

Novel B and T cell epitopes of chicken ovomucoid (Gal d 1) induce T cell secretion of IL-6, IL-13, and IFN- γ

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Summary

Background Chicken ovomucoid (OM, Gal d 1) has an important role in the pathogenesis of IgE-mediated allergic reactions to hen's egg white.

Objectives The purpose of this study was to clarify the mechanisms of T cell recognition of ovomucoid using intact OM and chemically modified, characterized and homogeneous solid phase synthetic peptides covering the whole molecule.

Methods Eighteen overlapping peptides were prepared by solid phase F-moc polyamide peptide synthesis (SPPS), characterized and high-pressure liquid chromatography (HPLC) purified. The peptides, together with intact, denatured and oxidized OM, were used to stimulate patient-derived cell cultures for mapping T cell epitopes. Proliferation responses, T cell phenotype and cytokine secretion using peripheral blood mononuclear cells (PBMC) from eight individuals and T cell lines (TCL) derived from six hen's egg-allergic patients, were examined. In addition, intact, denatured, oxidized and deglycosylated OM, as well as the peptides solely or with their keyhole limpet haemocyanin (KLH) complexes, were tested. For locating IgE and IgG B cell epitopes, seven egg-allergic patient sera and three OM-polyclonal sera were used. Healthy non-allergic individuals were included as controls.

Results Seven peptides were recognized by specific IgE, while OM-specific TCL recognized 10 peptides. Six of the OM peptides were commonly recognized both by patient S-IgE and blood-derived TCL. Among those, one novel epitope, peptide OM 61–74, had the ability to bind IgE. Another peptide, OM 101–114, was recognized by IgE and IgG sera, but not by any of the TCLs. In contrast, the peptides OM 41–56, OM 71–84, OM 131–144 and OM 171–186 were exclusively T cell epitopes with no affinity to specific antibodies. Abundant TCL secretion of IFN- γ , IL-6, IL-4, IL-13, IL-10 and TNF- α in response to OM stimulation indicates the contribution of Th2 as well as Th1/Th0 CD4⁺ cell subsets. For allergic patients moderate amounts of IFN- γ , IL-13, and high amounts of IL-6, were secreted in response to TCL stimulation by OM peptides. High amounts of IL-6 were secreted in response to all molecular forms of OM (intact-, modified-OM and the peptides 71–84 and 51–64) when TCLs from two non-allergic donors were used.

Conclusions One novel B cell epitope (OM 61–74) and 10 T cell epitopes have been identified. The most reactive epitopes of the OM molecule comprise the motifs 1–14 to 71–84, the overlapping peptide-pairs OM 121–134 and OM 131–144 and peptides OM 161–174 and 171–186. Peptides OM 1–14 and 171–186 are the only ones capable of inducing IL-4 secretion. Only one peptide (OM 11–24) induces IL-10 secretion. Those peptides recognized as both T and B cell epitopes or only T cell epitopes, have the potential to induce T cell secretion of moderate to high amounts of IL-13, IFN- γ and particularly IL-6.

Keywords: allergen specific T cell lines, B cell epitopes, cytokine profile, chicken egg white allergens, ovomucoid (Gal d 1), ovomucoid peptides, T cell epitopes

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Introduction

Hen's egg is an essential ingredient in human and animal food and frequently induces hypersensitivity reactions, especially in children [1]. Symptoms can arise due to ingestion of food not properly labelled, containing 'concealed' egg white allergens. Chicken ovomucoid (OM), ovalbumin (OA), ovotransferrin (Otr) and lysozyme (Lsz) are all allergens of hen's egg white [2–6]. OM was reported to be the major allergen [1,7]. The presence of hen's egg-specific IgE seemed to be a predictive marker at early infancy for allergic sensitization to common allergens that cause asthma, allergic rhinitis and atopic dermatitis [8–11].

OM (Gal d 1, 28 kd, Fig. 1), is a highly glycosylated protein comprising 186 amino acids arranged in three tandem domains: Gal d 1.1 (residue 1–64), Gal d 1.2 (residue 65–130) and Gal d 1.3 (residue 131–186). OM is highly homologous to pancreatic secretory trypsin inhibitor [12]. The carbohydrate moieties of OM represent about 25% of the molecule [13,14]. Characterization of OM-specific T cells has been very limited except for the investigation of PBMC proliferation responses following

OM, OM-fragment stimulation [11], and the generation of OM-specific TCL [15] and OM-domain-specific TCL [16]. T cell epitope mapping is therefore necessary to determine which regions are the major response epitopes of OM.

In this study, we screened the molecule for BT cell epitopes using sera, PBMC and OM-specific TCL derived from hen's egg-allergic patients. Native OM, chemically modified OM and 18 overlapping peptides solely and with their KLH carrier, complexes were investigated. In addition, phenotype investigations and cytokine release from the stimulated TCL were performed.

Materials and methods

Sera and cell donors

Diagnosis of egg allergy was based on history, skin prick test, assay of IgE and elimination of egg from the diet. Patients were selected from routine outpatient *in vitro* allergy diagnosis at the Central Laboratory, University Hospital Bergen. Patients donated blood samples after an

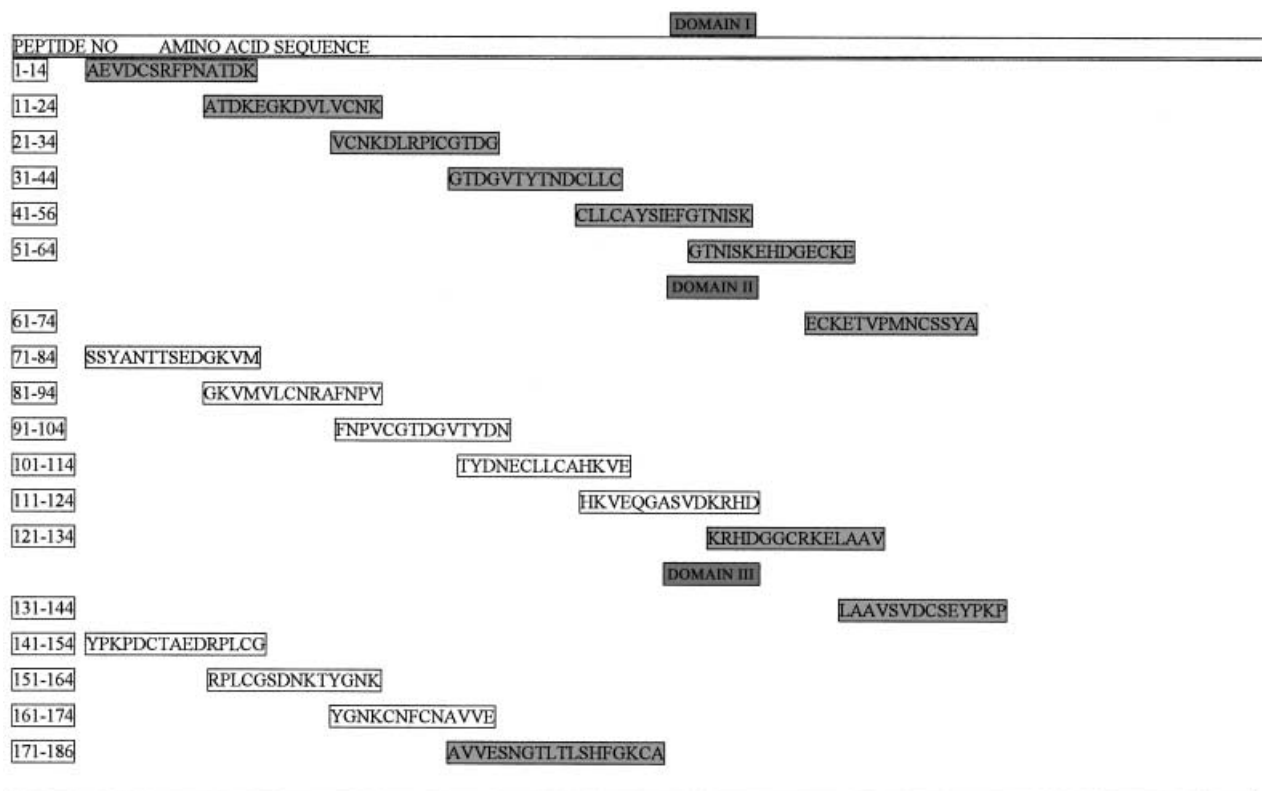


Fig. 1. Main allergenic areas along the OM molecule. Eighteen overlapping peptides are shown with five amino acid common windows. The yellow areas indicate OM-specific BT cell epitopes or T cell epitopes with the potential to induce abundant TCL secretion of IFN- γ , IL-6 and IL-13, in particular, and IL-4. The non-coloured epitopes are inactive. The three domains of the molecule are shown in blue boxes.

informed consent was obtained. *In vitro* diagnosis included serum-total (S-total) IgE and serum-specific (S-specific) IgE for hen's egg white, ovomucoid and ovalbumin as described later in 'Methods'.

Sera from seven patients (six males and one female) allergic to hen's egg white and two (females) non-allergic healthy adults (age range 28–63, mean 39.5 years) were collected. Radio-allergo-sorbent test (CAP/RAST) [17] indicated high titres of S-IgE; mean 3079 kU/L, range 187–8932 kU/L. S-specific IgE was assayed by CAP/RAST for egg white, ovalbumin and ovomucoid and expressed as class (0–6). Two non-allergic individuals were included as controls (S-IgE < 26 kU/L). The sera are further described in Table 1.

Denatured, oxidized and deglycosylated ovomucoid

The OM was trypsin inhibitor, type II-O, chicken egg white supplied by Sigma (St Louis, MO, USA) (T-9253). To produce denatured OM, type II-O OM (10 mg), 6 M Guanidine-HCl (2 mL, Fluka 50940, Buchs, Switzerland) 500 mM Tris-HCl (Sigma T-3253) and 10 mM EDTA (pH 8.6, Sigma ED2SS) were carefully shaken together. DL-dithiothreitol, Cleland's reagent (DDT) (4.6 mg, DL-DDT 99%, Sigma D-0632), were added, in molar excess (mole ratio DDT/OM = 100), to the mixture under N₂ gas for 2 min before incubation for 2 h at 37 °C. Iodoacetic acid (6.14 mg free acid, Sigma I-6250) freshly prepared in 100 µL 0.5 N NaOH was added, in molar excess with respect to DDT (mol ratio iodoacetic acid/DDT = 1.1), before shaking and incubation for 30 min, RT, in the dark. Finally, 1% of 2-mercaptoethanol (240 µL, Merck 805740, Dramstadt, Germany) was added slightly in excess of iodoacetic acid. The mixture was shaken then dialysed (12 kd cut-off) against 0.05 M NH₄HCO₃ (BDH 10302) overnight at

4 °C. The mixture was then lyophilized and stored at –20 °C until use.

Performic acid oxidation of OM OM type II-O, 100 mg, was treated with 70% formic acid, 10 mL, for 15 min. H₂O, 70 mL, was then added and the solution was lyophilized and stored.

Deglycosylation Cleavage of the carbohydrate moiety was performed using N-glycosidase F (20-units/1 mL PBS, pH 7.2, Boehringer Mannheim, Mannheim, Germany). Final solutions (enzyme/substrate ratio E/S): 10 units N-glycosidase F and 2.5 mg denatured OM in PBS were incubated for 24 h at RT. A further 10 units of the enzyme were added and the solution was incubated for 24 h at RT. The solution was then dialysed for 12 h against 0.05 NH₄HCO₃ and finally lyophilized.

Peptide synthesis

Eighteen overlapping (with five amino acid windows) peptides covering the whole OM-molecule were synthesized using solid phase F-moc SPPS and a semiautomatic outfit (Biolynx™ 4175, LKB, Uppsala, Sweden). The peptides were purified and their N-terminal amino acid sequences were ascertained (Applied Biosystem, Foster City, CA, USA) [6]. The SPPS peptides and OM 3 domains are shown in Fig. 1.

IgG polyclonal antibodies

Rabbit immunization was approved by the Norwegian Experimental Animal Committee, Oslo. Three rabbits, Ø-100Ø-101Ø-102 were immunized with OM Type II-O (Sigma T-9253) as follows: 0.5 mL OM (1 mg/mL sterile

Table 1. Patient and control sera. Nine sera were analysed using CAP/RAST for screening serum total and specific IgE antibodies for hen's egg white, ovalbumin (Gal d 2) and ovomucoid (Gal d 1)

Patients	Age/Sex	S-total IgE (Ku/L)	CAP/RAST S-specific IgE (Class 0–6)		
			Egg white	Ovalbumin	Ovomucoid
RB	29/M	1255	6	6	4
AL	22/M	5010	6	5	4
TK	30/M	2461	2	3	3
DS	42/M	187	4	3	3
HP	52/M	3340	5	5	5
BAA	40/M	8932	3	3	3
EST	50/F	373	0	0	3
AB, healthy	63/F	21	0	0	0
KH, healthy	28/F	26	0	0	0

NaCl, 0.9%) in suspension with 0.5 mL Freund's complete adjuvant was injected into each animal at week 1. In week 3 and 5, each animal was injected with 0.5 mL OM (1 µg/µL) in suspension with 0.5 mL Freund's incomplete adjuvant. The rabbit sera were tested for antibody production before each immunization and after the third immunization. The animals were bled out c. 14 days after the last immunization. Each serum was collected and was stored at -80 °C.

Carbodiimide coupling of KLH peptide

To facilitate binding of peptides to OM-specific IgE and IgG, respectively, the peptides were coupled to a carrier molecule, KLH, through conjugation with 1-ethyl-3[3-dimethylaminopropyl]carbodiimide HCl (EDC). Coupling was performed according to the manufacturer (Instruction sheet 0114: Inject Immunogen EDC Conjugation Kits, Cat. no. 77101, Pierce, Rockford, IL, USA). Briefly, 2 mg KLH and 2 mg peptide were dissolved in 200 µL deionized water and 0.5 mL conjugation-buffer (0.1 M MES, 0.9 M NaCl, 0.02% NaN₃, pH 4.7, respectively). The peptide solution was added to the carrier protein solution. One vial of EDC was dissolved in 1-mL dH₂O 50 µL of this solution and was immediately added to the carrier-peptide mixture. The reaction was allowed to continue for 2 h at RT before purification of the conjugate by gel filtration. Each column was washed with 3 × 5 mL of purification-buffer salt-solution (one bottle dissolved in 60 mL dH₂O). The peptide-carrier mixture (0.5–1 mL) was added. Aliquots of 0.5-mL purification buffer were added (repeated 20 times) and each fraction was collected separately. Absorbency of the fractions was measured (λ = 280 nm, Shimadzu Spectrophotometer UV-2501 PC) to locate the conjugated proteins. Fractions containing acceptable levels of the conjugate were pooled and stored at -20 °C until use.

Enzyme linked immunosorbent assay (ELISA)

Ninety-six-well, flexible round bottom, microtitre plates (Cat. no. 001-010-2401, Dynatech Laboratories, Oslo, Norway) were coated with 0.5 µg OM (Sigma) in 100 µL buffer/well. Before coating, OM was dissolved in 100 mM bicarbonate buffer (NaHCO₃: 8.401 g/L, Na₂CO₃: 5.30 g/500 mL, ratio 3 : 1, respectively, pH 9.6). Coating was allowed to proceed overnight with gentle shaking at 4 °C. The wells were then washed five times with 250 µL Tris-Tween buffer, pH 7.4 (Tris base: 12.11 g, NaCl: 5.844 g, MgCl₂ × 6H₂O: 0.508 g, Tween-20: 0.5 mL). Rabbit sera, diluted 1 : 125 000 in Tris-Tween buffer, pH 7.4, were added to the wells, 100 µL. Only 100-µL buffer were added to the control wells. The plates were incubated for 2 h at RT and the wells were subsequently washed as mentioned before. Anti-rabbit IgG alkaline phosphatase conjugate (100 µL, Sigma A8025, diluted

1 : 1000 in Tris-Tween) were added to each well. The plates were further incubated for 2 h at RT before repeated washings. Substrate (p-nitrophenyl phosphate, tablets 5 mg, N-9389, Sigma), 100 µL, at a concentration of 1 mg/mL in Tris buffer, pH 9.5, was added to each well before recording the absorbency (λ = 405 nm) using spectrophotometer (Labsystem Multiscan Bichromatic Type 348). Patients' sera were used undiluted, 100 µL/well; anti-human-IgE alkaline phosphatase conjugate (Sigma A-3525) diluted 1 : 1000 in Tris-Tween, pH 7.4, 100 µL/well, was used instead of the rabbit-IgG alkaline phosphatase conjugate. Inhibition was calculated according to:

$$100 - [\text{Absorbency of Ag/Ab complex} / \text{Absorbency buffer}]$$

Following the same ELISA procedure, 100 µL of intact, oxidized and denatured OM, at descending dilutions (5000–0.005 µg/mL), were used to coat the wells, 100 µL/well. Inhibition of specific S-IgE and polyclonal rabbit antibodies were detected for the evaluation of allergenicity/antigenicity of the three molecules.

Radio allergosorbent test (CAP/RAST) inhibition

Specific serum/pool, 35 µL, and allergen, 35 µL (5 mg/mL), or NaCl, 35 µL, were incubated for 2 h at 37 °C in Eppendorf tubes. The antigen/serum solutions are further investigated using CAP/RAST™ assay. Sample analysis was performed according to the manufacture's instructions (Pharmacia & Upjohn diagnostics AB, Uppsala, Sweden). S-specific IgE was expressed as CAP/RAST class (0–6).

$$\% \text{ Inhibition} = [\text{bf (kU/L)} - \text{sample (kU/L)} / \text{bf (kU/L)}] \times 100$$

where bf = buffer background; kU = 1000 × international unit of IgE.

Allergen-specific PBMC proliferation assay

PBMC was collected from the interphase after Ficoll-paque (Research Grade, code no. 17-0840-02 (Pharmacia Biotech AB, Uppsala, Sweden) centrifugation, washed and resuspended in complete RPMI 1640/AB⁺ medium [15]. PBMC (1–2 × 10⁶/well) were cultured in 24-well cell culture clusters (Corning and Costar, Corning Incorporation, New York, USA) with or without 100 µL allergen. The allergen concentrations were 0.11 or 10 mg/mL, at a final culture volume of 2 mL. Five days after initiating the cultures, 20 U/mL of rIL-2 (Boehringer Mannheim) were added to each well. On day 7 200 µL in triplicates from each 2 mL culture were seeded into 96-well clusters (Corning and Costar) and pulsed overnight

with 20 µL 0.05 mCi/mL [methyl-³H] thymidine (Amersham Pharmacia Biotech, Amersham, England). The cultures were harvested and assayed for proliferation responses (expressed in cpm). T cell blasts were isolated by Ficoll-paque centrifugation, and the interphase layer expanded in 24-well plates. Mitogen stimulation of the clusters was done by adding phytohaemagglutinin (PHA, 0.8 µg/mL), rIL-2 (20 U/mL) and supplement of APC (autologous irradiated, 25 Gy PBMC 1×10^5 /well).

Allergen-specific TLC Proliferation assay

Specific proliferation response was tested 14 days after the last addition of PHA and rIL-2. Irradiated (25 Gy) autologous PBMC (10^5 cells/well) as well as allergen (concentration = 0.5 mg/mL culture), were added to the T cell enriched wells ($1-2 \times 10^6$ /cell well) and cultured for 4 days. From the 24-well plates, 200 µL of each culture were seeded in 96-well plates and pulsed overnight by 20 µL, 0.05 mCi/mL [methyl-³H] thymidine. The radioactive uptake was measured and cpm given as means of triplicates. The 24-well cultures were harvested on day 5, pelleted cells were resuspended in cRPMI-1640 and phenotype markers were stated by flow cytometry. The culture filtrates were stored at -20°C for cytokine profile analysis.

Phenotype determination

Freshly prepared PBMC (day 1) and established TCL following specific allergen challenge (harvested on day 5) were analysed for the expression of dual $\text{CD4}^+/\text{CD8}^+$ and $\text{CD2}^+/\text{CD19}^+$ using Coulter Epics XL-MCL Flow Cytometer System II™ software, fully automated set-up supplied by Coulter Corporation, Luton, UK. Monoclonal antibody T4RD/T8-FITC (Coulter 6603802) and T11RD1/B4-FITC (Coulter 6603801), respectively, were used for the analysis.

Cytokine analysis

Estimations of IL-4, IFN- γ , IL-6, IL-10, IL-13 and TNF- α in culture filtrates of specific allergen-stimulated TCL were performed according to the manufacturer's instructions, using commercial enzyme-linked immunosorbent assay kits (PeliKine Compact™ human ELISA kits, CLB, Amsterdam, The Netherlands). The specificity and the sensitivity of the kits were given by the manufacturer. The lower detection limits for the different cytokines in pg/mL were as follows: 0.2 for IL-4, 0.2 for IL-6, 1 for IL-10, 0.5 for IL-1, 1 for TNF- α and 0.8 for IFN- γ . In order to calculate the results from the calibration curve, calibration data were plotted as the logarithm of the substrate concentration vs. absorbency, and an S-shaped pattern appeared. A curve was fitted to the calibration data points using the formula:

$$y = \left(\frac{\exp(Ax + B)}{1 + \exp(Ax + B)} \right) C + D$$

where x is the logarithm of the calibrator concentration in pg/mL, and y is the corresponding absorbency, read at $\lambda = 450$ nm, by a spectrophotometer (Labsystems Multiscan Bichromatic Type 348, Labsystems and Life Sciences International Ltd, UK). The best fit of the coefficients ABCD were determined by the KaleidaGraph™ program (version 2.1.3, 1991, Abelbeck Software). For all calibration curves, the correlation coefficient was > 0.999 . We then measured samples by calculating substrate concentrations from the absorbency using the above formula.

OM preparations and peptides

Eighteen synthetic peptides of OM, intact OM and the chemically modified OM were further purified and characterized using the Microseparation system (130 A, Applied Biosystem, Foster City, CA, USA). The retention times of all the investigated OM preparations/peptides were registered. OM peptide coupling to KLH was likewise confirmed (HPLC registrations not shown).

KLH-coupled peptides

The OM peptide-KLH carrier complexes were used, and rabbit and human sera in ELISA-inhibition protocol were used. Peptide-KLH coupling increased the inhibiting potential of several peptides by up to 50% as related to peptide alone (results not shown). However, some of the KLH-coupled peptides, when compared with their uncoupled counterparts, showed only slight or no increase in their inhibition values. Non-specific background inhibition by KLH alone was subtracted. Inhibition values above 15% were considered relevant, both for uncoupled and coupled peptides.

Results

Cell and sera donors

Cell and sera donors are described in Table 1. Serum total IgE, CAP/RAST class (0–6) for hen's egg white, ovalbumin (Gal d 2) and ovomucoid (Gal d 1) as well as patients' age and gender are shown.

Elisa

Figure 2 shows that the allergenicity of OM was substantially lowered following the denaturation procedure. The concentration necessary for 50% specific IgE inhibition (C50) for the denatured OM was 1700-fold higher (mean of six sera) compared with the intact and oxidized OM. For

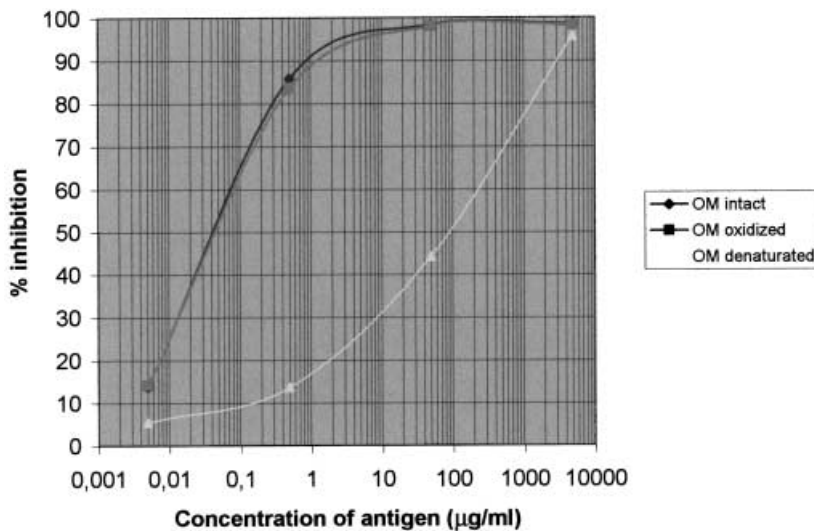


Fig. 2. Allergenicity of intact, oxidized and denatured OM. Mean specific IgE inhibition of seven patient sera, using the ELISA procedure, are shown. The allergenicity, compared with intact OM, was substantially lowered following the denaturation procedure. The concentration of denatured OM, necessary for 50% inhibition of specific IgE, was 1700-fold higher than that for intact OM. The oxidation of the native molecule did not affect the allergenicity.

the antigenicity, the corresponding C50 for IgG inhibition (mean of three different sera) was 1000-fold higher for the denatured OM.

Native and oxidized OM (0.5 mg/mL) have completely inhibited binding of rabbit-specific IgG in sera Ø100, Ø101 and Ø102 (inhibition range 98.3–100%). The binding of denatured OM (0.5 mg/mL) to rabbit antibodies was low: 37.3–45.3% inhibition was detected (results not shown). Only peptide 1–14 was recognized by all the three polyclonal sera (inhibition range 15.5–23.5%). Serum Ø100 recognized five OM peptides, namely 11–24

(23.3% inhibition), 32–44 (19.2%), 51–64 (13.1%), 61–74 (15.8%) and 101–114 (20.1%).

Likewise, intact, oxidized and deglycosylated OM (0.5 mg/mL) were recognized by specific IgE of all the seven patient sera tested (giving mean inhibition values of 99.9%, 98.5% and 95%, respectively). In addition, OM 1–14 and OM 11–24 were detected by IgE in six of the patient sera, while four of the sera recognized three OM-peptides, 31–44, 51–64 and 101–134. OM 61–74 and OM 121–134 were recognized by IgE of 31 patient sera, respectively (Fig. 3).

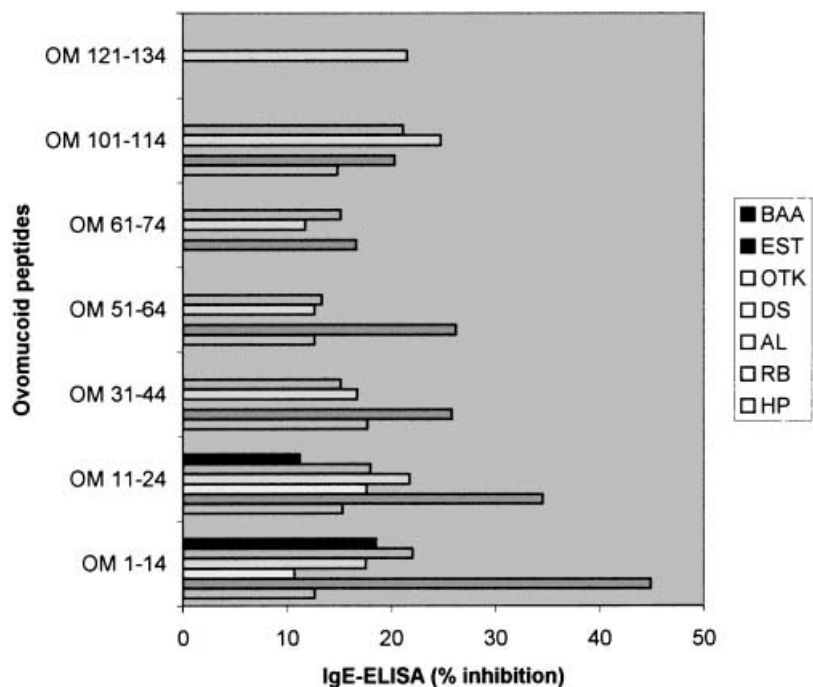


Fig. 3. OM peptides and patient sera IgE. Eighteen synthetic OM peptides were screened against seven individual patient sera to detect OM-specific IgE. Seven peptides that were recognized by patient IgE, using ELISA. 100% inhibition indicates complete recognition. Inhibition values above 15% were considered relevant. Pairs of OM peptides were often recognized simultaneously by the same patient sera: OM 1–14 and OM 11–24 were recognized by six sera, OM 51–64 and OM 61–74 were recognized by 43 sera, respectively. In addition, OM 101–114 was recognized by four patient sera, while OM 121–134 was recognized by the serum of one patient.

Table 2. Cultures of PBMC and allergen-specific TCL showing the CD4⁺ : CD8⁺ ratios. Mean CD4⁺ : CD8⁺ ratio for eight non-stimulated PBMC cultures and allergen-specific TCL of six patients

Culture type	Mean CD4 ⁺ : CD8 ⁺ ratios	Range
Non-stimulated PBMC	1.36	1.00–1.60 (<i>n</i> = 8)
Allergen-stimulated TCL	2.02	0.3–3.50 (<i>n</i> = 6)

T cell phenotype

The mean CD4⁺/CD8⁺ ratio of freshly prepared PBMC was compared with the ratio observed following OM-specific TCL stimulation. For the non-stimulated PBMC: a mean of *n* = 8, CD4⁺/CD8⁺ ratio 1.36 (range 1.0–1.60). CD4⁺/CD8⁺ ratio for allergen-specific TCL: mean value was 2.02 in six patients with a range 0.3–3.5, Table 2.

TCL stimulation

Native and modified OM preparations were added to OM-specific TCLs from seven allergic individuals and two healthy controls. Stimulating TCLs with native, oxidized and denatured OM gave mean responses of 70 018 cpm (range 624–17 2471), 18 637 cpm (range 310–59 299),

25 294 cpm (range 342–74 569), respectively. TCL from one patient (BAA) showed no stimulation response.

The same TCLs were also stimulated with 18 OM peptides. Patient T cells recognized 10 of the peptides (Fig. 4). Interestingly, two or three overlapping peptides were often recognized by one single TCL; OM 51–64 and OM 61–74 were recognized by three TCLs (HP, DS, AL). Other peptides in this region, namely OM 41–56 and OM 71–84, were also recognized by two TCLs (DS, EST) and one TCL (HP), respectively. In addition, OM 1–14 gave proliferation of three TCLs (RB, EST, BAA), while OM 11–24 was recognized by two TCLs (RB, AL). TCL proliferation responses were detected following OM 31–44 (RB), OM 121–134 (AL) and OM 131–144 (RB, BAA) stimulation. Finally, OM 171–186 was recognized by RB, DS, EST. OM preparations and peptides showed no proliferation responses using TCL derived from the two non-allergic individuals.

Comparing the BT cell epitopes, peptide amino acid sequence 104–114 was exclusively a B cell epitope, while peptides covering the amino acid sequences 41–56, 71–84, 131–144 and 171–186 were all exclusively T cell epitopes.

Cytokine assay

TCL cytokine secretion following stimulation with native and modified OM as well as the 18 OM peptides were investigated. Table 3 shows the cytokine secretion of OM-specific TCL (six patients) stimulated with native and

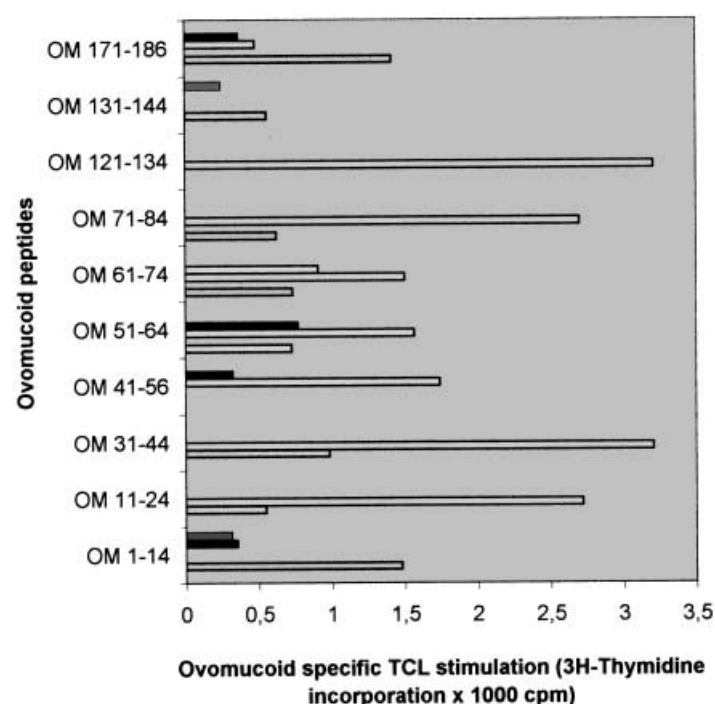


Fig. 4. OM peptides and patient T cell responses. OM-specific TCL derived from six allergic individuals and two healthy controls were added to 18 overlapping OM peptides. Ten peptides were recognized by TCLs. Cpm values for ³H thymidine incorporation, were adjusted for by respective OM-specific TCL added medium only (control cpm were generally < 100). Two healthy controls gave no proliferation responses towards the peptides.

Table 3. OM-specific TCL cytokine secretion following stimulation with rIL-2, OMn (OM native), OMo (OM oxidized), Omd (OM denaturated). Results are given as pg/mL and > indicate values above the highest standard of the kit

TCL	IL-4					IFN- γ					IL-6					IL-10				
	Med	rIL-2	OMn	OMo	Omd	Med	rIL-2	OMn	OMo	OMd	Med	rIL-2	OMn	OMo	Omd	Med	rIL-2	OMn	OMo	OMd
HP	16	170	44	16	154	7	300	153	63	33	8	30	> 450	> 450	13	0	60	32	9	9
RB	9	31	11	10	20	10	> 500	> 500	130	> 500	3	> 450	> 450	15	23	1	34	5	0	5
AL	14	55	4	3	11	2	140	23	8	6	50	> 450	100	> 450	> 450	3	200	23	3	0
DS	12	> 450	9	14	7	17	> 500	53	36	8	15	> 450	> 450	> 450	9	1	300	13	5	5
EST	9	15	16	11	15	19	80	31	6	6	45	90	> 450	45	> 450	1	14	19	5	2
BAA	9	120	0	0	0	15	300	0	1	0	6	> 450	> 450	> 450	7	1	300	1	0	0
AB, healthy	0	> 450	0	0	0	0	> 500	0	0	0	3	48	> 450	> 450	> 450	0	265	0	0	0
KH, healthy	0	> 450	0	0	0	0	> 500	0	0	0	5	174	> 450	> 450	> 450	0	42	0	0	0

TCL	IL-13					TFN- α				
	Med	rIL-2	OMn	OMo	Omd	Med	rIL-2	OMn	OMo	OMd
HP	2	> 125	> 125	> 125	100	0	0	0	0	0
RB	2	> 125	> 125	60	> 125	1	5	11	1	4
AL	2	> 125	26	11	11	0	2	5	1	1
DS	2	> 125	26	25	19	0	2	2	1	1
EST	2	> 125	67	35	28	1	2	4	4	1
BAA	2	> 125	0	0	0	1	5	0	0	0
AB, healthy	0	> 125	0	0	0	0	9	0	0	0
KH, healthy	0	> 125	0	0	0	0	12	0	0	0

Med = OM-specific TCL added medium only (control culture). The cytokine secretion of control cultures (Med) are subtracted from cytokine secretion of the stimulated cultures. The mixture of cytokines secreted indicate the presence of both Th1/Th0 and Th2-like subpopulations. Common for allergic and healthy derived TCL were high IL-6 secretion and a general secretion of all the measured cytokines, following rIL-2 stimulation.

Table 4. Cytokine secretion by TCL (six patients) following stimulation with 18 OM peptides. Cytokine secretions are shown in pg/mL. All the OM peptides tested on allergic-patient-derived TCL have the potential to induce IL-6 secretion. INF- γ and IL-13 were also secreted in response to OM peptide TCL stimulation. This indicates the presence of Th2 (IL-6, IL-13) but also Th1/Th0 (INF- γ)-like T cells in the cultures

Peptides	INF- γ (<i>n</i> = 5)	IL-6 (<i>n</i> = 6)	IL-13 (<i>n</i> = 5)
OM 1–14	9	450, 3, 5	
OM 11–24	2, 8, 6	3, 5	4
OM 21–34	5	3, 450, 45, 450	
OM 31–44	8, 3	2, 450, 54	2
OM 41–56	2, 2, 1		3
OM 51–64 §	6, 31	7, 450, 11, 31, 6	2
OM 61–74	6	2, 10, 6, 15	1
OM 71–84 *	6	6, 4, 450, 75, 450	
OM 81–94	10	450, 4, 5	
OM 91–104	8	8, 450, 7	
OM 101–114	5		
OM 111–127	5	450, 450	
OM 121–134	5, 1	20, 15	4
OM 131–144	6	4, 450, 20, 450, 20	3
OM 141–155	6	7, 1	
OM 151–164	6		
OM 161–174	86	9	
OM 171–186	106	450, 35, 450	3, 10

n = number of patients secreting specific cytokine. Open bars = no detectable cytokine secretion. § TCL from a non-allergic individual (KH) secreted IL-6 (72 pg/mL) in response to OM peptide 51–64. * TCL from two non-allergic individuals secreted IL-6 in response to OM peptide 71–84 (AB: > 450 pg/mL; KH: 88 pg/mL). IL-4 (*n* = 2) 39 pg/L for peptide (OM 1–14) and 4 pg/L for peptide (OM 171–186). IL-10 (*n* = 1) 3 pg/L for peptide (OM 11–24). TNF- α (*n* = 3) 1 pg/mL for each of peptides 21–34, OM 81–94 and OM 171–186.

chemically modified OM, rIL-2 and control with only cells and medium. Considerable amounts of all the investigated cytokines were measured. IL-6 secretion was high; in addition other Th2-like cytokines, IL-4, IL-13 and IL-10 were detected. Likewise, high INF- γ secretions emphasize the presence of Th1 cells as well. rIL-2 stimulated TCL donated by non-allergic individuals showed abundant cytokine secretion of INF- γ , IL-4, IL-6, IL-10, IL-13 and TNF- α . However, they secreted IL-6 in response to stimulation with native, denatured and oxidized OM preparation (Table 3).

Generally, when the peptides were used to stimulate patient-derived TCL (Table 4), cytokine secretion levels were much lower, except for IL-6. Almost all the OM peptides could provoke low to moderate INF- γ secretion

(five patients) and moderate to high IL-6 secretion (six patients). In addition, IL-13 was secreted in response to several OM peptides in five patients. IL-4 was detected in response to peptide OM 1–14 and 171–186 (for two patients). Hardly any IL-10 (one patient) and TNF- α (two patients) secretion could be measured in these TCL cultures. As shown in Table 4, no cytokines could be detected (with the exception of IL-6) upon treating the non-allergic derived TCL with the peptide panel. Patient KH secreted IL-6 in response to OM 51–64 (72 pg/mL) and both non-allergic derived TCLs secreted IL-6 in response to OM 71–84 (AB: > 450 pg/mL; KH 88 pg/mL).

Discussion

Knowledge of the primary structure of ovomucoid [12] allows the identification of OM-specific B cell epitopes. In previous studies OM-specific B cell epitopes were characterized by Cooke and Sampson [11]. Five IgE and seven IgG binding epitopes were reported. Zhang and Mine [18] reported IgE and IgG binding activities to all three OM domains, and the third domain in particular. Similarly, studies of Besler *et al.* [19] described two IgE binding epitopes (OM 90–121 and OM 134–186) produced by enzymatic cleavage of OM. T cell proliferation responses towards native or modified OM have been reported [1,11,15]. However, no distinct OM-specific T cell motifs have been described. Tables 5 and 6 show comparative overviews of the reported IgE binding epitopes and T cell epitopes of OM, respectively.

Using 18 overlapping OM peptides and patient TCL, we found 10 distinct T cell epitopes. Six of these peptides were also recognized by IgE, leaving four peptides to be exclusively T cell epitopes. Furthermore, seven peptides were recognized by IgE, including OM 101–114, solely a B cell epitope in the present study. OM 61–74, as a B cell epitope, has to our knowledge not been reported. The responses following OM peptide stimulation were relatively lower than stimulation with native/modified OM (< 3200 vs. 21018 and 172471 cpm), in accordance with previous findings [11,15,16]. Investigating PBMC responses to hen's egg white ovalbumin (*Gal d 2*), no correlation was found between ³H-thymidine incorporation and CAP-RAST value [20].

The same patient TCL often recognized adjacent overlapping peptide pairs (e.g. OM 51–64, OM 61–74 or OM 1–14, OM 11–24). This recognition pattern could mirror the presence of common epitope sequences having similar affinities for binding APC/MHC/TCR complex. Pfeifer *et al.* [21] reported that TCR-ligand density might determine the T cell response; high ligand density induces B cell help while low ligand density favours delayed type hypersensitivity. The nature of a peptide sequence is

Table 5. IgE binding epitopes of ovomucoid (Gal d 1). An overview

OM-Domains	Cooke & Sampson (1997)	Besler <i>et al.</i> (1999)	Holen <i>et al.</i> (2001)
Domain I: OM 1–64	OM 1–20 OM 49–50		OM 1–14 OM 11–24 OM 31–44 OM 51–64 OM 61–74
Domain II: OM 65–130	OM 85–96 OM 115–122	OM 90–121	OM 101–114 OM 121–134
Domain III: OM 131–186	OM 175–186	OM 134–186	

Cooke and Sampson [11] detected five IgE binding epitopes of OM, using dodecapeptides prepared by SPOTs kit and seven pooled egg-allergic patients' sera. Zhang and Mine [18] confirmed IgE binding activity of the three OM domains using OM-specific sera from children utilizing an ELISA procedure. Full allergenicity of OM needed the combination of all three OM domains. Besler *et al.* [19] investigated the peptide fragments following trypsin-, pepsin-, thermolysin- and CNBr-cleaved purified OM. All three OM domains showed IgE binding activity. Two distinct IgE binding epitopes were identified by amino acid sequence analysis following immunoblotting procedures, utilizing six OM-specific human sera and a pooled serum. Holen *et al.* detected seven IgE-binding epitopes following ELISA incubation of seven individual OM-specific patient sera and 18 overlapping synthetic peptides (Fmoc polyamide peptide synthesis), covering the whole OM molecule. Among these, patient sera IgE binding to OM 61–74 has not been reported before.

important for MHC binding, as successful MHC binding requires dominant motif amino acids. Peptides bound by MHC class II molecules are longer (13–15 amino acids) than those bound by MHC class I (mostly octa-, nona- or deca-peptides) [22]. We conclude that full allergenicity of

OM requires intact or nearly intact OM molecules. The allergenicity of OM was substantially lowered following the denaturation procedure, compared with intact or oxidized OM, as shown in Fig. 2. IgE and TCL from genetically non-related subjects recognized both different and common epitopes of OM. This was also reported by Eigenmann *et al.* testing OM compounds [16]. Furthermore, single individuals harbour multiple T cell specificity for OM. This was comparable with the findings using grass and birch pollen (Phl p1 and Bet v 1) [23–25].

The results show that allergen stimulation certainly modulates the CD4⁺ : CD8⁺ ratio. In a previous report, the CD4⁺ : CD8⁺ ratios were found to be significantly higher in AD patients sensitive to OA than in healthy control [20]. Other studies reported no differences found in the percentage of CD4⁺/CD8⁺ in atopic asthmatic children when compared with age-matched atopic non-asthmatic children [26]. It is now accepted that expansion of Th2-like cells plays a critical role in the pathogenesis of allergic diseases [27]. TCL stimulation using intact or chemically modified OM provoked considerable secretion of different cytokines. IL-2 and IL-6 in synergy with IL-13 and IL-4 promote IgE isotype switch. IL-4 and IL-10 are known to reduce production of IFN- γ , attenuating a Th2-response [27–32]. IL-10 reduces antigen-specific T cell proliferation and has been referred to as an 'anticytokine' [33]. IL-6 and TNF- α are multipotent non-specific cytokines produced in the context of inflammation and infection, and IL-6 induces Fc ϵ RI expression [34]. Since the cultures used in these

Table 6. T cell epitopes of ovomucoid (Gal d 1). An overview

OM domains	Eigenmann <i>et al.</i> (1999)	Holen <i>et al.</i> (2001)
Domain I: OM 1–64	Domain I: OM 1–64	OM 1–14 OM 11–24 OM 31–44 OM 41–56 OM 51–64 OM 61–74
Domain II: OM 65–130	Domain II: OM 65–130	OM 71–84 OM 121–134
Domain III: OM 131–186	Domain III: OM 131–186	OM 131–144 OM 171–186

T-cell proliferation in response to OM/OM fragment stimulation is depicted in references [1,11,15.] Eigenmann *et al.* [16] established OM-specific T cell lines and clones derived from four egg-allergic patient PBMC. Proliferation responses were detected towards all three OM domains in one or several TCL/TCC. Holen *et al.* report 10 novel T cell epitopes recognized by OM-specific T cell lines derived from six different hen's-egg-allergic patients. Six of these peptides were also recognized by patient sera.

studies are enriched T cells (> 98% T cells), we conclude that the observed cytokine secretion must be due to stimulation of T subset cells. Further evidence comes from intracellular cytokine staining in flow cytometer typing that will reveal the precise type of the cytokine secreting cell [35]. Thus, IL-4, IL-13 and IL-6 must be secreted by Th2 and/or Tc2 lymphocytes, while IFN- γ was produced by Th1/Th0 and/or Tc1 cells [36,37]. IL-10 and TNF- α could be produced by all T subset cells following activation.

Very high TCL proliferation induced by OM resulted in a general abundant cytokine secretion (Table 3). The results reflect the potent sensitizing ability of native OM and OM preparations. Using allergen-specific TCL, the various OM peptides provoked IFN- γ , IL-13 and particularly IL-6 secretion. In addition, some of the 'non-B non-T cell' epitopes of OM could trigger cytokine secretion, mainly IL-6 and IFN- γ . In addition, intact and modified OM and two OM peptides could provoke IL-6 secretion using TCL from the two healthy individuals. Common for allergic and non-allergic individuals were the abundant OM-triggered IL-6 secretions (regardless of any TCL proliferation responses) as well as a general cytokine secretion following rIL-2 stimulation. IL-6 secretion is allergen-specific; a common feature of several allergens (shown for α -lactalbumin, β -lactoglobulin, α S 1-casein, and gliadin, unpublished data). Distinct allergens/antigens or epitopes may elicit functionally distinct T cell responses. IFN- γ was secreted in response to stimulation with almost all the chemically modified OM and peptides, using allergic-patient-derived TCL.

The presence of IFN- γ in almost all cell culture supernatants following hen's egg allergen stimulation is in accordance with previous reports [15,38]. IFN- γ was also secreted following *Phelium pratense*, *Vespula* venom and *Parietaria officinalis* peptide stimulation [23,39,40]. Accumulating evidence suggests that PBMC from children in their first years of life have a 'naturally' diminished IFN- γ production and this is even more so in atopic neonates [41,42]. Stimulated PBMC/TCL from adult egg-allergic patients have the capacity to produce IFN- γ and the production of this Th1 cytokine may possibly be a marker of tolerance towards egg. It is known that T cells from allergic and non-allergic individuals could recognize the same antigenic epitopes leading to activation and cytokine production. However, it seems that the outcome of this TCL activation differs between allergic and non-allergic individuals.

Studies on T cell recognition of modified natural allergens and synthetic peptides could improve immunotherapy treatment of allergy by avoiding the inherent risk of conventional allergens.

Conclusion

The most allergen-reactive epitopes along the OM molecule could be identified and are shown in Fig. 1. Those areas are both T and B cell epitopes or T cell epitopes only, with the ability to provoke TCL cytokine secretion. Considerable amounts of all the cytokines analysed (IL-4, IFN- γ , IL-6, IL-10, IL-13 and TNF- α) were measured. The allergenicity and antigenicity were substantially declined by denaturing the OM molecule. Native, oxidized and deglycosylated OM were fully recognized by specific IgE. Full allergenicity of OM need the intact molecule, suggesting that rupturing adjacent epitope pairs gives variable TCL stimulation, as found using OM preparations and peptides. Allergen-stimulated TCL cultures gave higher mean ratios of CD4⁺ : CD8⁺ than those that were non-stimulated. TCL derived from non-allergic donors showed increased proliferative responses by OM or its modified products. Recombinant IL-2 stimulated TCL show abundant cytokine secretion. T cell epitopes, both synthetic and natural, can systematically be modified to optimize any desired property. Libraries of such peptides are expected to be instrumental for the development of synthetic vaccines, TCR antagonists or induction of MHC blockers.

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