



## Epitope characterization of ovalbumin in BALB/c mice using different entry routes

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### Abstract

Ovalbumin (OVA) is known as a major allergen in egg white. A number of studies have reported the partial T and B cell epitope mapping of OVA using murine models and allergic patients' sera. Recently, we have reported the IgE-binding regions of the entire OVA molecule using egg allergic patients' sera. However, the entire epitope mapping of OVA in a murine model has not been completed yet. In the present study, BALB/c mice were administered a solution of OVA using three different entry routes (oral, intraperitoneal and subcutaneous) with their respective adjuvant (cholera toxin, aluminum hydroxide and Freund's adjuvant). Two nitrocellulose membranes containing 188 overlapping synthetic peptides (with a length of 12 amino acids and an offset of two amino acids) covering the primary sequence of OVA, were probed with the three different BALB/c mice antisera. Antisera obtained from orally challenged mice identified eight IgE epitope regions, i.e. I53D60; V77R84; S103E108; G127T136; E275V280; G301F306; I323A332 and A375S384, while sera raised by intraperitoneal and subcutaneous injections exhibited two (K55D60 and K277L282) and five (K55R58; G127T136; K279L282; T303S308 and I323A332) IgE binding sequences, respectively. The residues critical for the epitope–paratope interactions were finely characterized using the oral immunization serum. Analysis of IgE binding epitopes in mice provides us with potential strategies for design of specific immunotherapy.

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**Keywords:** BALB/c mouse; Immunoglobulin E epitope mapping; Ovalbumin; Route of immunization; SPOTS membrane

### 1. Introduction

During the last 10 years, the prevalence of food allergies has increased significantly. Current data indicate that up to 8% of children under the age of ten and 4% of adults are affected [1]. The clinical symptoms of an allergic reaction range in severity from mildly irritating to life threatening [2]. The gut is exposed on a daily basis to a multitude of antigens, which do not usually elicit an overt immune response. The exact mechanisms that result in an allergic event are still unknown; however, considerable progress has been made [3–6]. During the onset of an allergic reaction, it has been shown that dietary antigens

can be captured by local cells, cross the intestinal membrane and trigger a specific immune response [7].

The most common form of food allergies, also known as type I, is characterized by the secretion of allergen-specific immunoglobulin E (IgE). The diagnosis of food allergies encompasses the measurement of allergen-specific IgE levels in susceptible individuals [8]. Immunoglobulin E plays a pivotal role in the induction of an allergic response as their cross-linking at the surface of mast cells is responsible for the release of inflammatory mediators such as histamine, prostaglandins and leukotrienes. The sites or regions of the dietary molecule(s) that IgE specifically recognizes are known as IgE epitopes or allergenic determinant.

In recent years, the B and T cell epitopes of the major food allergens have been characterized and provided better understanding of allergen recognition by the immune cells [3]. Epitope characterization has also given further insights into the development of pertinent experimental and therapeutic approaches for the prevention or the suppression of allergic disorders [9].

*Abbreviations:* APC, Antigen presenting cell; CFA, Complete Freund's adjuvant; DC, Dendritic cells; Fmoc, fluorenylmethoxycarbonyl; Ig, Immunoglobulins; IFA, Incomplete Freund's adjuvant; OVA, Ovalbumin; SPOTS, Simple Precise Original Test System; TBS, Tris-buffered saline; TBST, Tris buffered saline containing Tween-20

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Egg allergy is one of the three most prevalent forms of food allergies in children in the United States [4]. One of the major allergens in egg white, is ovalbumin (OVA) [10,11]. Ovalbumin is a 385 amino acid glycoprotein of approximately 45 kDa, which constitutes about 54% of the egg white proteins. The carbohydrate moieties of ovalbumin account for about 3% of its molecular weight [12].

A number of studies describe the allergenic and antigenic determinants of ovalbumin recognized by sera obtained from egg allergic patients and murine models. The fine mapping of ovalbumin IgE-epitope was initiated with the identification of the NH<sub>2</sub>-terminal fragment G1F10 by radioallergosorbent test (RAST) using sera obtained from egg-allergic patients [13]. Utilizing synthetic or cyanogen bromide (CNBr)-cleaved OVA peptides, subsequent studies demonstrated that the segments C11K19, I34E70 [14] and also V41M172, G301P385 [15] were reactive with patients' IgE antibodies. A different study also provided evidence that three overlapping haptenic peptides covering the sequence A347P385 were capable of specifically inhibiting histamine release from human basophils *in vitro* cultures, opening the door to innovative anti-allergic strategies [17].

The fragment I323R339 was initially identified as an immunodominant T-cell epitope [18]. This fragment was later shown to present binding activities to human IgE antibodies [19], and to be responsible for up to 50% of OVA-specific IgE response in BALB/c mice sensitized intraperitoneally [20]. The presence of both B and T cell epitopes [21,22] in the fragment I323R339 has led to its widespread use as a hapten model for the induction of oral tolerance [23] and the regulation of T cell subsets in the context of peptide-based immunotherapy [10,24]. This same fragment also resulted in the creation of a specific BALB/c transgenic strain of mice coding for TCR- $\alpha$  and - $\beta$  transgenes which together specifically recognize the OVA peptides 323–339 [25].

Additional studies also identified the region V41M172 and G301P385 as antigenic determinants of OVA, using specific anti-OVA monoclonal antibodies, containing IgG1 and IgM isotypes, produced from BALB/c mice [15]. A further study showed that irradiation of OVA could induce the formation of neoepitopes by using IgG1-monoclonal antibodies from BALB/c. The fragment V173M196 was specifically identified as an IgG1-antigenic determinant only found on the irradiated form of OVA [16].

However, none of the studies described above have produced a complete map of ovalbumin IgE epitopes recognized by allergic subjects or model animals. Very recently, we have completed the mapping of the linear IgE binding epitopes of the entire ovalbumin primary sequence using 18 egg allergic patients pooled sera. A total of five distinct regions i.e. L38T49, D95A102, E191V200, V243E248 and G251N260 were finely mapped and structurally characterized [26]. Information obtained from human studies yield limited information due to small sample sizes, ethics and genetic variations. On the other hand, more detailed and controlled analysis can be obtained from animal studies, in particular murine models. We therefore embarked on a study towards the entire IgE epitope map of

ovalbumin in the BALB/c mouse, a strain of mice previously used for the characterization of allergic responses to food proteins [27].

Animal studies commonly require attention to various factors such as the dose of allergens [28], the frequency of exposure [29,30], the animal genetic background [31], the adjuvant [32] and particularly the route of immunization [33]. Parenteral routes of administration are the most commonly used in experimental immunizations as they were shown to be the most efficient in triggering immune responses [33]. Oral administration would logically be the most appropriate route of immunization to mimic the phenomenon of food allergic disorders. But oral immunization generally fails to elicit an immune response, unless the oral antigen is co-administered with a mucosal adjuvant such as cholera toxin (CT) [34]. Numerous studies have focused on the influence of the route of immunization on the intensity of the humoral immune response [35] and the antibody isotypes generated [35,36], but very few reports have compared the antibody epitope specificity resulting from different routes of immunization [37].

In the present study, we have not only completed the entire map of IgE-binding epitopes present on the ovalbumin protein using BALB/c mouse antisera, but we have also demonstrated the existence of a substantial heterogeneity in IgE-binding epitopes, between groups of BALB/c mice sensitized to ovalbumin via three different routes of exposure i.e. oral, subcutaneous and intraperitoneal routes.

## 2. Materials and methods

### 2.1. Experimental animals

Female BALB/c mice were purchased from Charles River Laboratories (Montreal, QC, Canada) at 6–8 weeks of age. All animals were housed at the central animal facility, University of Guelph, in accordance with the Canadian Council on Animal Care (CCAC) guidelines. The mice were maintained under an ovalbumin-free diet (Teklad Global 14% protein and 3.5% fat) in a 12-h lighting cycle for the duration of the experiment. Food and water were available *ad libitum*.

### 2.2. Antigen sensitization procedure

Three sets of female BALB/c mice ( $n=5$ ) were respectively sensitized following three distinct protocols. The first set of mice was injected subcutaneously with 100  $\mu$ g (per mouse) of pure ovalbumin in 100  $\mu$ l of an emulsion of complete Freund's (Sigma, St. Louis, MO) adjuvant: saline 0.9% (1:1, v/v). After 4 weeks, a booster was given to each mouse with the same amount of antigen using incomplete Freund's adjuvant (Sigma, St. Louis, MO). The second set of mice was injected intraperitoneally with the same amount of pure ovalbumin. In this group, each mouse received two doses of 100  $\mu$ g of ovalbumin in a 100  $\mu$ l of aluminum hydroxide (Alhydrogel® 2%, Superfos Biosector, Frederikssund, Denmark) Al(OH)<sub>3</sub>: saline 0.9% (1:1, v/v) at an interval of 4 weeks. Seven days after the second injection, the mice from these two sets were euthanized in an atmosphere of CO<sub>2</sub>. Whole blood samples were collected by cardiac puncture in heparinized tubes. The antisera collected within each set were pooled and stored at –80 °C until analyzed.

The third set of mice was orally challenged twice a week with 1.0 mg of ovalbumin in 100  $\mu$ l of autoclaved distilled water containing 10  $\mu$ g of Cholera Toxin (CT, Calbiochem, San Diego, CA) for a duration of 4 weeks. After the last gavage, each mouse was injected intraperitoneally with 1.0 mg of ovalbumin in 100  $\mu$ l of Al(OH)<sub>3</sub>: saline 0.9% (50:50) to induce anaphylaxis. After 30 min, mice were euthanized with an atmosphere of CO<sub>2</sub>. Whole blood samples were

collected by cardiac puncture; antisera were pooled and stored at  $-80^{\circ}\text{C}$  until analyzed. For each set, two control mice underwent the same respective treatment but were administered placebo solutions.

Ovalbumin was co-administered with three distinct adjuvants specific to the route of administration. Complete/incomplete Freund's adjuvant (CFA/IFA) and aluminum hydroxide are both routinely used as parenteral adjuvants [38]. In accordance with the CCAC guidelines for immunization of research animals, injections containing Freund's adjuvant could only be administered subcutaneously, for this reason aluminum hydroxide, was used intraperitoneally. Cholera toxin (CT) is known as the most potent mucosal adjuvants [38,39], it has since been used routinely to induce allergic responses in mice [40–45] and it was therefore orally co-administered with OVA.

### 2.3. Determination of antibody titers

Ovalbumin specific IgE antibodies were measured by a sandwich ELISA as previously described [46]. Briefly, an ELISA 96-well microplate (Corning, Cambridge, MA) was coated with 100  $\mu\text{l}$  of ovalbumin (5  $\mu\text{g}/\text{well}$ ) in 100 mM bicarbonate buffer pH 9.6 and incubated overnight at  $4^{\circ}\text{C}$ . The plate was then washed three times with TBST (50 mM Tris, pH 8.0, 136 mM NaCl, 2.7 mM KCl and 0.05% Tween-20), blocked with 200  $\mu\text{l}/\text{well}$  of TBS containing 2% bovine serum albumin (BSA, Fisher Scientific, Pittsburgh, PA) and incubated at  $37^{\circ}\text{C}$  for 2 h. BALB/c mice serum samples were diluted 1:20 (oral and intraperitoneal) or 1:500 (subcutaneous) with a solution of TBS containing 1% BSA. One hundred microliters (per well) of each diluted serum were added onto the plate in duplicate wells, and the plate was incubated overnight at room temperature. The plate was then washed four times with TBST. A solution of biotin-conjugated monoclonal anti-mouse IgE antibodies (Sigma, St. Louis, MO) was diluted in TBS containing 1% BSA (1:1,000). This solution was added into the wells (100  $\mu\text{l}/\text{well}$ ) and the plate was incubated at  $37^{\circ}\text{C}$  for 2 h. Thereafter, the plate was washed five times and was incubated at  $37^{\circ}\text{C}$  with 100  $\mu\text{l}/\text{well}$  of alkaline-phosphatase (AP) conjugated to avidin (1:10,000; Sigma, St. Louis) prepared in TBS containing 1% BSA. After 1 h of incubation, the plate was washed five times and color was allowed to develop with the addition of 100  $\mu\text{l}/\text{well}$  of  $p$ -nitrophenol phosphate (1.0 mg/ml, Sigma) in a 0.1 M diethanolamine buffer (pH 9.8). The plate was incubated for 30 min at  $37^{\circ}\text{C}$  and the absorbance was measured at 405 nm with a Bio-Rad microplate reader. The antibody levels were expressed as the average optical density (OD) of duplicate wells.

### 2.4. Peptides synthesis on SPOTS membranes

One hundred and eighty-eight overlapping dodecapeptides (with a two amino acid offset) covering the entire primary sequence of OVA (385 amino acids) were synthesized on two  $\beta$ -alanine  $\text{NH}_2$ -derivatized cellulose membrane (SPOTS Membrane, Sigma-Genosys, The Woodlands, TX, USA) following the manufacturer's instructions and based on the Fmoc (fluorenylmethoxycarbonyl)-chemistry. Spots containing a single glycine residue served as a negative control, and spots containing a known epitope of the human epidermal growth factor receptor (EGF-R) with the sequence CAHYID served as positive control to validate the overall synthesis.

### 2.5. SPOTS membranes probing and analysis

The dried membranes were removed from storage at  $-30^{\circ}\text{C}$ , allowed to reach room temperature and rinsed with methanol. The membranes were washed three times with TBS and incubated overnight at room temperature in 20 ml of SPOTS blocking buffer (Sigma-Genosys) diluted in TBST containing 5% sucrose.

Thereafter, membranes were washed once with TBST and incubated at room temperature in 10 ml of mice pooled sera (diluted 1:20 in SPOTS blocking buffer). After 4 h of incubation, the membranes were washed three times with TBST, and each membrane was incubated in 10 ml of biotin-conjugated monoclonal anti-mouse IgE (1:1000, Sigma) for 2 h at room temperature. The membranes were washed three times, and a final incubation step with alkaline phosphatase-avidin conjugate (1:15,000, Sigma) was carried

out for 1 h at room temperature. The membranes were again thoroughly washed three times with TBST, and were allowed to equilibrate for 10 min in detection buffer (0.1 M NaCl in 0.1 M Tris/HCl, pH 9.5). Membrane-bound antibodies were detected using a 1:100 solution of chemiluminescent CDP-Star substrate (Boehringer Mannheim, Ingelheim, Germany) and Nitro-Block II enhancer (Tropix, Bedford, MA, USA) diluted in detection buffer. The chemiluminescence was visualized with a molecular light imager (EG-G Berthold, Bad Wildbad, Germany). Image analysis was performed using the WinLight software (EG-G Berthold) and spot intensity was expressed in counts per second (cts/sec).

### 2.6. Single amino acid substitution analysis

In order to identify the amino acid residues critical for IgE-binding activity, a total of 13 peptides (12-amino acid long) comprising the native epitope sequence (WT) and 12 other sequences comprising single amino acid substitution, were synthesized on a derivatized SPOTS membrane. For each epitope identified after oral sensitization, the amino acids present in the native sequence were sequentially replaced by either Glycine (G), Alanine (A), Methionine (M) or Asparagine (N). These single substitutions were carried out in the order G, A, M and N, for example if the native sequence did not contain any G residues, the single substitutions were carried out with G residues. However, if the peptide contained G residues, the peptide was sequentially mutated with A residues, likewise if the native sequence contained both A and G residues, the amino acid sequence was sequentially substituted with M residues. The pooled serum obtained from the orally sensitized mice was used to probe these multiple peptides and compare the IgE binding ability of the mutant sequences with that of the wild-type (WT) sequence.

### 2.7. SPOTS membranes regeneration

After each probing cycle, the membranes were regenerated by washing three times (20 min each) with an aqueous solution of 8 M urea containing 1% SDS (w/v) and 0.1% 2-mercaptoethanol (v/v), followed by three washes (20 min each) in an aqueous solution containing 50% of ethanol (v/v) and 10% of acetic acid (v/v). Membranes were then rinsed in methanol and stored at  $-30^{\circ}\text{C}$  until use.

## 3. Results

### 3.1. BALB/c mice humoral response

Three groups of BALB/c mice ( $n=5$ ) were immunized with ovalbumin using three distinct routes of administration and their respective adjuvant. ELISA assays were performed following the protocol described above. OVA specific IgE absorbance readings at 405 nm gave values ranging from 0.380 to 0.452 OD units. Similar to previous reports [30], the IgE antibody titers obtained after subcutaneous injection were higher than the titers obtained intraperitoneally or orally (data not shown). The background level was determined by testing control mice sera; the  $\text{OD}_{405}$  reading values were less than 0.1 absorbance unit. Based on our previous data, it is known that the absorbance readings obtained in these experiments coincide with specific IgE titers sufficient for membrane probing purposes.

One study reported the production of specific cholera toxin antibodies in mice when orally co-administered with an antigen [47]. Therefore, to exclude this possibility, the pooled sera obtained from oral immunization were tested, and no specific binding activity was detected against cholera toxin (data not shown).

### 3.2. OVA immunoglobulin E epitopes mapped by SPOTS assay

One hundred and eighty-eight overlapping peptides were synthesized to determine which regions of the OVA protein were recognized by BALB/c mouse IgE antibodies. These peptides were subsequently exposed to the pooled sera obtained from orally, subcutaneously and intraperitoneally sensitized groups of mice. Serum from control mice did not recognize any of the synthesized peptides. In contrast, multiple IgE binding epitopes were recognized by the OVA-sensitized mice. Fig. 1 shows the SPOTS membranes probed with the pooled sera obtained from (A) orally, (B) intraperitoneally and (C) subcutaneously sensitized mice. Each IgE binding epitope is represented by a series of 2 to 5 dark contiguous spots (chemiluminescence stain). Each spot represents a single peptide sequence which overlaps the adjacent peptide by 10 amino acids. Each native epitope sequence was defined as the amino acid sequence common to all the peptides present in a group of contiguous stained spots.

A total of eight prominent epitope regions were identified after probing with the orally sensitized mice sera (Fig. 1A). Strong signals were detected on spot numbers 25–27[A-1], 37–39[A-2], 49–52[A-3], 63–64[A-4], 135–138[A-5], 148–151[A-6], 161–162[A-7] and 187–188[A-8]. In contrast, only two major IgE-binding epitopes were elicited by intraperitoneal immunization (Fig. 1B), and correspond to high intensity spots number 25–28[B-1] and 136–139[B-5]. Interestingly, these two epitopes overlap the regions recognized by the specific IgE contained in the orally sensitized mice sera. Lastly, results (Fig. 1C) obtained with subcutaneously sensitized mice sera indicated five distinct binding regions. Spots number 24–28[C-1] and 136–140[C-5] displayed a high IgE binding intensity while spots 63–64[C-4]; 149–152[C-6] and 161–162[C-7] indicated a lower binding intensity. The overall results obtained from the SPOTS membranes probing are summarized in Table 1. Interestingly, the peptide sequences comprised within spots 24–28 and spots 135–140 have not only generated the highest intensity signals within

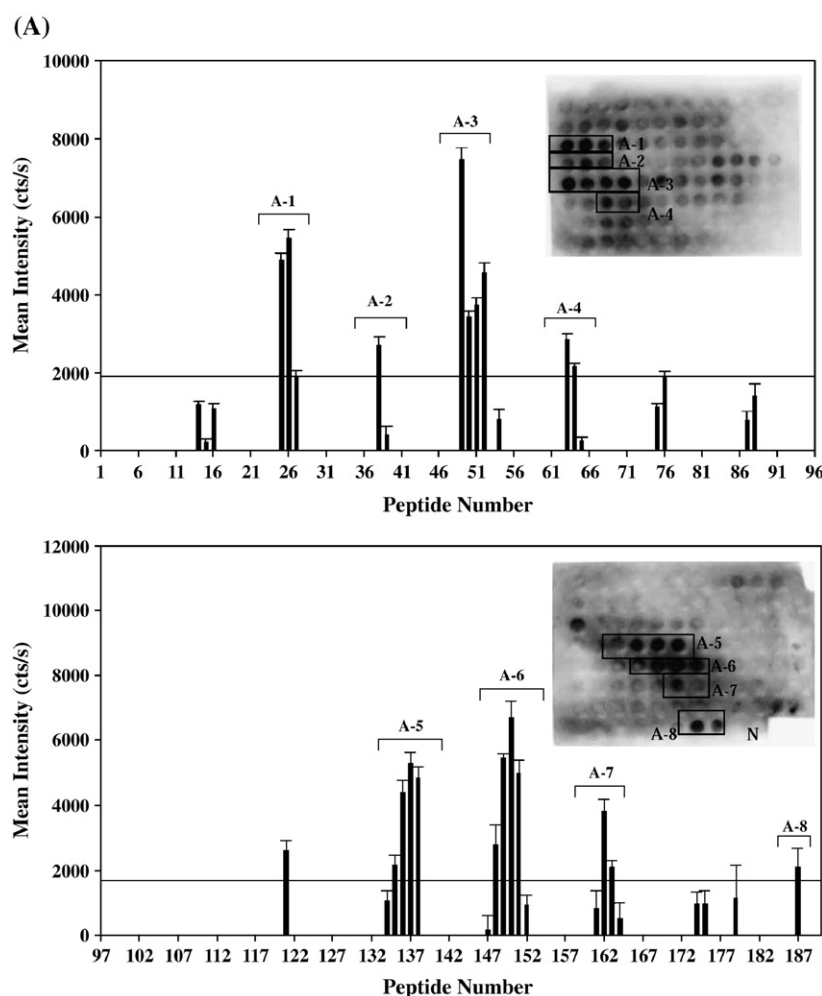


Fig. 1. Sequential epitope mapping of OVA. Two SPOTS membranes were subsequently probed with pooled sera obtained from female BALB/c mice which were orally (Fig. 1A), intraperitoneally (Fig. 1B) and subcutaneously (Fig. 1C) sensitized with OVA protein co-administered with a specific adjuvant (see Materials and methods). The negative control spot (N), composed of a single glycine residue, is labelled at the bottom right corner of the second membrane for each probing. The horizontal line indicates the threshold value below which the spots intensities were not significant compared to the prominent epitope regions.

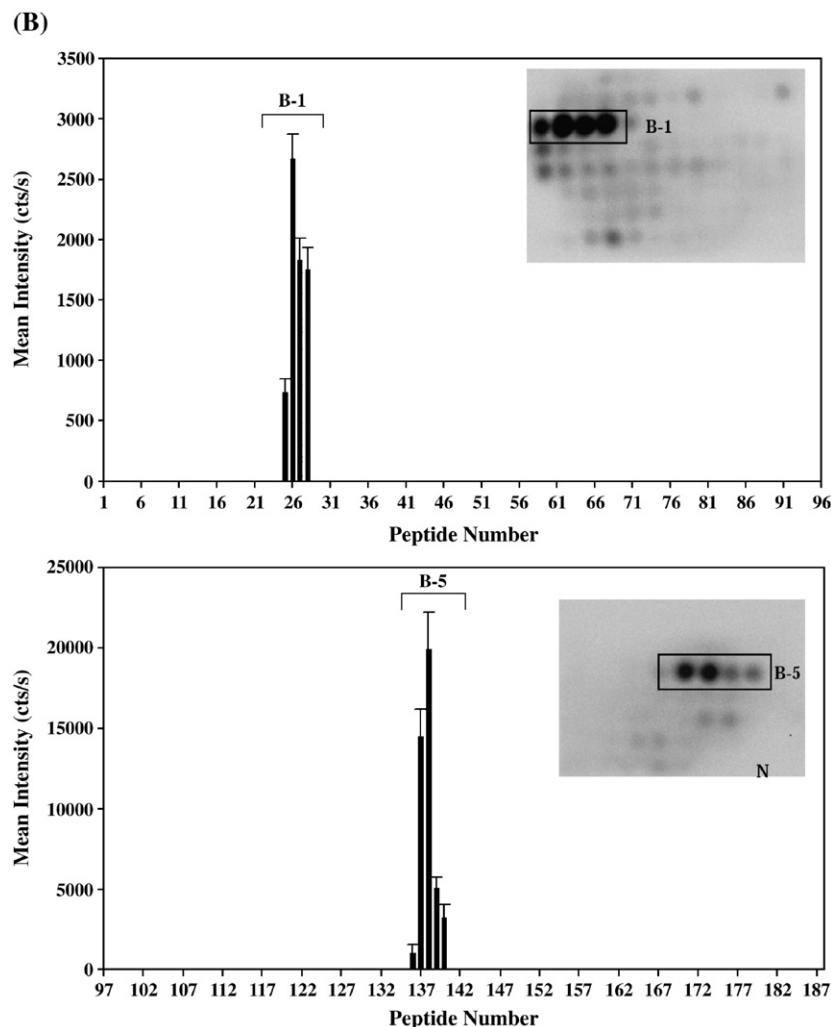


Fig. 1 (continued).

the three types of sera, but were, more significantly, common to all three sera.

Fig. 2 shows the results obtained with orally sensitized mice sera. Eight epitope regions were distributed along the length of the ovalbumin amino acid sequence. These regions were designated as I53D60, V77R84, S103E108, G127T136, E275V280, G301F306, I323A332 and A375S384 respectively.

### 3.3. Single amino acid substitution analysis

The amino acids essential for the binding of IgE were determined by synthesizing each IgE epitope recognized by the orally sensitized-mice sera with single amino acid substitution performed at each position. A representative SPOTS membrane strip containing the wild-type (WT) and mutated peptides corresponding to the residue sequence I53D60 is presented in Fig. 3. It shows that mouse IgE-binding activity was either eliminated or significantly reduced, when the amino acids K55, V56, V57, R58 and F59 were substituted by a glycine (G) residue. All IgE epitopes (oral sensitization) were tested in an identical manner. Single mutations in epitope regions A1, A3,

A5 and A8 resulted in decreased binding activity of OVA-specific IgE but no significant variation was observed for epitope regions A2, A4, A6 and A7. The critical residues identified in epitope regions A1, A3, A5 and A8 are summarized in Table 2.

### 3.4. Epitopes hydrophobicity profile

It is generally hypothesized that a sequence region which is rich in charged residues is likely to be surface exposed in the folded protein, and would therefore be more likely to form a B cell epitope. The amino acids composing the eight epitope regions determined after oral sensitization were classified as hydrophobic, polar or charged (Figs. 4). Solely considering the oral sensitization, a total of 64 epitope specific amino acids were enumerated. Hydrophobic residues were found to be the most frequently occurring (34/64), followed by polar residues (17/64) and then charged residues (13/64). In increasing order, the residues valine (V), alanine (A) and phenylalanine (F) appeared the most frequently, followed by isoleucine (I), serine (S) and arginine (R) at the same frequency.

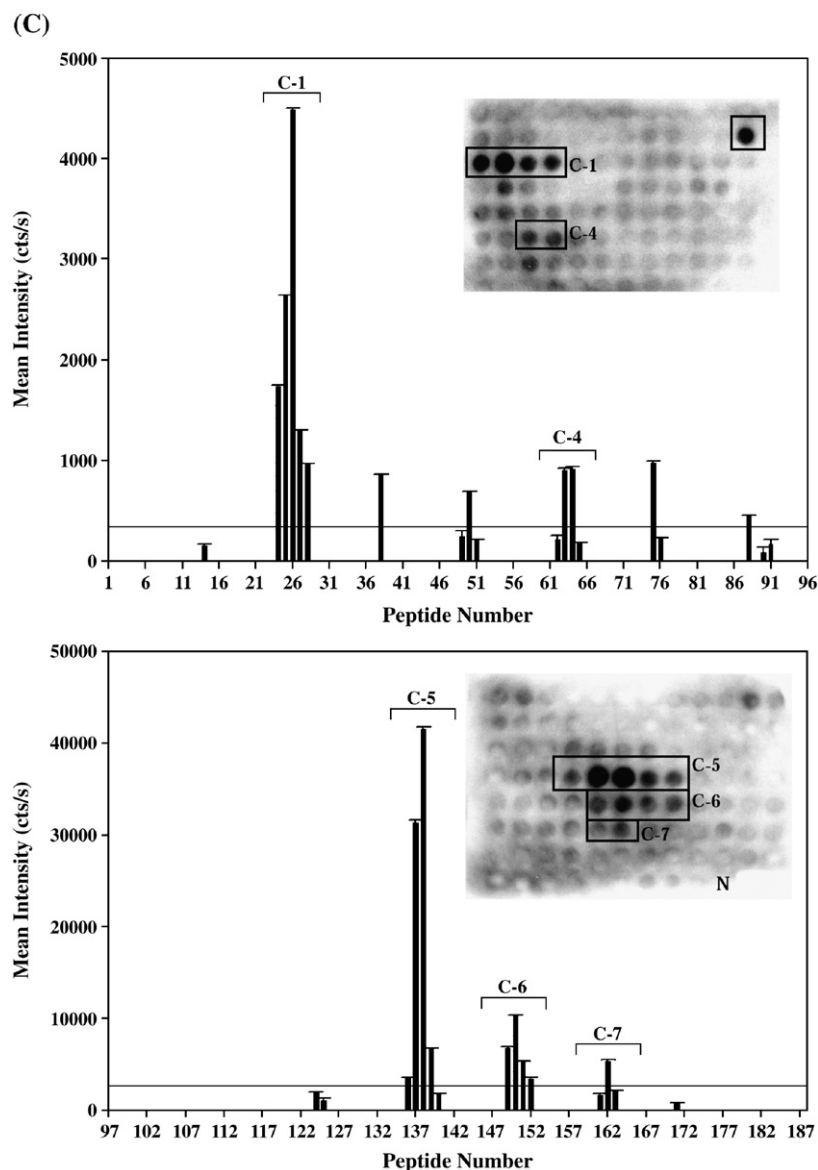


Fig. 1 (continued).

Most of the critical residues belonged to the hydrophobic (A, V and F) and charged classes of residues (K and R). Relative epitope hydrophobicities, as determined by the method of Hopp and Woods [48], identified two IgE epitopes (G301T306 [A6] and A375S384 [A8]) as relatively hydrophobic regions of the protein, while the other six regions tend to be more hydrophilic (Fig. 5).

### 3.5. Immunoglobulin E epitopes localization on a 3D model

A model of the tertiary structure of OVA was generated to display the location of the epitopes identified by the orally sensitized mice sera (Fig. 6). The epitope I53-D60 [A1] is located on  $\alpha$ -helix B (54–60). V77-R84 [A2] is made up of a portion of  $\alpha$ -helix C (77–80) and a  $\beta$ -turn type I (82–84). S103-E108 [A3] is made up of a random structure located at the surface of the protein, G127-T136 [A4] of  $\alpha$ -helix E (128–136),

E275-V280 [A5] of a  $\beta$ -turn type III associated with  $\alpha$ -helix (275–280), G301-F306 [A6] of  $\alpha$ -helix I (301–305). The epitope I323-A332 [A7] is composed a  $\beta$ -turn type I (324–327) plus random, then a portion of  $\beta$ -sheet s5A (329–332), whereas A375-S384 [A8] is made of a  $\beta$ -sheet s4B (377–376) plus  $\alpha$ -turn (376–380) plus a portion of  $\beta$ -sheet s5B (382–384).

As expected, most of the epitopes are found on the surface on the OVA native structure or partially buried, and mainly consist of  $\beta$ -sheets and  $\beta$ -turns (Fig. 6). On the other hand, the two epitope regions I53D60 [A1] and V77R84 [A2] are present on  $\alpha$ -helices (red and green colored-ribbons). One epitope in particular A375S384 [A8] (pink), is buried within the groove of the OVA molecule. The two prominent epitopes previously indicated as regions I53D60 [A1] (red) and E275V280 [A5] (dark blue), are respectively located on  $\alpha$ -helix and  $\beta$ -sheet structures. It is interesting to note that both these regions are the most easily accessible sites when compared to the remaining

Table 1  
Disparity in OVA epitope recognition pattern

| Oral entry route      |                                          | Intraperitoneal entry route       | Subcutaneous entry route             |                                          |                                          |
|-----------------------|------------------------------------------|-----------------------------------|--------------------------------------|------------------------------------------|------------------------------------------|
| 1.0 mg OVA + 10 μg CT |                                          | 0.1 mg OVA in Al(OH) <sub>3</sub> | 0.1 mg OVA in CFA/IFA                |                                          |                                          |
| A1                    | <sup>53</sup> INKVVRFD <sup>60</sup>     | B1                                | <sup>55</sup> KVVR <sup>58</sup>     |                                          |                                          |
| A2                    | <sup>77</sup> VNVHSSLR <sup>84</sup>     | —                                 | —                                    |                                          |                                          |
| A3                    | <sup>103</sup> SRLYAE <sup>108</sup>     | —                                 | —                                    |                                          |                                          |
| A4                    | <sup>127</sup> GGLEPINFQT <sup>136</sup> | —                                 | C4                                   | <sup>127</sup> GGLEPINFQT <sup>136</sup> |                                          |
| A5                    | <sup>275</sup> ERKIKV <sup>280</sup>     | B5                                | <sup>277</sup> KIKVYL <sup>282</sup> | C5                                       | <sup>279</sup> KVYL <sup>282</sup>       |
| A6                    | <sup>301</sup> GITDVF <sup>306</sup>     | —                                 | —                                    | C6                                       | <sup>303</sup> TDVFSS <sup>308</sup>     |
| A7                    | <sup>323</sup> ISQAVHAAHA <sup>332</sup> | —                                 | —                                    | C7                                       | <sup>323</sup> ISQAVHAAHA <sup>332</sup> |
| A8                    | <sup>375</sup> AVLFFGRCVS <sup>384</sup> | —                                 | —                                    | —                                        |                                          |

The sequences A1–A8, B1, B5 and C1, C4–C8 correspond to the epitope regions identified after probing the SPOTS membranes with BALB/c mice sera (Fig. 1), following oral (A), intraperitoneal (B) and subcutaneous sensitization (C). Superscript numbers represent the amino acid position on the OVA primary sequence. Dotted lines (–) indicate that there was no IgE binding activity on the designated regions as assayed by SPOTS membranes.

epitopes and probably explains their prominence. Overall, the 3D localization of the epitope regions indicates that the major structural components of BALB/c mouse IgE epitopes in ovalbumin contain  $\beta$ -sheets and  $\alpha$ -helices.

#### 4. Discussion

Identification of the molecular basis of allergen epitopes contributes to an understanding of the aetiology of allergic disorders, but most of all, it offers a rationale for experimental and therapeutic strategies. Such information can for example be exploited in the development of peptide-based vaccines [49], the production of recombinant hypoallergenic molecules [50] or the determination of cross-reactivity between food allergens [51].

The current study was undertaken to investigate the effects of immunization by different routes and their respective

OVA EPITOPE A-1 (I53D60)



Fig. 3. Substitution analysis of sequential OVA epitope I53D60 in orally sensitized mice. The critical residues were identified by sequentially substituting each amino acid of the native peptide sequence with glycine (G). The SPOTS membrane was then probed using the pooled antisera obtained from orally sensitized Balb/c mice ( $n=5$ ), and visualized by the addition of chemiluminescent substrate. Amino acid substitutions are indicated on the figure: the letter refers to the amino acid position at which substitution was carried out. The letters and numbers across the top of the panel indicate the one-letter amino acid code for the residue normally at that position, the position of each residue in the OVA protein, and the amino acid that was substituted at that position. WT, indicates the wild-type peptide (no amino acid substitution).

adjuvant on allergen B cell epitope recognition. To the best of our knowledge, this is the first research study that not only entirely mapped BALB/c mouse IgE epitopes present on the ovalbumin protein, but also characterized the structural heterogeneity of IgE epitopes generated after exposure to an allergen through different routes i.e. oral, intraperitoneal and subcutaneous.

Of particular interest are the results obtained from the orally challenged set of mice, as an oral challenge represents the natural route of human sensitization to food allergens. The elicitation of an allergic response in mouse by oral administration provides clear ties to the human condition [52,53], for this reason, we compared the OVA IgE epitope map obtained in this study to the one obtained in our previous study using human patients' sera [26]. Table 3 presents the similarities and differences between the two species.

On the one hand, mouse and human epitopes have similar epitope sizes as well as very similar epitope physicochemical

|     |                             |            |                           |                           |             |
|-----|-----------------------------|------------|---------------------------|---------------------------|-------------|
| 1   | GSIGAASMEF                  | CFDVFKEKLV | HHANENIFYC                | PIAIMSALAM                | VYLGAKDSTR  |
|     | A-1                         |            | A-2                       |                           |             |
| 51  | TQ <b><u>INKVVRFD</u></b>   | KLPGFGDSIE | AQCGTS <b><u>VNVH</u></b> | <b><u>SSLR</u></b> DILNQI | TKPNDVYSFS  |
|     | A-3                         |            | A-4                       |                           |             |
| 101 | LAS <b><u>SRLYAE</u></b> ER | YPILPEYLQC | VKELYR <b><u>GGLE</u></b> | <b><u>PINFQT</u></b> AADQ | ARELINSWVE  |
| 151 | SQTNGIIRNV                  | LQPSSVDSQT | AMVLVNIAIVF               | KGLWEKAFKD                | EDTQAMPFRV  |
| 201 | TEQESKPVQM                  | MYQIGLFRVA | SMASEKMKIL                | ELPFASGTMS                | MLVLLPDEVS  |
|     |                             |            | A-5                       |                           |             |
| 251 | GLEQLESIIN                  | FEKLTEWTSS | NVME <b><u>ERKIKV</u></b> | YLPRMKMEEK                | YNLTSVLMAM  |
|     | A-6                         |            | A-7                       |                           |             |
| 301 | <b><u>GITDVF</u></b> SSSA   | NLSGISSAES | LK <b><u>ISQAVHAA</u></b> | <b><u>HAE</u></b> INEAGRE | VVGSAAEAGVD |
|     |                             |            | A-8                       |                           |             |
| 351 | AASVSEEFRA                  | DHPFLFCIKH | IATN <b><u>AVLFFG</u></b> | <b><u>RCVSP</u></b>       |             |

Fig. 2. Epitope localization on OVA primary sequence (oral sensitization). Amino acid residues in bold and underlined correspond to the sequence of the eight IgE epitope regions identified by the oral antiserum and are indicated A-1 to A-8. Numbers on the left indicate the position of the amino acid sequence in native ovalbumin.

Table 2  
Critical amino acids in the immunodominant OVA epitopes

| Epitope region | Amino acid sequence                                     |
|----------------|---------------------------------------------------------|
| A-1            | <sup>53</sup> <b>INKVVRFD</b> <sup>60</sup>             |
| A-3            | <sup>103</sup> <b>SRLYAE</b> <sup>108</sup>             |
| A-5            | <sup>275</sup> <b>ERKIKV</b> <sup>280</sup>             |
| A-8            | <sup>375</sup> <b>AVLFFGR</b> <sup>384</sup> <u>CVS</u> |

Amino acid residues in bold and underlined correspond to residues found critical for the binding activity of BALB/c mouse OVA specific IgE.

characteristics. Epitope secondary structures ( $\beta$ -sheets) and major residues critical for binding (hydrophobic and charged) are also shared by both species. These observations are not surprising, since the overall structure and function of the immune system are known to be very comparable between mice and humans [54].

On the other hand, there are no common IgE binding regions between the two species and in fact, we identified a higher number of IgE epitopes in BALB/c mice (oral sensitization). We had previously observed that our results using human patients' sera were different from results reported in earlier studies also involving human patients' sera [26]. We had attributed these differences to variations within the human population e.g. genetic background, sample size and outgrown vs. persistent allergic patients. Therefore, since IgE epitope maps in humans do not correlate well from one study to another, it is not surprising at first that two different species may recognize distinct epitopes on the same molecule. However, when we analyzed our BALB/c results against the other studies involving human egg allergic patients' sera (Fig. 7), we observed that all epitope regions identified in our study (oral sensitization) – except for epitope E275L280 – do in fact overlap with the reported human IgE epitopes i.e. sequences I34E70 [14] and also V41M172, G301P385 [15]. Therefore, these epitope similarities do suggest that the mouse represents a valid model for the understanding of human food allergy [54,55].

It was also interesting to compare our epitope map results to previous studies using the same mouse strain (Fig. 7B). To the best of our knowledge, I323R339 is currently the only sequence that has been reported as a major OVA IgE epitope in BALB/c mice [13–23]; and we have identified the epitope sequence, I323A332, after both oral and subcutaneous exposure (Table 1). Two additional studies have identified the regions V41M172, G301P385 [15] and V173M196 [16] as antigenic determinants of OVA, using BALB/c monoclonal antibodies composed of IgG1 and IgM isotypes. In our study, we have determined the binding activities of IgE and yet, the results indicate that the IgE epitopes we identified, do overlap with these IgG1/IgM antigenic regions (Fig. 7B). This suggests that the sequences V41M172, G301P385 and V173M196 contain structures that are very immunogenic in BALB/c mice. The only region identified in our study and not found in previous reports is the epitope sequence E275L280. This can be explained by differences linked to experimental conditions, and by the fact that different antibody isotypes (IgE vs. IgG1/IgM) are likely to generate different clone specificities.

In the present study, our main objective was to identify and compare the ovalbumin IgE-epitopes generated by three distinct modes of administration. Our first observation is that each immunization protocol has resulted in the recognition of multiple epitopes, which has also been reported in previous studies [56,57]. Secondly, results showed that the three types of sera shared common IgE epitope regions (Table 1). Indeed, the subcutaneous and oral immunizations have generated five overlapping epitope regions (i.e. K55R58, G127T136, K279L282, T303S308 and I323A332) and, most interestingly, all three sera shared two prominent epitope regions (i.e. E53D60 and E275L282). These two regions displayed the highest binding signal intensity in all three types of sera, suggesting these regions may represent immunodominant epitopes (Table 1, Fig. 1).

On the other hand, significant heterogeneity was also observed dependent on the mode of administration. These variations were characterized by differences in number of epitopes generated, epitope primary sequences, epitope lengths, and epitope locations on the tertiary structure (Table 1 and Fig. 6). We speculate that at least, three factors have contributed to these differences: (1) the nature of the adjuvant, (2) the local immune environment (i.e. the phenotype of the antigen presenting cells first encountering the antigen) and (3) the body compartments traversed by the antigen.

First, we will consider the nature of the adjuvant. The co-administration of adjuvants aimed at potentiating the IgE antibody response [58] as these adjuvants are known to favour a specific Th2-like cellular response [59–61]. Recent reports have assessed the influence of adjuvants on epitope specificity [62,63], but none have included a detailed comparison of their influence on epitope structure.

The antigen conformational changes possibly caused by adjuvants remain unknown, however, the potential mechanisms of their immunostimulating activities have been reviewed [64–66]. Immunological adjuvants are usually classified into

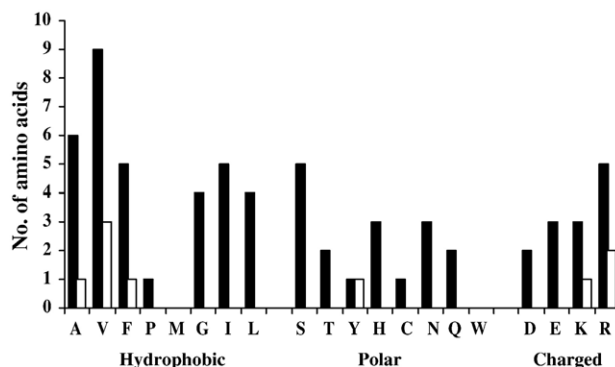


Fig. 4. Classification of amino acids in the OVA sequential epitopes (oral sensitization). The amino acids composing the eight sequential OVA epitopes were classified as hydrophobic, polar and charged residues. The frequency with which each of them occurred is represented below. Black bars represent the amino acids found in the epitope regions identified with the antisera from orally-sensitized mice, and the white bars indicate the critical amino acids identified after probing with this same antisera.

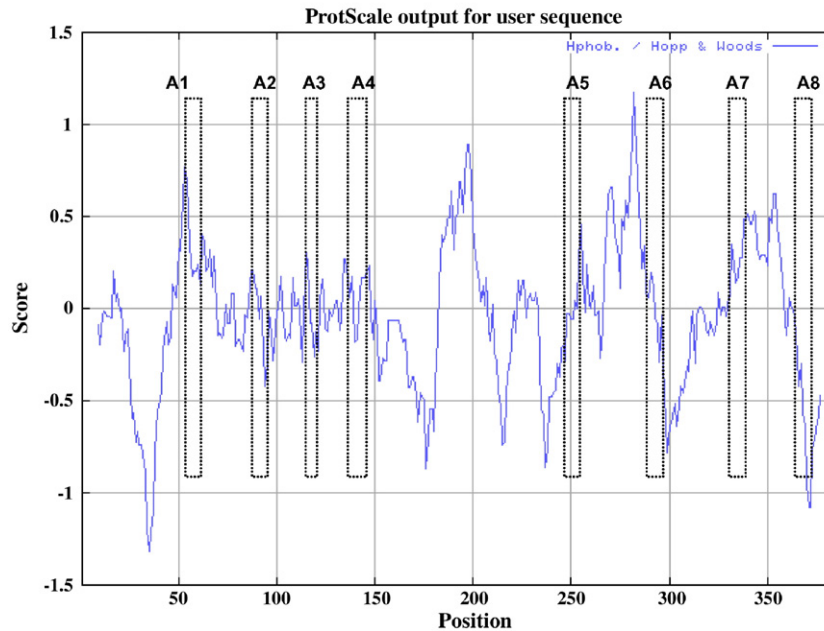


Fig. 5. Hydropathy profile of OVA. A hydropathy plot was prepared in the ExPASy ProtScale web site using the Hopp and Woods method of calculating [40]. Amino acids were scanned over a window length of 17 amino acids, and the average hydropathy values were plotted. Hydrophilic regions are indicated by positive hydropathy values, and hydrophobic regions by negative values. The amino acids corresponding to the OVA primary sequence are numbered along the x-axis. Stretches corresponding to each of the OVA epitopes are indicated by boxes. Numbers A1 to A8 refer to epitopes identified after probing with the orally-sensitized BALB/c mice antisera i.e. I53D60, V77R84, S103E108, G127T136, E275V280, G301F306, I323A332, and A375S384 respectively.

two groups: the first, known as “vehicle” adjuvants, create a depot effect and transport the antigen from the injection site to local lymphoid tissues. The second class of adjuvants, known as immunomodulators, can activate antigen presenting cells (APC) or other immune cells to produce cytokines in a non-antigen specific manner. Most of them contained or are

derived from bacterial components [67,68]. While aluminium hydroxide constitutes a typical example of vehicle adjuvant, CFA is an oil-in-water emulsion which contains minute amount of killed *Mycobacterium tuberculosis*; therefore CFA, as well as cholera toxin (CT), belong to the second category. On the basis of their immunostimulatory capacities, it is

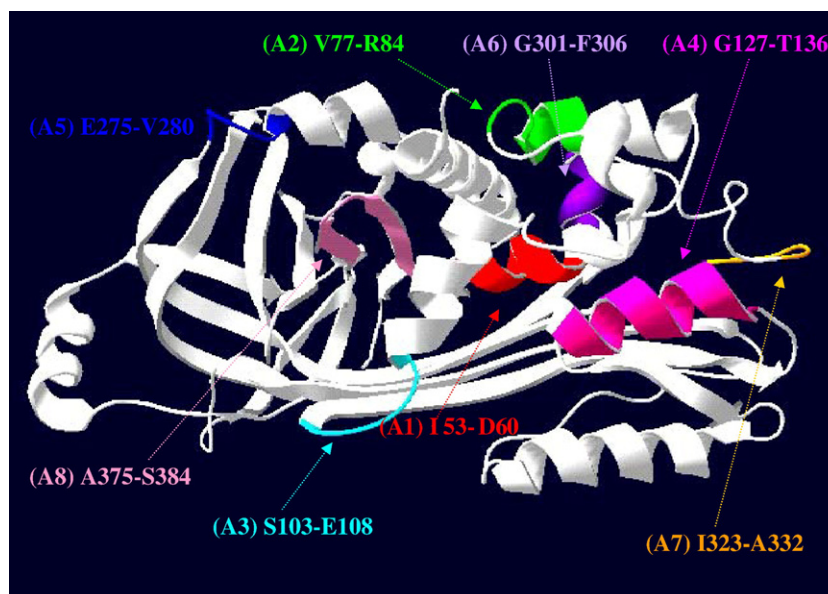


Fig. 6. Representation of OVA sequential epitopes on a 3D model. The picture represents the ribbon diagram tertiary structure of hen ovalbumin. The eight epitope regions identified after oral sensitization are represented in eight different colors on the ribbon structure. The structure was drawn with Dep View/Swiss-Pdb Viewer version 3.7 from Glaxo Smithkline (Geneva, Switzerland).

Table 3  
Comparison of Human vs. BALB/c mouse OVA IgE sequential epitopes

|                                                   | Human egg allergic patients [26]                      | BALB/c mouse oral sensitization                                                          |
|---------------------------------------------------|-------------------------------------------------------|------------------------------------------------------------------------------------------|
| Number of epitope regions                         | Five                                                  | Eight                                                                                    |
| Epitope regions on OVA primary sequence           | L38T49<br>D95A102<br>E191V200<br>V243E248<br>G251N260 | I53D60<br>V77R84<br>S103E108<br>G127T136<br>E275V280<br>G301F306<br>I323A332<br>A375S384 |
| Number of residues per epitope                    | 6 to 12 residues                                      | 6 to 10 residues                                                                         |
| Epitopes physicochemical composition <sup>a</sup> | 54.3% hydrophobic<br><br>23.9% polar<br>21.7% charged | 53.1% hydrophobic<br><br>26.6% polar<br>20.3% charged                                    |
| Main residues critical for IgE binding activity   | Hydrophobic and charged                               | Hydrophobic and charged                                                                  |
| Epitopes main secondary structures                | $\beta$ -sheets and $\beta$ -turns                    | $\beta$ -sheets and $\alpha$ -helices                                                    |

<sup>a</sup> Percentage of total residues.

therefore suggested that CT and CFA are capable to generate additional antibody specificities compared to aluminium hydroxide, a more inert mineral-based adjuvant. This may explain why eight and five IgE epitopes were generated after oral and subcutaneous injection, as opposed to only two IgE epitopes after intraperitoneal exposure to the same protein (Table 1).

One study compared the effects of the two adjuvants, dextran sulphate (DS) and CFA in five different strains of mice [69]. It showed that CFA was capable of inducing allergen-specific antibody of additional specificity compared to DS. Although DS was not used in our study, these findings support the fact that adjuvants have a considerable effect on the process of epitope recognition.

Secondly, the route of exposure also defines the phenotypes of antigen presenting cells (APC) to which the antigen will be first exposed [30]. Previous reports have already suggested that the processing and subsequent presentation of protein antigens were important factors in the quality of an immune response e.g. antibody specificity and response intensity [70]. Indeed, it is known that different phenotypes of circulating APC are distributed along the peripheral and mucosal immune systems e.g. macrophages and dendritic cells (DC) in the peritoneal area, Langerhans cells in the epidermis, and microfold M cells together with the Peyer's patches in the intestinal mucosa. Cells of the DC lineage predominate in the skin and in the Peyer's patches, whereas macrophages are more abundant in the peritoneum [37]. Previous studies have shown that some APC differ in their chemical content i.e. distinct proteases content and MHC molecules expression [70]. Although it is unclear how, this disparity in APC phenotype may significantly account for the structural heterogeneity observed in our epitope map results.

Third, it is coherent that an antigen orally administered will transit through compartments distinct from a parenterally introduced antigen. Indeed, a subcutaneous or intraperitoneal exposure will result in the antigen being rapidly routed to the closest immune tissues e.g. draining lymph nodes or spleen, via the blood or the lymphatic stream. In contrast, orally administered antigens (ovalbumin in our study) will transit through the gastrointestinal compartment, encountering acidic pH and enzymatic activities, before reaching the intestinal mucosa where the antigen is taken up, processed and presented by the underlying immune cells. A widely accepted view is that the resistance of a food protein to the gastrointestinal tract accounts for its allergenic nature [3,71]. However it is not unreasonable to expect partial degradation of the OVA protein may occur in this harsh environment, which does not affect the overall allergenic properties of the protein [72]. Such partial degradation or unfolding of the protein may account for the larger number of epitopes generated after oral administration (compared to the parenteral routes), as previously buried sites are exposed. Supporting this rational, a previous study demonstrated that *in vitro* gastrointestinal digestion of a plant allergen resulted in fragmentation of the protein, without affected its overall IgE reactivity when compared to its non-digested counterpart [73].

The question arises, which of the above factor exerts the major influence on epitope recognition. We believe that the epitope heterogeneity found between the three routes cannot be attributed to a single factor, but rather a combination of effects exerted by both the nature of the adjuvant and the entry route.

Mutational analysis of the IgE-binding epitopes identified on the ovalbumin molecule revealed that single amino acid changes within the epitope native sequence could decrease or eliminate IgE-binding activity. These residues are likely to represent anchor residues within the antibody contact region of the epitope. This suggests the possibility of using an altered form of OVA protein or OVA-derived peptides in a context of allergy immunotherapy.

The potential for animal models such as mice to mimic human disease processes makes them invaluable tools for testing treatment regimens. The present report has described, in detail, the entire epitope map of ovalbumin in BALB/c mouse, a widely used food allergy model. We have demonstrated that the nature of the adjuvant and the entry route generated significant heterogeneity in epitope recognition, especially in the number of IgE epitopes generated. We anticipate that an incomplete identification of epitopes, through the use of suboptimal immunization schemes, may lead to failure in the abolition or inhibition of an allergic response. Our study suggests that the oral route may be the optimal route of administration when exploring immunotherapeutic strategies against food allergy.

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**(A) Ovalbumin allergenic (IgE) epitopes reported in human egg allergic patients***OVA primary sequence*

1 \_\_\_\_\_ 385

(a) 38\_\_49 95\_\_102 191\_\_200 243\_\_248 251\_\_260

(b) 1\_10

(c) 11\_19 33\_\_\_\_77

(d) 41\_\_\_\_\_171 301\_\_\_\_\_385

(e) 347\_\_385

(a): Mine and Rupa [26]

(b): Elsayed, et al. [13]

(c): Elsayed and Stavseng [14]

(d): Kahlert, et al. [15]

(e): Honma, et al. [17]

**(B) Ovalbumin allergenic (IgE) and antigenic (IgG1/IgM) epitopes reported in BALB/c Mouse***OVA primary sequence*

1 \_\_\_\_\_ 385

(a) 53\_60 77\_84 103\_108 127\_136 275\_\_280 301\_306 323\_332 375\_384

(b) 41\_\_\_\_\_171 301\_\_\_\_\_385

(c) 173\_\_\_\_196

(d) 323\_\_339

(a): Present study (Oral sensitization) - IgE isotype

(b): Kahlert, et al. [15] - IgG1/IgM isotypes (monoclonal)

(c): Masuda, et al. [16] - IgG1 isotype (monoclonal)

(d): Renz, et al. [20] - IgE isotype

Fig. 7. Schematic representation of the epitope regions of ovalbumin, as reported by different groups in human egg allergic patients (A) and in BALB/c mouse (B). The numbers refer to the amino acid positions on the primary sequence of ovalbumin, and the lines represent the length of the epitope regions.

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