

The [173–196] fragment of ovalbumin suppresses ovalbumin-specific rat IgE responses

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Abstract

Peptides and protein hydrolysates are attractive tools for the induction of tolerance or regulation of targeted B and/or T cell responses. In vivo, peptides are mainly produced by the action of digestive enzymes or following the processing of exogenous antigens by antigen-presenting cells (APCs). In vitro, these molecules are generally produced by enzymatic digestion and chemical hydrolysis of proteins. We investigated the T and B cell determinants of the major food allergen ovalbumin (nOVA) in rat by analyzing (1) the stimulatory effect of nOVA peptides generated by cyanogen bromide (CNBr) cleavage on nOVA-specific T cells, and (2) the potential of CNBr-derived OVA fractions to induce oral tolerance to nOVA. Peptide fractions of the CNBr-hydrolysed OVA were isolated by high-pressure liquid chromatography and tested for their ability to stimulate nOVA-specific T cells isolated from rats parenterally immunized with nOVA. The nOVA fractions containing the stimulatory determinants were then intragastrically administered to rat to test their potential to induce oral tolerance. The whole CNBr hydrolysate stimulated proliferation of nOVA-specific T cells. Three out of the five HPLC-purified peptidic fractions were also able to stimulate proliferation and cytokine production by nOVA-specific T cells. A peptide fraction exhibiting a single peak by HPLC contained the 173–196 nOVA segment and stimulated nOVA-specific T cells. This segment also promoted oral tolerance to nOVA and reduced IgE responses. CNBr hydrolysis releases several peptides with stimulatory effect on nOVA-specific T cells including a new nOVA [173–196] T cell determinant which induces oral tolerance to nOVA.

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Abbreviations: CNBr, cyanogen bromide; PTH, phenyl-thiohydantoin; RCM, reduction and carboxymethylation; nOVA, native ovalbumin; HPLC, high-pressure liquid chromatography.

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1. Introduction

Identification of protein sequences with B or T cell specificity led to the use of peptides for the analysis of immune responses and as tools for therapeutic

immune regulations. In fact, peptides are now widely used as vaccine antigens to promote targeted cytotoxic T cell responses [1–3]. Similarly, peptides are being considered for the induction of tolerance for protection against autoimmune diseases [4]. For example, tolerance to milk proteins was reported after oral administration of partially hydrolyzed milk proteins [5] or peptides obtained by enzymatic hydrolysis of milk proteins [6]. These findings strongly suggest that peptides represent an attractive strategy for manipulation of unwanted immune responses to food proteins.

Ovalbumin is a common food ingredient known to induce allergic symptoms in a number of individuals. Immunoblot and histamine release assays have identified an allergenic B cell epitope of nOVA in the C-terminal region comprising residues 347–385 [7]. Efforts have also been dedicated at identifying T cell epitopes of nOVA since IgE responses are controlled by cytokines (i.e., IL-4 and IL-13) produced by T helper cells [8,9]. In this regard, a nOVA-specific T cell line established from a patient with hen egg allergy was reported to respond to a synthetic OVA 323–339 peptide [10], suggesting the presence of T cell epitopes in this fragment of the nOVA molecule. Another study has shown that T cells from hen egg allergic patients could also recognize a synthetic peptide corresponding to the 105–122 residues of OVA [11]. Homologous proteins with known amino acid substitutions [12,13] or fragments resulting from the chemical or enzymatic cleavage of proteins of interest [14–16] were proven effective at mapping effector B and T cell epitopes for a number of protein antigens. However, distinct routes of antigen delivery can generate a diversity of T cell epitopes and influence their hierarchy. The molecular characteristics of dietary proteins after digestion are usually ignored although it is generally admitted that only a very small amount of the ingested protein is absorbed in adults. Furthermore, the induction of oral tolerance seems to require bioactive peptides of adequate sizes [17–20]. Therefore, identification of all potential B or T cell determinants on a given protein may require the use of several hydrolysis methods. We have investigated the presence of T cell determinants in nOVA fragments generated by CNBr hydrolysis. Our results show that the protein fragment corresponding to the 173–196 segment of nOVA stimulates nOVA-specific T cells

and induces oral tolerance with suppression of nOVA-specific IgE responses.

2. Materials and methods

2.1. Animals

Male Brown Norway and Wistar female rats were purchased from the CSEAL-CNRS (Orléans, FRANCE) and bred at the animal facility of the Centre de Recherche de Jouy-en-Josas, INRA. Parents and F1 hybrids (BWF1) rats were maintained under standard pathogen-free conditions and provided food and water ad libitum. Animals used in experiments were female BWF1 rats and weighed 125–160 g.

2.2. Immunizations and induction of oral tolerance

The antigens nOVA and KLH were purchased from Sigma (Saint Quentin Fallavier, France). Rats (four by groups) were immunized by subcutaneous injection at the base of the tail. The antigens were emulsified (v/v) in complete Freund's adjuvant containing 1 mg/ml of killed *Mycobacterium tuberculosis* strain H37Ra (Sigma) and rats were injected 75 µl of emulsion containing 200 µg of antigen. For oral tolerance studies, rats were immunized with alum as adjuvant well known for inducing high IgE responses. More specifically, groups of five rats received intragastrically by gavage injection, 1 ml of saline only (control group) or saline containing 25 mg of nOVA (0.555 mM), or 2 mg (0.673 mM) of the stimulating nOVA fraction. Ten days later, the rats injected intraperitoneally with 300 µl of saline containing 50 µg of nOVA adsorbed on 2 mg of alum. The extent of systemic unresponsiveness including IgE Abs was evaluated on sera from blood samples collected at day 20 (10 days after the systemic immunization) and at day 34 when the animals were sacrificed. All sera were kept at -70°C until analyzed.

2.3. Carboxymethylation and CNBr cleavage of OVA

The CNBr cleavage of nOVA (MW 45,000) was performed after reduction of disulfide bridges and

carboxymethylation (RCM-OVA) as previously reported [21]. Briefly, nOVA was dissolved in 6 M deionised urea, 0.36 M Tris–HCl buffer, 5.3 mM EDTA and 90 mM 2-mercaptoethanol. After 4 h of incubation at room temperature in the dark, 1 ml of 1.44 M iodoacetate was added to 7.5 ml of the solution and the reaction was allowed for 10 min. The carboxymethylated nOVA (RCM-OVA) was then dialyzed against HPLC quality water in the dark. Cyanogen bromide cleavage was performed on the RCM-OVA by adding 510 mg of protein to 50 ml 70% formic acid and 1.85 g CNBr (Sigma). This procedure yielded a CNBr/methionine molar ratio of 98. After overnight incubation in the dark at room temperature, the reaction was halted by addition of 50 ml HPLC quality water. The product of CNBr cleavage was then purified by evaporation and six further washes in a vacuum evaporator.

2.4. High-pressure liquid chromatography purification of OVA peptides

Separation of CNBr-derived peptides of nOVA was performed on a C-18 column (Nucleosil 170 × 4.5 mm, 5 µm porosity) under reversed-phase conditions and UV monitoring of eluates at 214 nm. The column was equilibrated with HPLC quality water containing 0.07% trifluoroacetate. One milligram of the CNBr hydrollysate of nOVA was dissolved in 6 M urea, injected into the column and eluted at a 1 ml/min flow rate with an increasing concentration gradient of acetonitrile, from 15% up to 50%. The fractions collected were dried by evaporation and washed in a vacuum evaporator. The sequence of peptides in some HPLC fractions was determined as previously reported [22]. Briefly, fractions were subjected to the first step of the Edman manual sequencing [23] in order to identify the NH₂ terminal amino acid. In this method, the phenyl-thio-hydantoin (PTH) derivatives were separated on a C-18 column (Spherisorb ODS.2, 150 × 4.6 mm, 3 µm porosity) at 40 °C. PTH derivatives were eluted (1 ml/min) with a discontinuous linear gradient of isopropanol and were detected at 269 nm. The full amino acid composition was performed after acidic hydrolysis (HCL 5.7 N, 24 h, 110 °C under vacuum) and amino acids were separated on a cation exchange chromatography column (BTC 2710, 200 × 3.2) using a biotronic LC-500

analyser. Automated Edman degradation was performed using an applied Biosystem Model 470 A sequencer [24]. PTHs were separated on a C-8 column (Superspher RP8 Merck, 250 × 4 mm, 4 µm porosity).

2.5. T cell proliferation assay

T cell proliferation assays were performed as previously reported [25] on inguinal lymph node (LN) cells isolated 10 days after immunization. LN were aseptically removed, teased and passed through a mesh to obtain a single cell suspension. Mononuclear cells were then washed twice in culture medium consisting of RPMI-1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum. Cells were resuspended at 5×10^6 viable cells/ml in RPMI supplemented culture media and 100 µl was dispensed into 96-well flat-bottomed microtiter plates. Antigens were added in a volume of 100 µl and the plates were incubated at 37 °C in a 5% CO₂ humidified atmosphere. In selected experiments, cell suspensions were enriched for T cell by passage through nylon wool column. The T cells were stimulated as indicated above in the presence of 10% gamma-irradiated (or mitomycin-treated) autologous spleen cells as antigen-presenting cells.

The monoclonal antibody MRC OX-6 directed against rat MHC class II molecules (Biosys, France), which blocks antigen presentation via MHC class II molecules, was added to selected cultures. After 2 days, the cultures were pulsed with 2.77×10^4 Bq of [³H]-thymidine (specific activity 185 GBq/mmol, Amersham, Saclay, France) in a volume of 20 µl/well. Cells were harvested 18 h later on glass filter papers (Nunc) and thymidine incorporation measured by using a liquid scintillation counter (Packard).

2.6. Bioassay for detection of IL-2 in culture supernatants

IL-2 production by nOVA-specific cells was evaluated by the ability of their culture supernatants to support the growth of the IL-2-dependent CTL-FD mouse cytolytic T lymphocyte line (a kind gift of C. leclerc, Pasteur Institute, France) [26]. Briefly, culture supernatants of nOVA-specific cells were collected after 24-h culture and 100 µl of cell-free su-

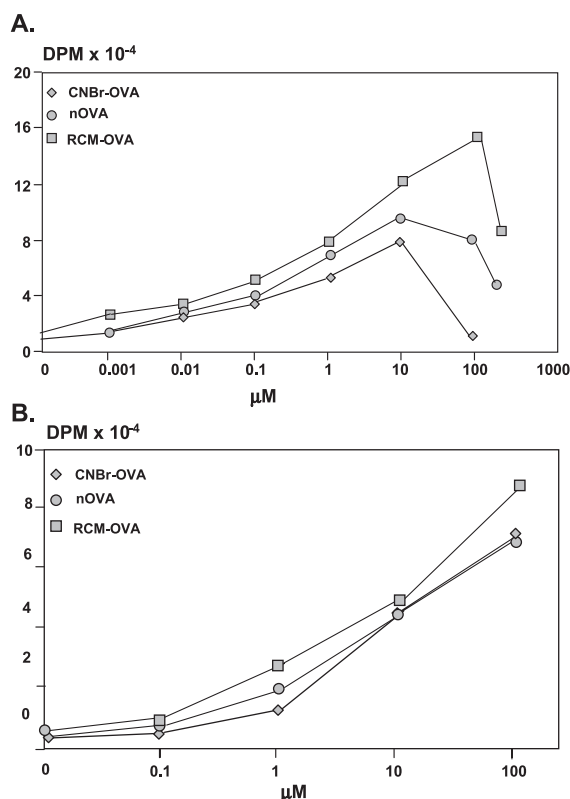


Fig. 1. Proliferative responses and IL-2 secretion by nOVA-specific T cells following in vitro restimulation with native nOVA, carboxymethylated OVA (RCM-OVA) or nOVA CNBr hydrolysate (RCM-CNBr-OVA). Inguinal lymph node cells were isolated from rats immunized 10 days earlier with nOVA. Triplicate cultures of cells from individual rats were restimulated in vitro with increasing doses of nOVA, RCM-OVA or RCM-CNBr-OVA. Panel A: Proliferative responses were determined by [³H]-thymidine incorporation after 3 days of cultures. Panel B: IL-2 secretion was evaluated by the ability of cell-free 24-h culture supernatants to maintain the growth of the IL-2-dependent CTL-FD cell line. Results are expressed as mean DPM of three rats and are representative of three separate experiments. In all these experiments, the standard deviation of [³H]-thymidine incorporation between triplicate cultures was lower than 20%.

pernatants was dispensed into flat-bottomed 96-well microtiter plates containing 2.25×10^4 CTL-FD cells/well. The plates were incubated for 24 h at 37 °C in a 5% CO₂ humidified atmosphere. Tritiated thymidine was then added to cultures and cells harvested 18 h later. In selected experiments, we measured proliferative responses of CTL-FD cells cultured with known amounts of IL-2. Consistent with previous

studies [26], stimulatory indices (DPM stimulated/DPM control unstimulated) of 10–12 were measured when CTL-FD cells were cultured in the presence of 250 mU/ml of IL-2.

2.7. Passive cutaneous anaphylaxis

For passive cutaneous anaphylaxis reactions, sera (100 μl) were injected intradermally in the shaved back of male Wistar rats [27]. Twenty four hours later, rats were injected 1 ml of saline containing 1% of Evans blue and the antigen nOVA (300 μg/animal) in the caudal vein. The animals were sacrificed 15 min

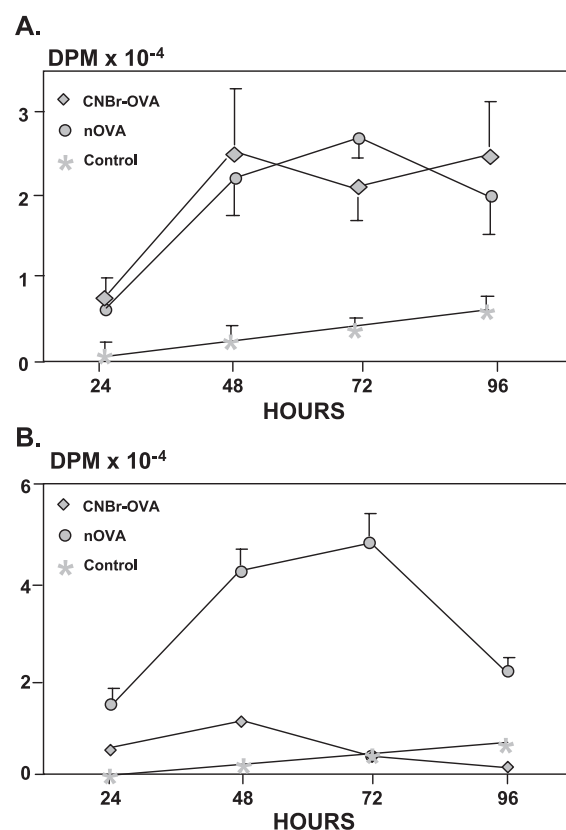


Fig. 2. Effect of antigen doses on the kinetic of nOVA-specific T cell proliferation in vitro. Inguinal lymph node cells from individual rats immunized with nOVA were restimulated in vitro with 1 μM (Panel A) or 100 μM (Panel B) of nOVA, or RCM-CNBr-OVA. Proliferative responses of triplicate cultures of antigen-stimulated and control unstimulated cells were measured after 24, 48, 72 or 96 h of culture. Results are expressed as mean DPM ± S.D. of three rats and are representative of three separate experiments.

Table 1

Inhibition of OVA-, RMC-OVA-, and RMC-CNBr-OVA-induced proliferation of OVA-specific T cells by anti-rat MHC class II antibody

Dose anti-MHC class II Ab (MRC OX-6)	Proliferative responses (DPM $\times 10^{-4}$)		
	nOVA (1 μ M)	RMC-OVA (1 μ M)	RMC-CNBr-OVA (1 μ M)
None	4.27 $\pm$ 0.86	4.05 $\pm$ 0.17	3.77 $\pm$ 0.26
1:5000	4.45 $\pm$ 0.29	3.83 $\pm$ 0.59	3.45 $\pm$ 0.57
1:1250	3.98 $\pm$ 0.86	3.61 $\pm$ 0.74	2.98 $\pm$ 0.68
1:625	3.48 $\pm$ 0.15	2.98 $\pm$ 0.53	2.71 $\pm$ 0.45
1:312	2.63 $\pm$ 0.23	2.29 $\pm$ 0.77	2.66 $\pm$ 0.93
1:156	1.97 $\pm$ 0.82	1.55 $\pm$ 0.42	1.06 $\pm$ 0.28

Inguinal lymph node cells from rats immunized with nOVA were restimulated for 3 days in vitro in the presence of graded doses of anti-MHC class II antibody. Proliferative responses were determined by [^{3} H]-thymidine incorporation during the last 24 h of cultures. Results are expressed as mean DPM $\pm$ S.D. and are representative of four rats.

later by cervical dislocation and PCA titers were determined as the last serum dilutions that induced a spot >5 mm diameter.

2.8. Statistics

Results were compared using an unpaired Student's two-tailed *t*-test and by the Mann–Whitney *U*-test of unpaired samples (**p* < 0.01; ***p* < 0.05).

3. Results

3.1. The carboxymethylated derivative and the whole OVA CNBr hydrolysate stimulate OVA-specific cells in vitro

We first investigated whether chemical modifications that resulted from carboxymethylation of nOVA affected the ability of this protein to stimulate proliferation of nOVA-specific T cells in vitro. It was also important to test whether the whole CNBr hydrolysate retained the stimulatory effect of nOVA on nOVA-specific cells. For this purpose, inguinal lymph node (LN) cells from rat immunized with nOVA were restimulated in vitro with nOVA, its carboxymethylated derivative (RCM-OVA) or the whole nOVA CNBr hydrolysate (RCM-CNBr-OVA). None of these stimuli affected the viability or stimulated the proliferation of LN cells from naïve rats or rats primed with the irrelevant protein KLH (data not shown). However, they were all able to stimulate proliferation of LN cells in a dose-dependent manner with characteristic bell-shaped curves (Fig. 1A). No difference was seen between proliferative responses of LN mononuclear cells and cell preparations enriched in T cells by passage through nylon column (data not shown). Reduced proliferative responses were seen with the 100 μ M dose CNBr-OVA and to a lower extent with

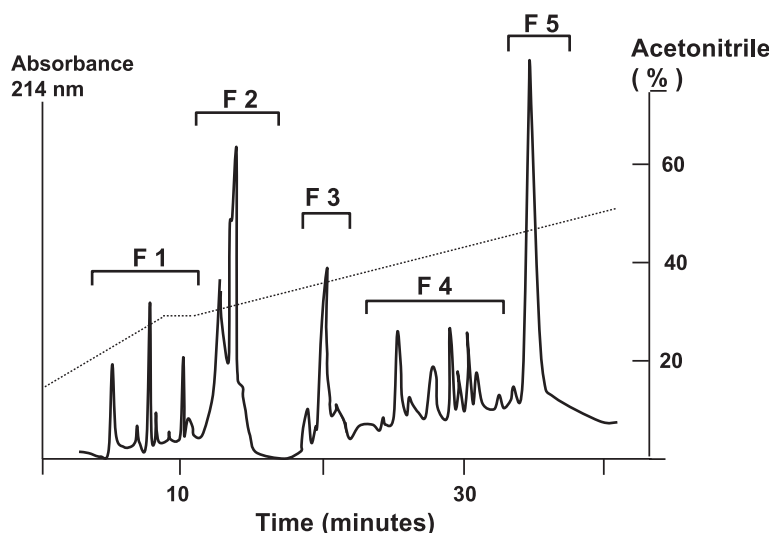


Fig. 3. Reversed-phase HPLC separation of RCM-CNBr-OVA fractions. The main fractions of nOVA fragments were separated using an increasing gradient of acetonitrile. The five fractions (F1 to F5) are indicated on the elution profile.

nOVA (Fig. 1A). However, higher doses (300 μ M) of nOVA or RCM-OVA were needed to inhibit the proliferative responses of OVA-specific cells in vitro.

The nOVA-specific proliferative responses were also associated with secretion of IL-2 by LN cells (Fig. 1B). Interestingly, IL-2 responses were not significantly different between the three groups despite the inhibitory effect seen with the high RCM-CNBr-OVA dose on the proliferative responses (Fig. 1A). Therefore, to rule out the possibility that different kinetics of T cell proliferation were achieved by low and high antigen concentrations, we analyzed the proliferative responses to low (1 μ M) and high (100 μ M) antigen doses 24 h after initiation of the cultures. Comparable levels of proliferation were seen when nOVA-specific cells were stimulated with 1 μ M of nOVA or RCM-CNBr-OVA (Fig. 2A). Cells stimulated with the 100 μ M of RCM-CNBr-OVA exhibited reduced proliferative responses when compared with cells activated with the same dose of nOVA (Fig. 2B). However, proliferative responses were significantly impaired when OVA-specific cells were stimulated with 300 μ M of nOVA (not shown).

We also asked if stimulation of OVA-specific cells by nOVA, RCM-OVA, or RCM-CNBr-OVA involved the same antigen presentation pathways through MHC class II molecules. This issue was addressed by analyzing the effect that the MRC OX-6 mAb directed against rat MHC class II molecules would have on the ability of nOVA, RCM-OVA, and RCM-CNBr-OVA to stimulate nOVA-specific T cells in vitro. Results in Table 1 show that the MRC OX-6 mAb inhibits nOVA-induced proliferation in a dose-dependent fashion. Similar results were obtained when the same dilutions of mAbs were added to LN cell cultures stimulated with 1 μ M RCM-OVA or RCM-CNBr-OVA.

3.2. HPLC separation and OVA-specific activity of cyanogen bromide-derived OVA peptides

Reversed-phase HPLC analysis of the CNBr-digested RCM-OVA showed a complex profile in which the peptides could be separated in 40 min using an acetonitrile gradient (Fig. 3). The effluent was fractionated into five fractions which were subsequently subjected to another round of chromatography to further remove the contaminating peptides from the other fractions. The fraction 3 (F3OVA) exhibited a

single peak while multiple peaks were seen in the F1 to F4 fractions. When CNBr-derived fractions were tested for their ability to stimulate nOVA-specific T cells, we found no significant stimulatory effect in fractions F1 and F2 (Fig. 4A). In contrast, the fractions F3, F4 and F5 promoted significant proliferative

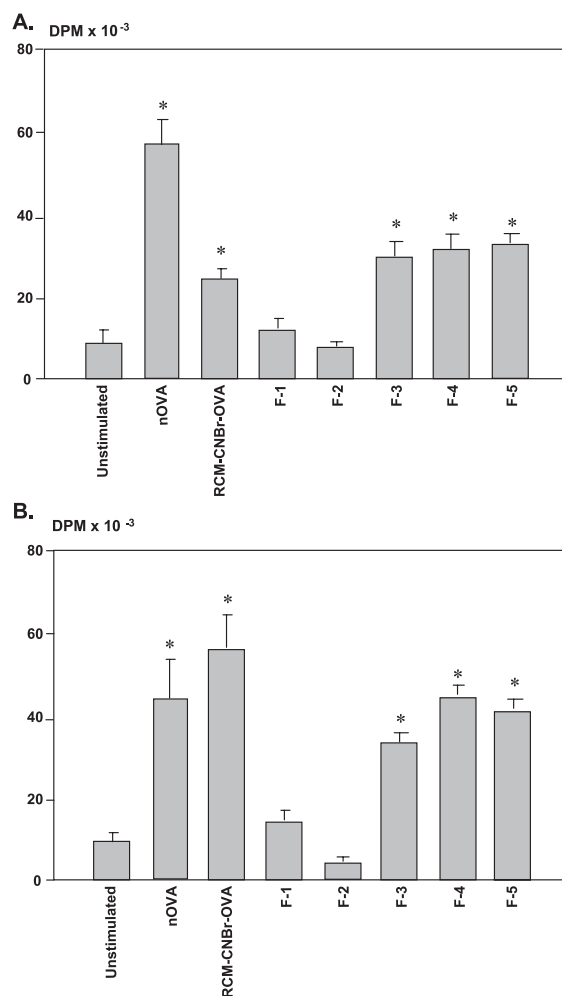


Fig. 4. Proliferative responses and IL-2 secretion by nOVA-specific T cells after in vitro restimulation with 10 μ M of nOVA, RCM-CNBr-OVA or nOVA HPLC-purified fractions. Proliferative responses by individual animals were measured by thymidine incorporation during the last 24 h of 3-day cultures (Panel A). Culture supernatants were collected after 24-h cultures for the evaluation of IL-2 secretion by the CTL-FD cell line bioassay (Panel B). All the results are expressed as mean DPM $\pm$ S.D. of individual rats and are representative of three separate experiments with three rats per group. *Significantly different ($p < 0.01$) from control unstimulated cultures as determined by Student's *t*-test.

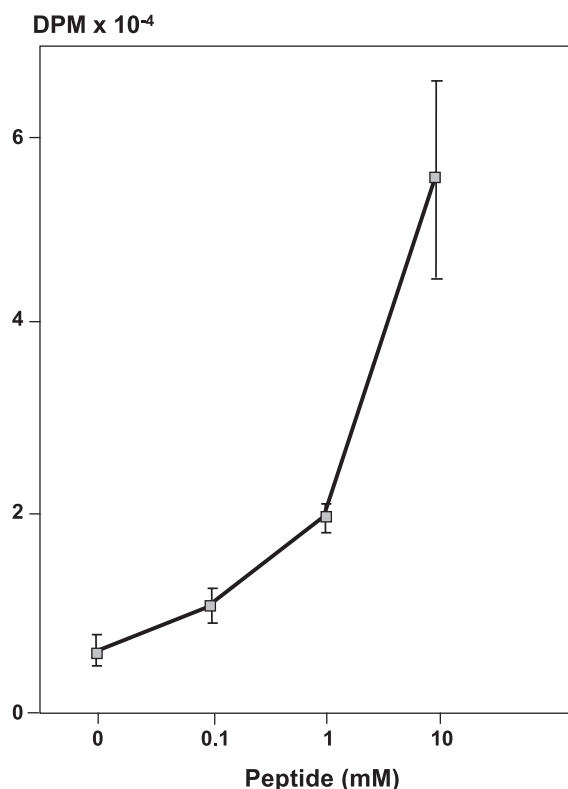


Fig. 5. Proliferative responses of nOVA-specific T cells after in vitro restimulation with various doses of F3OVA. Proliferative responses were measured by thymidine incorporation in triplicate cultures after 3 days of culture. Results are expressed as mean DPM $\pm$ S.D. of individual rats and are representative of three experiments with three rats per group.

responses which were of the same magnitude than those induced by the hole RCM-CNBr-OVA (Fig. 4A). These stimulatory fractions also promoted IL-2 secretion by nOVA-specific T cells (Fig. 4B), although these IL-2 responses did not completely mirror the pattern of proliferative responses.

3.3. A cyanogen bromide-derived nOVA peptide contains a new T cell determinant

In order to confirm that the stimulatory effect of fraction F3OVA on nOVA-specific T cells was due to the major peak seen in the elution profile, we further confirm this peak and grading doses were added to nOVA-specific T cell cultures. As seen in Fig. 5, the

product of this major peak stimulated nOVA-specific T cells in a dose-dependent fashion. We then analyzed the amino acid composition of this stimulatory fraction F3OVA. The first step of the Edman manual sequencing of one OD-absorbing peak showed a PTH corresponding to valine. The CNBr cleavage of nOVA produced only two peptides with a valine as the NH₂ terminal amino acid, namely peptide 41–172 and peptide 173–196 (MW 2971). The amino acid composition of the fraction F3OVA suggested that the peptide 173–196 was the main component of this fraction (Table 2). This result was confirmed by an automated Edman degradation which also indicated a slight contamination by the peptide P9–35 (<20% depending on the preparations). These findings suggest that the CNBr-derived 173–196 peptide is a new T cell determinant of nOVA.

Table 2

Amino acid composition of the major peptide in the stimulatory F3 fraction of OVA

Amino acid residues	Number of residues	
	Determined by amino acid analysis ^a	Corrected
N+D	2.9	3
T	1.0	1
S	0	0
E+G	3.6 ^b	3
P	0.1	0
G	1.0	1
A	2.9	3
C	0	0
V	2.2	3
M	0.0 ^b	1
I	0.7	1
L	1.9	2
Y	0.0	0
F	2.1	2
H	0.4	0
K	2.9	3
R	0.0	0

^a The amino acid composition was determined by cation exchange chromatography as described in the Materials and methods, after acidic hydrolysis. Note that the tryptophane does not survive the acidic hydrolysis.

^b High E+G value were obtained by the analysis of amino acid composition. This could be due to conversion of methionine into homoserine during CNBr hydrolysis and its subsequent detection as glutamic acid.

3.4. Oral treatment with the CNBr-derived [173–196] OVA peptide suppresses nOVA-specific T cell and IgE responses

To further demonstrate that the [173–196] nOVA fragment containing F3OVA fraction could regulate immune responses to nOVA *in vivo*, we tested its potential to induce oral tolerance. As expected, the proliferative responses to *in vitro* restimulation with nOVA was significantly reduced in LN cells isolated from rats that received an intragastric dose of nOVA prior to systemic immunization with nOVA (Fig. 6). Interestingly, LN cells from rat treated with a single intragastric dose of the [173–196] OVA peptide fraction F3OVA before intraperitoneal immunization with nOVA also showed reduced proliferation to *in*

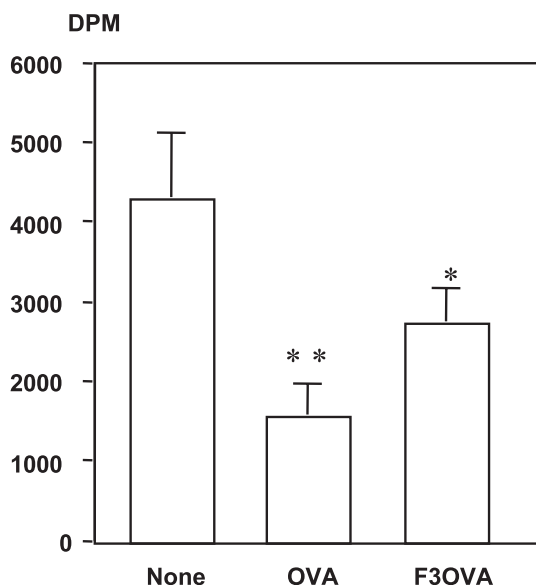


Fig. 6. Proliferative responses of nOVA-specific T cells from rat that received an intragastric dose of nOVA or derivatives. Groups of five rats received intragastrically by gavage injection 25 mg of nOVA in 1 ml saline or 2 mg of the fragment 173–196 containing F3OVA fraction in 1 ml saline or 1 ml saline alone (control group) and were parenterally immunized 10 days later with nOVA in alum (50 µg nOVA and 2 mg alum). Animals were sacrificed at day 34. Inguinal lymph node cells of individual animals were collected and separately restimulated *in vitro* for 3 days with nOVA. Proliferative responses were determined by [³H]-thymidine incorporation during the last 24 h of cultures. Results are expressed as mean DPM ± S.D. of five animals per group and are representative of three separate experiments (**p* < 0.01; ***p* < 0.05).

Table 3

Inhibition of OVA-specific IgE responses in rat intragastrically administered F3OVA fraction containing the 173–196 peptide

Oral treatment	Day 20		Day 34	
	PCA titers (log ₁₀)	Percent variation	PCA titers (log ₁₀)	Percent variation
None	1.925 ± 0.34	–	1.857 ± 0.43	–
nOVA	0.820 ± 0.24*	– 57.41	1.175 ± 0.38**	– 36.73
F3OVA	1.494 ± 0.22**	– 22.39	1.573 ± 0.32 ^{ns}	– 15.30

Groups of five rats were intragastrically administered by gavage 25 mg of nOVA in 1 ml saline, 2 mg of the fragment 173–196 containing F3OVA fraction in 1 ml saline, or 1 ml saline alone and parenterally immunized 10 days later with OVA in alum (50 µg nOVA and 2 mg alum). Individual serum was collected on day 20 and day 34 and IgE levels determined by PCA. Results are expressed as mean PCA titers ± S.D. and are from three separate experiments with five animals per group.

The percent of variation was calculated as follows:

$$100 - \left[\frac{\text{mean PCA titer in experimental group}}{\text{mean PCA titer in control group}} \times 100 \right]$$

ns: not significant.

**p* < 0.01.

***p* < 0.05.

in vitro restimulation with nOVA (Fig. 6), suggesting that F3OVA could promote oral tolerance to OVA.

The intraperitoneal immunization of rat with nOVA in alum promoted high IgE responses which were significantly inhibited by prior gavage injection of nOVA (Table 3). The groups of rats intragastrically administered the [173–196] nOVA fragment by gavage also showed significant suppression of anti-nOVA reagenic IgE responses on day 20 (Table 3). The rats intragastrically administered nOVA by gavage maintained low IgE levels at day 34 (Table 3). In contrast, IgE responses were only transiently impaired in rats treated with the [173–196] nOVA fragment and rose to reach levels not significantly different from those seen in control rats at day 34 (Table 3).

4. Discussion

Ovalbumin is a major allergen of hen egg white which frequently induces hypersensitivity symptoms among egg allergic individuals. The primary sequence and the secondary structure corresponding to the initial region of nOVA have been reported [28,29].

A number of studies have also been dedicated to establish nOVA peptides recognized by nOVA-specific IgE antibodies [7,30,31]. However, only limited reports are available on T cell epitopes contained in this major allergen. Similarly, few studies have been addressed to the use of nOVA peptides for induction of tolerance. We have investigated the ability of CNBr hydrolysis-derived nOVA peptides to stimulate nOVA-specific rat T cells and induce oral tolerance. Among the CNBr-derived nOVA fragments, we have identified the peptide 173–196 which displays stimulatory effects on nOVA-specific rat T cells. This fragment was also able to suppress both nOVA-specific T cell and IgE responses when orally administered to rat before immunization.

Carboxymethylation of nOVA was reported to generate a product with impaired ability to bind IgE and which exhibited only 20% of the RAST inhibition activity of the native protein [32]. Here, we report that the chemical changes after carboxymethylation of nOVA do not affect the ability of this molecules to stimulate nOVA-specific T cells. Thus, carboxymethylation of nOVA could affect recognition of B cell epitopes but not that of MHC class II-restricted T cells. A CNBr hydrolysate of nOVA was reported to sensitize target cells for nOVA-specific CTL lysis *in vitro* [33]. These authors have identified the nOVA 258–276 fragment as the key determinant for the cytolytic activity. We have now reported that the whole CNBr hydrolysate of nOVA can also stimulate MHC-restricted nOVA-specific T cells. Interestingly, proliferative responses of nOVA-specific T cells are inhibited by high concentrations of RCM-CNBr-OVA but not RCM-OVA or nOVA. The reasons for this discrepancy remain unclear, although inhibition of T cell proliferation but not IL-2 production by high antigen concentrations has been previously reported by others [34–36]. One could hypothesize that the inhibitory effect is not a unique feature of RCM-CNBr-OVA. Because of the large difference between the number T cell determinants exposed in the RCM-CNBr-OVA and RCM-OVA or nOVA, the inhibition observed with a 100 μ M concentration of RCM-CNBr-OVA only reflects what occur if cells were stimulated with higher concentrations of nOVA. In this regard, proliferative responses were also inhibited in cultures stimulated with ≥ 300 μ M of nOVA.

We have shown that three active peptide fractions could be separated by HPLC from the CNBr hydrolysate of nOVA. These results strongly suggest that several T cell-stimulating sites are exposed after CNBr hydrolysis. One of these sites could include the previously reported OVA 323–339 sequence [11,14]. In this regard, antigenic sites can be seen by T cells from multiple MHC types of several species [37]. Thus, the OVA 323–339 sequence which was reported to react with mouse [14] and human T cells [11] could also react with rat T cells. More importantly, we have characterized a single-peak HPLC-eluted fraction with stimulatory activity on T cells. This fraction contained the peptide P173–196 which could represent new T cell determinant in nOVA.

Peptides have been tested as tools for inducing oral tolerance to a number of antigens [6,17–20]. We have shown here that oral priming with the 173–196 peptide containing F3OVA fraction suppressed nOVA-specific T cell and IgE responses, suggesting that oral treatment with peptides could represent a potential strategy for prevention and/or treatment of egg allergy. In contrast with the oral tolerance induced by nOVA, a transient suppression of systemic immunity was induced by intragastric feeding of the F3OVA fraction. One can hypothesize that distinct processing and presentation pathways occur after intragastric gavage with nOVA and F3OVA. It is also possible that nOVA more efficiently promote oral tolerance via the involvement of several other T epitopes, in addition to F3OVA. Future studies will address experimental procedures that will help prolong the state of systemic unresponsiveness after oral treatment with whole protein or nOVA peptides such as the [173–196] fragment.

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