

# Comparison between Ovalbumin and Ovalbumin Peptide 323–339 Responses in Allergic Mice: Humoral and Cellular Aspects

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## Abstract

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Ovalbumin (OVA) is widely used in allergy research. OVA peptide 323–339 has been reported to be responsible for 25–35% of isolated BALB/c mouse T-cell response to intact OVA. An investigation of whether OVA and OVA 323–339 molecules can induce equivalent *in vivo* and *in vitro* immune responses was conducted. Eight-week-old BALB/c mice were randomly divided into three groups: OVA, OVA 323–339 and saline. On days 0, 7, 14, mice were intraperitoneally injected with 25 µg OVA or OVA 323–339 absorbed on 300 µg Alum, or saline; on days 21–23, all groups were challenged intranasally with either 20 µl of 1% OVA, 1% OVA 323–339 or saline. On day 28, after killing, splenocytes were isolated and cultured under the stimulus of each allergen or medium. Evaluated by hematoxylin/eosin and major basic protein immunohistochemical stainings, OVA and OVA 323–339 induced similar lung inflammation. Interestingly, significant serum total IgE and OVA-specific IgE were observed in OVA mice when compared to saline control. OVA 323–339 mice showed higher serum OVA-specific IgE, OVA 323–339-specific IgE, IL-4 and lower IFN-γ similar to OVA mice. The proliferative response to OVA was found in cultured splenocytes of both OVA and OVA 323–339 mice, while the similar proliferative response to OVA 323–339 was only observed in the splenocytes of OVA 323–339-sensitized and challenged mice. Although OVA 323–339 induced a Th2-like response in the mouse model as did OVA, OVA 323–339 has clearly limited immunogenic potency to activate OVA-sensitized and challenged mice splenocytes, unlike OVA.

## Introduction

Hen eggs are an essential ingredient in a variety of processed food products and are therefore difficult to eliminate from the diet. The proteins of hen's egg white, like ovalbumin (OVA), frequently induce hypersensitivity symptoms among egg allergic individuals [1, 2].

OVA, which constitutes approximately 58% (w/w) of the entire hen's egg white extract, is the most dominant ingredient of the five major allergens of egg white and is universally used as the main allergen in establishing different animal models of asthma, food and dermal allergy. OVA's allergenic epitopes were mainly determined by the primary structure and are dependent on a certain peptide chain length [1, 3, 4]. Among many egg white proteins, OVA 323–339 and OVA 1–10, as well as intact OVA, were reported to encompass B-cell epitopes which

were recognized by specific IgE antibodies. The OVA 323–339 sequence was also demonstrated to include CD4<sup>+</sup> T cell epitopes, which were restricted by the MHC class I-A<sup>d</sup> molecule in mice [2, 5, 6], and was considered to encompass at least one B cell epitope [7, 8]. Furthermore, the OVA-specific T-cell line from hen egg allergic patients was able to recognize OVA 323–339 presented by HLA-DR10 [2, 9].

OVA 323–339 has been used extensively to study the nature of class II MHC-peptide binding and T-cell activation [10–14]. It has been reported that OVA peptide 323–339 was responsible for 25–35% of the BALB/c T-cell response to the intact OVA, and it is highly probable that OVA 323–339 was closely related to the naturally created peptide by the antigen presenting cell (APC) during OVA processing [4, 5, 8, 15]. However, Janssen *et al.* [16] demonstrated that the immunodo-

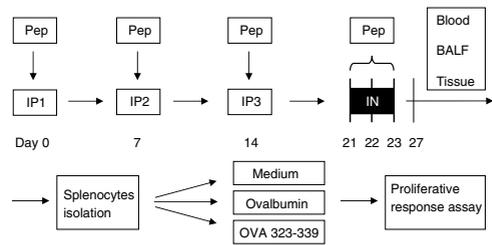
nant epitope of OVA, e.g. OVA 323-339, accounts for 50% of the OVA-specific B-cell response and 60–70% of the OVA-specific T-cell response. So far, there is only a small amount of available information on *in vivo* immune responses in OVA 323-339-treated animals. Kearney *et al.* [17] reported that systemic injection of OVA or OVA 323-339 in a T-cell transfer model induced a rapid and strong, but transient DO11.10 T-cell proliferation. They also described that the induction of more local and less transient response by the subcutaneous administration of OVA 323-339 in Freund's Complete Adjuvant resulted in a significantly enhanced T-cell response upon *in vitro* stimulation with OVA 323-339 [17, 18]. Knowledge on the immunogenicity of allergens is important and mandatory for establishing modern therapeutic methods for the treatment of allergy.

In this study, our aim was to investigate whether synthetic OVA peptide 323-339 has parallel *in vivo* immunogenicity with OVA in a BALB/c mouse allergy model. In addition, using these OVA or OVA 323-339-sensitized and challenged mice, *in vitro* splenocyte stimulation was examined.

## Materials and methods

**Allergens.** Ovalbumin (OVA, electrophoretic purity 99%, lot 81F-8230) was purchased from Sigma (St. Louis, MO, USA), and the OVA peptide with the amino acid residues 323-339 was synthesized using semi-automatic peptide synthesizer (LKB Biochrom, Ltd, Cambridge, England) as described by Johnsen and Elsayed [4]. Briefly, the solid-phase peptide synthesis of OVA 323-339 was prepared manually, and the preparations were cleaved from the resin by the use of HBr and trifluoroacetic acid (TFA). The purification of the peptides by gel filtration chromatography was performed using Biogel P2 column (dimensions 61 × 1 cm) with 200–400 mesh resin (Bio-Rad, Richmond, CA, USA) and the void volume peak contained the peptide in question. High-performance liquid chromatography was performed using an ion-exchange column (Bio Siol TSK DEAE 2-SW, dimension 250 × 4.6 mm; Bio-Rad) with a routine solvent system (20 mmol/l Tris/HCl, and 20 mmol/l Tris/HCl in 500 mmol/l NaCl) at pH 6.2, which was run at a linear gradient ratio for 14 min. Aliquots of 100 µg peptide were originally run, and the absorbance was monitored at 220 nm. The initial concentration of the peptides (2 µM) was used in sixfold serial dilutions for examining their activity.

**Animals.** Female BALB/c mice (8-week old) were obtained from Taconic (4140 Borup, Denmark) and housed at the Vivarium animal house, Haukeland University Hospital (Bergen, Norway). They were kept in a conventional animal room with 12-h dark/light cycles and received water and pelleted pathogen-free food



**Figure 1** Protocols of the *in vitro* and *in vivo* studies. Mice were sensitized with intraperitoneal injection (IP) and then challenged with the respective allergens or saline intranasally (IN). Three different study groups were used: Pep (mice sensitized and challenged with OVA 323-339), OVA and saline. The Pep group is presented as an example to explain the protocols.

(Special Diet Services, Witham, Essex, England) *ad libitum*. The experimental procedure was approved by the local Ethical Committee for Animal Experiments in Bergen, Norway.

***In vivo sensitization and challenge of mice.*** Fifty-one 8-week-old female BALB/c mice were randomly divided into three groups: OVA, OVA 323-339 and saline. They were intraperitoneally injected with 25 µg OVA or OVA 323-339 absorbed on 300 µg Alum (Imject®, lot E164 107, Pierce Biotechnology Inc., Rockford, IL, USA) or saline on days 0, 7, 14. On days 21–23, all groups were challenged intranasally with 20 µl of 1% OVA, 1% OVA 323-339 and saline, respectively. The protocol is illustrated in Fig. 1.

***Serum and bronchoalveolar lavage fluid (BALF) collection.*** On day 28, the mice were given intraperitoneal injections with 50 mg/kg of Pentothal (Abbott Scandinavian AB, Solna, Sweden) and killed by right ventricular exsanguinations. The blood was collected and left to coagulate at room temperature for at least 30 min, and then centrifuged at 1000 g for 10 min. The serum samples were stored at –20 °C until use. After right ventricular exsanguinations, the trachea was cannulated with a 20G catheter (Vasofix®, B.BRAUN Melsungen AG, Melsungen, Germany), and the lungs were lavaged three times with aliquots of 0.5 ml of sterile saline per mouse. The collected lavage fluid was centrifuged at 400 g, and the supernatant was stored at –20 °C for cytokine and antibody analysis.

***Histology.*** The lung was inflated with similar volume of phosphate-buffered 10% formaldehyde (600 µl) at a constant pressure and then immediately soaked in the same solution for approximately 24 h. Longitudinally oriented trachea, a horizontal slice from the middle zone of the single left lobe of the lung was then embedded in paraffin, 5-µm sections were cut and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). Bronchial airway wall thickness was measured with the method described previously [19].

**Immunohistochemistry.** For the detection of extracellular major basic protein (MBP) in tissues, rabbit polyclonal antiserum to mouse MBP was used, which was kindly provided by Dr. James Lee (Mayo Clinic, Scottsdale, AZ, USA). Anti-MBP was diluted 1:300 in antibody dilution buffer (DakoCytomation, CA, USA) and incubated for 1 h with 5- $\mu$ m paraffin sections at room temperature. Cross-reactivity was blocked with HP-block (DakoCytomation), followed by a reaction with horseradish peroxidase (HRP)-linked swine anti-rabbit diluted 1:500, and then the antibody-captured cells were detected with 3, 3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin.

**Quantification of total IgE.** Total IgE was measured by mouse ELISA quantification kits (Bethyl Laboratories, Montgomery, TX, USA) as previously described [19]. In brief, the plates were coated with affinity-purified goat anti-mouse IgE overnight at 4 °C and then blocked with bovine serum albumin in 10-mM phosphate-buffered saline (PBS) at pH 7.4. The threefold diluted serum samples or undiluted BALF samples and appropriate dilutions of a standard IgE preparation were used. The bound IgE was detected with polyclonal goat anti-IgE antibody (incubation for 1 h at 37 °C), followed by HRP-conjugated goat anti-mouse antibody.

The plates were developed by the addition of tetramethylbenzidine (TMB) and read with an ELISA plate reader (Labsystem Multiskan Bichromatic, Helsinki, Finland) at a wavelength of 450 nm. The kit detection range was 3.9–250 ng/ml.

**Detection of OVA-/OVA 323-339-specific IgE, IgG<sub>1</sub> and IgG<sub>2A</sub> in serum.** Specific immunoglobulin were assayed by in-housed ELISA as previously described [20]. In brief, microtitre plates were coated with OVA (fraction V, Sigma, 2  $\mu$ g/ml) or OVA 323-339 in 0.05 M NaHCO<sub>3</sub> buffer pH 9.6, and the serum samples were diluted 1:5 for OVA-specific IgE, 1:10 for IgG<sub>2A</sub> and 1:200 for IgG<sub>1</sub> in 10-mM phosphate buffer solution. Commercially available biotin-labelled anti-mouse isotype-specific secondary antibodies were then diluted to 2  $\mu$ g/ml (BD Pharmingen, San Jose, CA, USA).

As for the detection of specific IgE to OVA 323-339, microtitre plates were coated with rat anti-mouse IgE (clone R35-72, 2  $\mu$ g/ml), and the serum samples were diluted 1:10 in 10 mM phosphate-buffered saline. Biotinylated OVA 323-339-BSA conjugate was then applied. The biotinylations were performed according to the manufacturer's instructions (EZ-Link NHHS-PEO4-Biotinylation Kit; Pierce, Biotechnology, Rockford, IL, USA) [3, 20].

Streptavidin-horseradish peroxidase was diluted into 1:4000 (BD Pharmingen), and peroxidase substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) were used for immunodetection.

The results were measured at 405 nm wavelength (Spectra max plus, Sunnyvale, CA, USA) and expressed as optical density (OD).

**Determination of cytokine levels.** IL-4 and IFN- $\gamma$  in serum and BALF samples were measured with commercial ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocols [19].

**Cell culture.** The spleens were minced into small pieces, splenocytes were dissected free and then collected from interface after Ficoll-paque (GE Healthcare, Uppsala, Sweden) centrifugation. The viability of the collected cells was  $\geq 95\%$  by the use of trypan blue exclusion method. Separated splenocytes were cultured on 24-well cell culture clusters with 100  $\mu$ l or 200  $\mu$ l of saline or allergens (10  $\mu$ g/ml or 20  $\mu$ g/ml) in RPMI-1640 (Gibco BRL, Paisley, UK) at 37 °C 5% CO<sub>2</sub>, final volume of 2 ml. Three days after initiating the culture, 100  $\mu$ l of recombinant IL-2 (rIL-2) (1  $\mu$ g/ml) was added to each well. On day 4, 200  $\mu$ l of cells ( $2.5 \times 10^6$  cells/well) in duplicate were cultured in 96-wells overnight by adding 20  $\mu$ l of 0.05 mCi/ml [methyl-<sup>3</sup>H] thymidine.

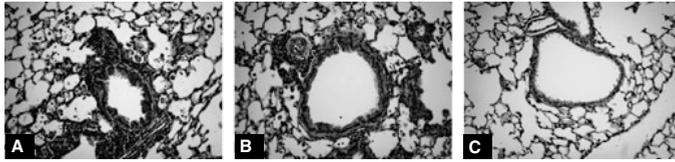
**Measurement of splenocyte proliferation.** Following stimulation, the cultures were investigated for primary proliferation responses as measured by <sup>3</sup>H-thymidine uptake. The cultured well contents were then harvested onto filter mats using a cell harvester. The discs representing each well were transferred from the filter mats into 5-ml volumes of scintillation fluid, and the incorporation of [<sup>3</sup>H] thymidine into the cultured cells was measured using standard scintillation counting procedures by 1414 liquid scintillation counter (Wallac, Turku, Finland). The results were expressed as counts per minute (cpm) and stimulation index (SI).

**Statistics.** Data were presented as mean  $\pm$  SD. Statistical analyses were performed with ANOVA using saline as a reference category, and followed by *post hoc* tests with adjustments for multiple testing by the approach of Dunnett's T3 where equal variances within groups are not assumed. Overall values  $P < 0.05$  were considered significant. All tests were two-tailed, and the data were analysed with SPSS statistical software (SPSS Inc., Chicago, IL, USA).

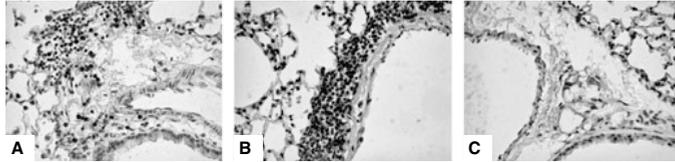
## Results

### Local inflammatory cell infiltration and airway wall remodelling

The saline control mice demonstrated normal lung morphology. In contrast, an eosinophil-dominant inflammatory cell infiltration including macrophages, a few neutrophils and lymphocytes were mainly observed around bronchi and vessels in the OVA- and OVA 323-339-treated mice. The hypertrophy of cells lining the airways was also shown in lung tissues with hematoxylin-eosin



**Figure 2** Photomicrographs of the lung tissues in hematoxylin-eosin staining. A: mice sensitized and challenged with peptide OVA 323-339; B: mice sensitized and challenged with OVA; C: saline-treated control mice. OVA and OVA 323-339 mice showed eosinophil-dominant inflammatory cells infiltration and an increased airway thickness. Original magnification: 200 $\times$ .



**Figure 3** Sections of airways stained immunohistochemically with major basic protein (MBP)-antibody. The MBP-positive cells are stained in brown in the airways of the allergen or saline-treated mice. A: OVA 323-339-treated mice; B: OVA-treated mice; C: saline-treated control mice. After killing, lung tissues were inflated with buffered formalin and lung sections were stained with anti-MBP. Note the increased density of eosinophils in the parenchyma in which MBP staining shows apical surfaces of respiratory epithelial cells in the bronchiole of OVA mice and OVA 323-339 mice. In contrast, significantly fewer eosinophils were present in saline-treated control animals in which no MBP-positive cells were detected in the airways. The figure is representative of five animals per group. Original magnification: 200 $\times$ .

staining. The eosinophil-dominant inflammation was clearly visualized by MBP immunohistochemical staining. Furthermore, thickenings of the bronchial airway walls and increased mucus production were found in the OVA 323-339 mice, as well as the OVA mice (Figs 2 and 3).

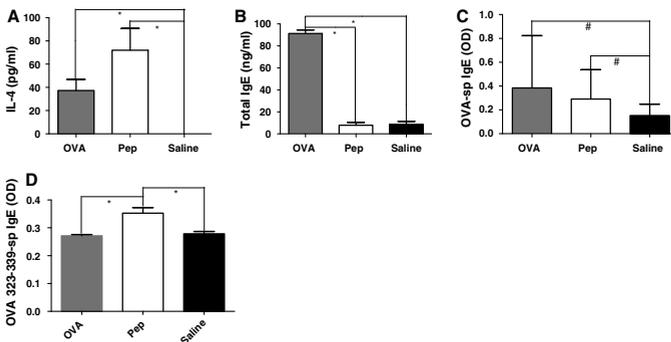
#### IL-4 and IFN- $\gamma$ profiles

An altered balance in the levels of inflammatory cytokines is an indicator of systemic, but not local inflammatory status; therefore, we measured both serum and BALF IL-4 and IFN- $\gamma$  levels. In serum we found increased IL-4 levels in both the OVA and OVA 323-339-treated mice ( $37.3 \pm 47.9$  pg/ml and  $71.9 \pm 93.9$  pg/ml, respectively) versus non-detectable levels in

the control mice (Fig. 4A). Serum IFN- $\gamma$  levels were under the detection limit in all samples of the three mice groups. In BALF samples, IFN- $\gamma$ , IL-4 and total IgE all levels were equally under the detection limits.

#### Total IgE, OVA and OVA 323-339-specific IgE/IgG<sub>1</sub>/IgG<sub>2A</sub> levels in serum and BALF

Allergen-specific IgE antibodies will increase after sensitization, whereas different subclasses of IgG antibodies can be studied for an evaluation of immune tolerance. We detected higher serum total IgE levels ( $91.3 \pm 9.2$  ng/ml) in the OVA-treated mice than that in the control ( $8.7 \pm 7.3$  ng/ml). Also in support of this, we observed a significant increase in OVA-specific IgE in OVA-treated mice ( $0.39 \pm 0.44$  OD) when compared to



**Figure 4** Cytokine and IgE levels in serum. A: IL-4, B: total IgE, C: OVA-specific IgE and D: OVA 323-339-specific IgE. OVA: mice were sensitized and challenged with ovalbumin; Pep: mice were sensitized and challenged with OVA 323-339; saline: mice were saline sham-sensitized and challenged with physiological saline. Statistical significance is presented with \* $P < 0.01$ , # $P < 0.05$ .

**Table 1** Serum-specific IgG antibody levels in the studied research groups.

	OVA	OVA 323-339	Saline control	<i>n</i>
OVA sp. IgG <sub>1</sub>	0.90 ± 1.21	1.25 ± 1.41	0.82 ± 1.20	17
OVA sp. IgG <sub>2A</sub>	0.69 ± 0.67	0.50 ± 0.44	0.45 ± 0.24	17
OVA 323-339 sp. IgG <sub>1</sub>	1.04 ± 0.60	0.76 ± 0.62	0.80 ± 0.47	17
OVA 323-339 sp. IgG <sub>2A</sub>	0.35 ± 0.45	0.32 ± 0.34	0.28 ± 0.11	17

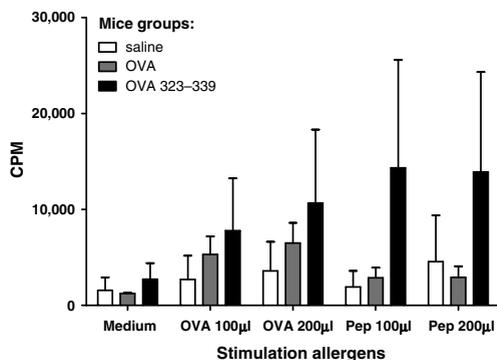
The results are expressed in optical density values (OD) of the means ± standard deviations (SD). ANOVA was performed for the data analyses, and the comparisons were studied between allergens groups and saline-treated control group,  $P < 0.05$  was considered as significant difference. No difference was found in antibody levels between the groups. Sp., specific; Saline, saline-treated control group; *n*, the number of individuals.

the control mice ( $0.15 \pm 0.96$  OD), (Fig. 4B, C). Unfortunately, only an insignificant total IgE production ( $7.9 \pm 7.1$  ng/ml) in OVA 323-339 mice was developed when compared to the control ( $8.7 \pm 7.3$  ng/ml). Importantly, we also found a significantly increased OVA-specific-IgE level in the OVA 323-339 mice ( $0.29 \pm 0.25$  OD) when compared to the control group ( $0.15 \pm 0.96$  OD), (Fig. 4C). Similarly, increased OVA 323-339-specific IgE ( $0.35 \pm 0.95$  OD) was measured in the OVA 323-339-treated mice versus the controls ( $0.28 \pm 0.03$  OD), but significantly increased OVA 323-339-specific IgE was not observed in the OVA mice group ( $0.27 \pm 0.02$  OD,  $P > 0.05$ ) (Fig. 4D). No significant increase in the serum levels of OVA-specific IgG<sub>1</sub> and IgG<sub>2A</sub>, OVA 323-339-specific IgG<sub>1</sub> and IgG<sub>2A</sub> were found when compared with the control group levels (Table 1).

In BALF, total IgE levels were under the detection limit.

#### The OVA and OVA 323-339-specific proliferation assay: OVA 323-339 epitope cannot activate OVA-sensitized and challenged mice splenocytes

To evaluate whether these allergen structures could activate T cells in cell culture, we isolated the splenocytes of each group and stimulated them with OVA or OVA 323-339. Splenocytes of both the OVA- and OVA 323-339-treated mice displayed a strong proliferative response after stimulation by OVA ( $5312 \pm 1886$  cpm and  $7792 \pm 5451$  cpm,  $P < 0.05$ ) when compared to the saline-treated control mice splenocyte response to OVA ( $2704 \pm 2472$  cpm). Medium as a control stimulant did not cause any significant difference in response between allergen-treated groups and saline-treated groups (OVA  $1231 \pm 86$  cpm and OVA 323-339 group  $2737 \pm 1664$  cpm vs. saline-treated control mice  $1582 \pm 1334$  cpm,  $P > 0.05$ , respectively). Stronger proliferative responses were induced by OVA 323-339 stimulation in splenocytes of OVA



**Figure 5** Proliferative response of splenocytes to different allergens at different doses. After the isolation of splenocytes from all the study groups, mononuclear splenocytes ( $2 \times 10^6$ /ml) in the presence of rIL-2 were stimulated with 100 µl or 200 µl (10 µg/ml) of OVA 323-339, 100 µl or 200 µl (10 µg/ml)/well of OVA and 100 µl medium per well, respectively, in the presence of rIL-2. On day 4, splenocytes were incubated with 20 µl of <sup>3</sup>H-labelled thymidine overnight, and incorporation of <sup>3</sup>H was measured in cpm as the indicator of the T-cell-stimulating growth. Spleen mononuclear cells from OVA or OVA 323-339 mice showed higher cpm values after stimulation with OVA or OVA 323-339 when compared to the medium stimulation. On the x-axis, the medium or allergens that were used in the *in vitro* stimulations are presented in each of the *in vivo* study groups, respectively.

323-339-treated mice ( $14343 \pm 11232$  cpm versus saline-treated control mice splenocyte response to OVA 323-339  $1926 \pm 1678$  cpm,  $P < 0.01$ ).

We also studied whether the readily processed epitope of OVA 323-339 could activate OVA or saline-treated mice splenocytes in culture. There was no significantly increased response to OVA 323-339 stimulus in the OVA-treated mice ( $2879 \pm 1049$  cpm,  $P > 0.05$ ) versus saline-treated mice control ( $1926 \pm 1678$  cpm). In addition, there were no obvious differences observed in the cpm values between 100 µl or 200 µl of allergens which were used to stimulate the isolated splenocytes (Fig. 5).

## Discussion

The isolated splenocytes from OVA 323-339 or OVA-sensitized and challenged mice groups, when stimulated in cell culture with their respective allergens did demonstrate T-cell proliferation. However, unlike OVA, OVA 323-339 could not stimulate T-cell division of OVA-sensitized and challenged mice. Our *in vivo* results showed that after repeated intranasal challenge, both OVA 323-339 and OVA-treated mice groups developed a significantly increased inflammatory response in the target organ, which in this model is the airway. The specific IgE immunoglobulin levels were likewise increased by a significant difference when compared to the control.

A similar expression of morphology such as an increased eosinophil-dominant inflammation around the bronchi and vessels, and thickenings of bronchial walls and mucus over-secretion, was found in both OVA and OVA 323-339-sensitized and challenged mice in lung tissue histology and MBP immunohistochemistry. Augmented serum IL-4 production levels in mice treated with OVA and OVA 323-339 were also observed. Although no IFN- $\gamma$  production could be detected in serum or BALF samples. Our results suggest that both OVA and OVA 323-339 were *in vivo* capable of producing cytokine-induced systemic and local inflammation. OVA-treated mice illustrated an increased total and OVA-specific IgE production, while OVA 323-339 mice only showed a significant increase in the production of OVA 323-339-specific IgE. A possible explanation for the decline of total IgE production using the peptide is that the entire OVA with multi immunogenic structures could display stronger immunogenicity than its peptides. However, higher OVA-specific IgE was also observed in OVA 323-339-treated mice and, in contrast, OVA 323-339-specific IgE level in OVA mice was much lower than that in OVA 323-339 mice, and no difference was found when compared to that of the control. An *in vivo* molar ratio between OVA 323-339 and OVA is more than 20:1. This may suggest that the production of peptide-specific cross-antibody to OVA could be a dose-dependent process which requires a certain threshold amount of the immunogen (in other words, a high dose of OVA may be needed) to get the target peptide sequence of intact OVA presented and expressed on the surface of APC cells to induce a detectable IgE production. Other structures from intact OVA may be considered to be dominant immunogenic structures, e.g. OVA 1-10. Of course, the impact of species variation or strain difference in the production of epitope-specific antibodies, of course, could not be ignored [19].

Immune responses to protein antigens are strongly influenced by the nature of the helper T lymphocytes (Th) subsets involved. Th1 cells can evoke cell-dependent immunity by IFN- $\gamma$  production and inhibit the production of IL-4 by Th2 cells and Th2 cell proliferation *in vitro* [21]. Th1 cells also promote immunoglobulin class switching from specific IgG<sub>1</sub> to specific IgG<sub>2A</sub> [22], which is dependent on the presence of activating Fc $\gamma$  receptors [23, 24]. Therefore, it is not surprising that the *in vivo* serum levels of specific IgG<sub>2A</sub> did not show an increase when compared to the control mice as quite low IFN- $\gamma$  levels were developed in our model.

Th2 cells have been recognized to induce bronchial inflammation as a result of enhanced secretion of Th2 cytokines [25-29]. IL-4 is the exclusive IgE isotype switching factor, responsible for the over-production of IgE, and can promote immunoglobulin class switching to IgE and IgG<sub>1</sub> [22]. In our experiment, an increased IgE

without paralleled IgG<sub>1</sub> production was possibly because of the short-term experiment protocol.

IgE has the capacity to bind high-affinity receptors (Fc $\epsilon$ RI) on mast cells and basophils, in addition to low-affinity receptors (Fc $\epsilon$ RII) on lymphocytes and other inflammatory cells such as eosinophils, monocytes and platelets [3, 30]. The number of IgE binding epitopes varies in different allergens. Although the minimal requirements of an allergenic peptide were demonstrated to be four amino acids, a molecular size of 12-15 amino acids may be necessary for antibody binding which may be related to a requirement for helical conformation [7, 31]. OVA 323-339 is composed of 17 amino acids, which as expected, induced higher OVA 323-339-specific IgE [3].

Splenocyte stimulation with OVA induced an increased proliferative response as observed in both OVA and OVA 323-339-sensitized and challenged mice, while proliferation to OVA 323-339 only occurred in splenocytes of the OVA 323-339 mice and not in the OVA mice. Accordingly, cells stimulated with control culture medium demonstrated very low thymidine incorporation when compared with the responses to OVA and OVA 323-339 stimulation. The presented proliferative results are consistent with the results of Janssen *et al.* [32] who demonstrated that OVA 323-339 induced a proliferative response in cell cultures from OVA-challenged animals. From Figure 4, we can find no significant difference in proliferative responses between using 100  $\mu$ l and 200  $\mu$ l of allergens when cells were stimulated with identical allergens. This could imply that the *in vitro* proliferative response to OVA or OVA 323-339 is not dose dependent or our doses have already reached the upper limit for full splenocyte response.

The proliferative response assay demonstrated that OVA 323-339 *in vitro* stimulation did lead to splenocyte proliferation and activation in cultivated cells. This could suggest that small peptides such as OVA 323-339 can play their role by being presented and expressed on the membrane surface of activated splenocytes in the same way as OVA, which is consistent with previous ideas: OVA and OVA 323-339 share at least one common epitope structure [7, 8]. Importantly, the cellular proliferative responses to allergens were accompanied by increased humoral allergen-specific IgE antibody production, which strongly suggested that the splenocyte proliferative responses most probably were IgE-related.

To conclude, we suggest that OVA 323-339 can induce Th2-dominant immune responses, and that the splenocyte proliferative response to OVA323-339 is possibly IgE-related much like that of OVA.

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## References

- Langeland T, Harbitz O. A clinical and immunological study of allergy to hen's egg white. V. Purification and identification of a major allergen (antigen 22) in hen's egg white. *Allergy* 1983;38:131-9.
- Holen E, Elsayed S. Specific T cell lines for ovalbumin, ovomucoid, lysozyme and two OA synthetic epitopes, generated from egg allergic patients' PBMC. *Clin Exp Allergy* 1996;26:1080-8.
- Elsayed S, Apold J, Holen E, Vik H, Florvaag E, Dybendal T. The structural requirements of epitopes with IgE binding capacity demonstrated by three major allergens from fish, egg and tree pollen. *Scand J Clin Lab Invest* 1991;204:17-31.
- Johnsen G, Elsayed S. Antigenic and allergenic determinants of ovalbumin—III. MHC Ia-binding peptide (OA 323-339) interacts with human and rabbit specific antibodies. *Mol Immunol* 1990;27:821-7.
- Buus S, Sette A, Grey HM. The interaction between protein-derived immunogenic peptides and Ia. *Immunol Rev* 1987;98:115-41.
- Bijker MS, van den Eeden SJ, Franken KL, Melief CJ, Offringa R, van der Burg SH. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J Immunol* 2007;15:179.
- Mazur G, Baur X, Modrow S, Becker WM. A common epitope on major allergens from non-biting midges (Chironomidae). *Mol Immunol* 1988;25:1005-10.
- Taneichi M, Tanaka Y, Kasai M et al. Induction of differential T-cell epitope by plain- and liposome-coupled antigen. *Bioconj Chem* 2006;17:899-904.
- Shimojo N, Katsuki T, Coligan JE et al. Identification of the disease-related T cell epitope of ovalbumin and epitope-targeted T cell inactivation in egg allergy. *Int Arch Allergy Immunol* 1994;105:155-61.
- Smith CM, Rosa GT, May JS et al. CD4+ T cells specific for a model latency-associated antigen fail to control a gammaherpesvirus in vivo. *Eur J Immunol* 2006;36:3186-97.
- Bickert T, Wohlleben G, Brinkman M et al. Murine polyomavirus-like particles induce maturation of bone marrow-derived dendritic cells and proliferation of T cells. *Med Microbiol Immunol* 2007;196:31-9.
- Szymczak-Workman AL, Workman CJ, Vignali DA. Cutting edge: regulatory T cells do not require stimulation through their TCR to suppress. *J Immunol* 2009;1:182.
- Sun JB, Flach CF, Czerkinsky C, Holmgren J. B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit. *J Immunol* 2008;15:181.
- Culshaw S, Millington OR, Brewer JM, McInnes IB. Murine neutrophils present Class II restricted antigen. *Immunol Lett* 2008;15:118.
- Buus S, Colon S, Smith C, Freed JH, Miles C, Grey HM. Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc Natl Acad Sci USA* 1986;83:3968-71.
- Janssen EM, Wauben MH, Jonker EH et al. Opposite effects of immunotherapy with ovalbumin and the immunodominant T-cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1999; 21:21-9.
- Kearney ER, Pape KA, Loh DY, Jenkins MK. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity* 1994;1:327-39.
- Pape KA, Kearney ER, Khoruts A et al. Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo. *Immunol Rev* 1997;156:67-78.
- Sun LZ, Elsayed S, Bronstad AM et al. Airway inflammation and bronchial remodelling in toluene diisocyanate-exposed BALB/c mouse model. *Scand J Immunol* 2007;65:118-25.
- Vaali K, Puimalainen TJ, Lehto M et al. Murine model of food allergy after epicutaneous sensitization: role of mucosal mast cell protease-1. *Scand J Gastroenterol* 2006;41:1405-13.
- Fang SP, Tanaka T, Tago F, Okamoto T, Kojima S. Immunomodulatory effects of gyokuhifusan on INF-gamma/IL-4 (Th1/Th2) balance in ovalbumin (OVA)-induced asthma model mice. *Biol Pharm Bull* 2005;28:829-33.
- Randolph DA, Stephens R, Carruthers CJ, Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest* 1999;104:1021-9.
- Getahun A, Heyman B. How antibodies act as natural adjuvants. *Immunol Lett* 2006;15:104.
- Getahun A, Dahlstrom J, Wernersson S, Heyman B. IgG2a-mediated enhancement of antibody and T cell responses and its relation to inhibitory and activating Fc gamma receptors. *J Immunol* 2004;1:172.
- Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003;111:291-7.
- Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002;3:715-20.
- Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255-81.
- Nakamura Y, Ghaffar O, Olivenstein R et al. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *J Allergy Clin Immunol* 1999;103:215-22.
- Kuperman DA, Huang X, Koth LL et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;8:885-9.
- Vercelli D, Geha RS. The IgE system. *Ann Allergy* 1989;63:4-11.
- Elsayed S, Staveng L. Epitope mapping of region 11-70 of ovalbumin (Gal d I) using five synthetic peptides. *Int Arch Allergy Immunol* 1994;104:65-71.
- Janssen EM, Wauben MH, Nijkamp FP, van Eden W, van Oosterhout AJ. Immunomodulatory effects of antigen-pulsed macrophages in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 2002;27:257-64.