swoboda, N. De Weerd, P.L. Bhalla, V. Niederberger, W.R. Sperr, P. Valent, H. Kahlert, H. Fiebig, P. Verdino, W. Keller, C. Ebner, S. Spitzauer, R. Valenta, M.B. Singh, Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE-binding capacity: candidates for grass pollen-specific immunotherapy, *Eur. J. Immunol.* 32 (2002) 270–280.
- [25] R.J.J. Van Neerven, T. Wikborg, G. Lund, B. Jacobsen, A. Brinch-Neilsen, J. Arned, H. Ipsen, Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4⁺ T Cells by inhibiting serum-IgE-facilitated allergen presentation, *J. Immunol.* 163 (1999) 2944–2952.
- [26] H.J. Peng, S.N. Su, Z.N. Chang, P.L. Chao, S.W. Kuo, L.C. Tsai, Induction of specific Th1 response and suppression of IgE antibody by vaccination with plasmid DNA encoding Der f 11, *Vaccine* 20 (2002) 1761–1768.