

Chemical Denaturation of Ovalbumin Abrogates the Induction of Oral Tolerance of Mouse Reaginic Antibody Responses

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The effect of chemical denaturation of ovalbumin (OVA) on the induction of oral tolerance of reaginic antibody responses was studied. Both urea-denatured OVA (UD-OVA) and carboxymethylated UD-OVA (CM-OVA) were purified by centrifugation. When compared with OVA and UD-OVA, CM-OVA had the least sensitizing capacity and allergenicity in IgE responses to OVA. BALB/c IgE, IgG1 and IgG antibody responses were suppressed by OVA, but not by UD-OVA or CM-OVA, fed prior to sensitization with OVA, UD-OVA, or CM-OVA in alum, respectively. The priming effect of specific IgG and IgG1 antibody responses was induced by CM-OVA fed prior to sensitization with OVA or CM-OVA. The proliferation of BALB/c spleen cells and their secretion of T helper type 2 (Th2) cytokines interleukin-4 (IL-4) and IL-5 were also orally tolerized by OVA, but not by denatured OVA. Although denatured OVA is hypoallergenic, the present result indicates that denaturation of a soluble protein prevents the induction of oral tolerance of Th2 responses.

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INTRODUCTION

Oral tolerance is the state of systemic immune hyporesponsiveness induced after feeding soluble proteins to unsensitized animals and humans [1–6]. The induction of oral tolerance may play an important role to prevent food allergy. Early studies have shown that the breakdown of oral tolerance may lead to hypersensitivity reactions to protein antigens [3–6]. Animal studies have further shown that oral autoantigens can suppress experimental autoimmune diseases [7–9]. This natural route of tolerance induction has been applied to clinical trials [10–13]. As the results of the clinical trials up till now are inconclusive, animal studies of oral tolerance induction are still needed.

Dietary proteins are usually heat-denatured. The secondary structure and allergenicity of denatured proteins may be changed due to different treatments. Because of the report that heat-treated cow's milk has decreased oral-sensitizing capacity in guinea-pigs [14, 15], denatured proteins have been recognized as hypoallergenic foods [14–16]. Since guinea-pigs develop lethal anaphylaxis to food proteins [14–16], murine studies of oral tolerance induction may be more helpful to understand human reactivity to dietary proteins. However, studies of effects of feeding denatured proteins to mice are few and controversial

[8, 17–20]. We have recently shown that feeding of denatured ovalbumin (OVA) fails to tolerize delayed-type hypersensitivity (DTH) and IgG antibody responses in mice [20]. Therefore, the effect of oral presentation of denatured OVA on reaginic antibody responses is further investigated.

MATERIALS AND METHODS

Animals. BALB/c mice (H-2d) and male Sprague–Dawley rats (350–450 g) were purchased from the Animal Centre, National Yang-Ming University (Taipei, Taiwan). They were maintained on a chicken egg-free diet (Lab diet; PMI feeds, St. Louis, MO, USA) in the Animal House of Veterans General Hospital-Taipei. Groups of three or six female mice were first used at 6–8 weeks of age.

Denaturation of ovalbumin. OVA (grade V) was purchased from Sigma (St. Louis, MO, USA). Urea-denatured OVA (UD-OVA) and carboxymethylated UD-OVA (CM-OVA) were prepared as described previously [20]. After extensive dialysis against distilled water, both UD-OVA and CM-OVA were precipitated when the pH of crude mixture was adjusted to 4.7. The pellets of denatured OVA washed four times with deionized water by centrifugation (12000 *g* for 30 min at 4°C) were suspended in 0.9% saline or lyophilized.

Oral administration of antigen. Native and denatured OVA were suspended in 0.9% saline (40 mg/ml) for feeding. Mice were fed 0.5 ml

of saline, or 20 mg native or denatured OVA as described previously [20].

Systemic sensitization. Mice were injected intraperitoneally (i.p.) with 10 µg OVA, UD-OVA, or CM-OVA adsorbed onto 4 mg alum and suspended in 0.5 ml of PBS. The day of systemic sensitization was referred to as day 0 and the days of treatments were given relative to it. Mice were bled 14 days after sensitization.

Assessment of specific IgG and IgG1 antibody responses. Serum-specific IgG and IgG1 antibody responses were assayed by an ELISA as previously described [20]. Briefly, 96-well Vinyl plates (Costar, Cambridge, MA, USA) were coated with OVA, UD-OVA, or CM-OVA (100 µg/ml) overnight at 4°C. After blocking, serum samples and standards (mouse IgG specific for OVA or sensitized sera specific for UD-OVA or CM-OVA) were incubated in duplicate for 6 h at room temperature. After washes, horseradish peroxidase-conjugated goat anti-mouse IgG or IgG1 (1/4000; SBA, Birmingham, AL, USA) were added and the plates were incubated overnight at 4°C. Colour development was performed and read at 492 nm as previously described [20].

Passive cutaneous anaphylaxis (PCA). Serum IgE antibody responses specific for OVA, UD-OVA, or CM-OVA were tritiated in triplicate by PCA reactions in male Sprague–Dawley rats. Serially double-diluted serum samples [1/5–1/1280] were injected intradermally (i.d.) in 0.1 ml volumes. All rats were challenged 48 h later by an intravenous (i.v.) injection of 1 ml of 0.9% saline solution containing 2 mg antigens and 5 mg Evans Blue. The reaction was read 30 min after challenge. The PCA titre was the highest dilution giving a positive reaction of at least 5 mm in diameter and expressed as means $\pm$ 1 SEM.

Spleen cell proliferative responses. Two weeks after sensitization, BALB/c spleen cells were prepared and cultured at 4×10^5 cells in 0.2 ml of RPMI-1640 with 10% fetal calf serum (Gibco, BRL, Grand Island, NY, USA) in 96-well flat-bottomed microtitre plates (Costar). OVA, UD-OVA, or CM-OVA in RPMI-1640 were added in triplicate (0.1 mg/ml). On day 4 of culture, 1 µCi of tritiated thymidine was added to each well for a further 16-h incubation. The cells were harvested and the total amount of thymidine incorporation was assessed using a β counter (Beckman, Fullerton, CA, USA).

Spleen cell production of interleukin-4 (IL-4) and IL-5 in vitro. BALB/c spleen cells collected as above were cultured in complete medium (5×10^6 cells/ml) with or without OVA (0.1 mg/ml) in 24-well flat-bottomed microtitre plates (1 ml/well; Costar). The supernatants were harvested after 4 days of culture, separated from cells by centrifugation at 7200 g for 5 min and store at -20°C until required. The production of IL-4 and IL-5 was assayed in duplicated by ELISA kits (Endogen, Cambridge, MA, USA).

Statistical analysis. Specific IgG and IgG1 antibody titres were presented as scatter plots with the median lines indicated. All group comparisons for specific IgG and IgG1 antibody responses were made using two-tailed Wilcoxon tests.

RESULTS

Sensitizing capacity and allergenicity of OVA, UD-OVA and CM-OVA

BALB/c mice (six per group) were sensitized twice with OVA, UD-OVA, or CM-OVA in alum, respectively, on day 0 and day 7. They were bled on day 14 and the sera collected from each group were pooled separately. Their allergenic reactions to OVA, UD-OVA and CM-OVA were assessed using PCA tests in

triplicate (Table 1). The sensitizing capacity of CM-OVA was much lower than that of OVA and UD-OVA. The mice sensitized with OVA and UD-OVA had decreased allergenic reactions to CM-OVA.

Feeding denatured OVA fails to induce oral tolerance of reagenic antibody responses to OVA

The effect of feeding denatured OVA prior to sensitization with OVA was investigated. Groups of mice (six per group) were fed 20 mg OVA, UD-OVA, or CM-OVA. The controls were fed saline only. They were sensitized with 10 µg OVA in alum 1 week later. Their reagenic antibody responses were assayed 2 weeks after sensitization (Fig. 1). Compared with controls, those fed denatured OVA showed no suppression of serum IgE, IgG1 and IgG antibody responses to OVA. In contrast, the group fed OVA showed suppression of IgG, IgG1 and IgE antibody responses to OVA. Moreover, the mice fed CM-OVA showed increased IgG and IgG1 antibody responses to OVA.

Feeding denatured OVA fails to induce oral tolerance of reagenic antibody responses to UD-OVA

The effect of feeding denatured OVA prior to sensitization with UD-OVA was further examined. Groups of mice (six per group) were fed 20 mg OVA, UD-OVA, CM-OVA, or saline only. One week later, they were sensitized with 10 µg UD-OVA in alum. Their reagenic antibody responses were assessed 2 weeks after sensitization (Fig. 2). Compared with saline-fed controls, those fed denatured OVA showed no suppression of IgE, IgG1 and IgG antibody responses to UD-OVA. In contrast, the group fed OVA showed profound suppression of IgG, IgG1 and IgE antibody responses to UD-OVA.

Feeding denatured OVA fails to induce oral tolerance of specific reagenic antibody responses to CM-OVA

The effect of feeding denatured OVA prior to sensitization with CM-OVA was also elucidated. Groups of mice (six per group) were fed 20 mg OVA, UD-OVA, CM-OVA, or saline only. One week later, they were sensitized with 10 µg CM-OVA in alum. Their reagenic antibody responses were tested 2 weeks after sensitization (Fig. 3). Compared with saline-fed controls, those

Table 1. Sensitizing capacity and allergenicity between native and denatured OVA

PCA reactions to sensitization	OVA	UD-OVA	CM-OVA
OVA/alum	1280 $\pm$ 0	640 $\pm$ 0	23 $\pm$ 11
UD-OVA/alum	1280 $\pm$ 0	1066 $\pm$ 369	40 $\pm$ 0
CM-OVA/alum	<2.5	13 $\pm$ 6	67 $\pm$ 18

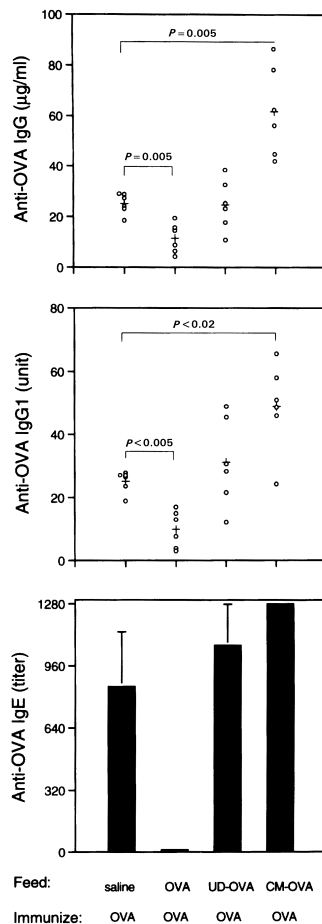


Fig. 1. Oral tolerance of reagenic antibody responses to OVA was induced by OVA, but not by UD-OVA or CM-OVA. Groups of six mice were fed 20 mg OVA, UD-OVA, CM-OVA, or saline only on day -7. They were sensitized with 10 µg OVA in alum on day 0 and their IgE, IgG1 and IgG antibody responses to OVA were tested 2 weeks later.

fed denatured OVA showed no suppression of IgE, IgG1 and IgG antibody responses to CM-OVA, but those fed CM-OVA showed enhancement of IgG and IgG1 antibody responses. In contrast, the mice fed OVA showed significant suppression of IgG and IgG1 antibody responses. Whereas the mice fed OVA or saline only showed negative PCA reactions to CM-OVA, those fed CM-OVA had weak positive PCA reactions to CM-OVA.

Feeding denatured OVA fails to tolerize T helper type 2 (Th2) cytokine production

Groups of mice (three per group) fed as above were sensitized 1 week later with 10 µg OVA, UD-OVA, or CM-OVA in alum. Their cytokine IL-4 and IL-5 production in spleen cell cultures was investigated 12 days after sensitization (Table 2). Compared with the control group, the group fed UD-OVA or CM-OVA did not show a reduction in spleen cell secretion of IL-4 and IL-5. In contrast, the mice fed OVA had decreased production of the Th2 cytokines IL-4 and IL-5.

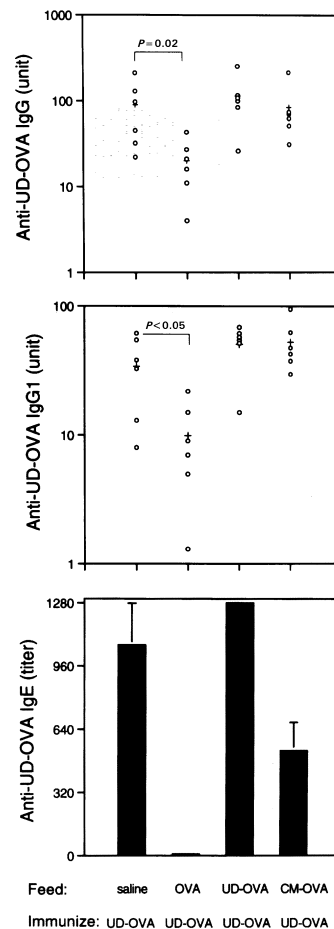


Fig. 2. Oral tolerance of reagenic antibody responses to UD-OVA was induced by OVA, but not by UD-OVA or CM-OVA. Groups of six mice were fed 20 mg OVA, UD-OVA, CM-OVA, or saline only on day -7. They were sensitized with 10 µg UD-OVA in alum on day 0 and their IgE, IgG1 and IgG antibody responses to UD-OVA were tested 2 weeks later.

Feeding denatured OVA fails to tolerize spleen cell proliferative responses

Groups of mice (three per group) fed as above were sensitized 7 days later with 10 µg OVA, UD-OVA, or CM-OVA in alum. Their spleen cell proliferative responses were tested 12 days after sensitization (Table 3). When compared with the control group, those fed denatured OVA did not have tolerance of spleen cell proliferation, but those fed OVA had decreased [^{3} H]thymidine uptake.

DISCUSSION

Consistent with our recent report that ingestion of chemically denatured OVA failed to suppress systemic DTH and IgG antibody responses [20], we further demonstrated here that mouse IgE and IgG1 antibody responses could be orally tolerized by OVA, but not by chemically denatured OVA. In addition, we also showed that spleen cell proliferation and production of Th2

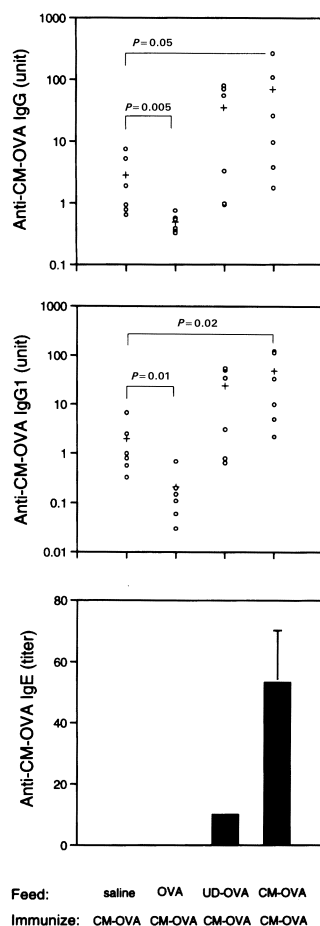


Fig. 3. Oral tolerance of reagenic antibody responses to CM-OVA was induced by OVA, but not by UD-OVA or CM-OVA. Groups of six mice were fed 20 mg OVA, UD-OVA, CM-OVA, or saline only on day -7. They were sensitized with 10 μ g CM-OVA in alum on day 0 and their IgE, IgG1 and IgG antibody responses to OVA were tested 2 weeks later.

cytokines were orally tolerized by OVA, but not by chemically denatured OVA. The present result suggests that the oral tolerogenicity of a protein is highly affected by its physicochemical structure.

It has been shown that denaturation of proteins leads to

Table 2. Ingestion of denatured OVA failed to suppress spleen cell production of IL-4 and IL-5 (pg/ml)

Feed	Sensitize	IL-4 secretion		IL-5 secretion	
		OVA*	Medium only	OVA*	Medium only
Saline	OVA	97.3	6.2	980.2	52.5
OVA	OVA	6.8	3.7	194.2	41.7
UD-OVA	OVA	78.7	5.5	541.0	45.3
CM-OVA	OVA	92.4	9.9	1835.2	98.6

*0.1 mg/ml of OVA.

Table 3. Ingestion of denatured OVA failed to suppress spleen cell proliferative responses to OVA

Feed (d -7)	Sensitize (d 0)	OVA* (d 12†; c.p.m.)	Medium only (d 12†; c.p.m.)	Stimulation index
<i>Exp. 1</i>				
Saline	OVA	32396	8978	3.6
OVA	OVA	7044	7672	0.9
UD-OVA	OVA	40121	9882	4.1
CM-OVA	OVA	34277	7854	4.5
<i>Exp. 2</i>				
Saline	UD-OVA	33769	8348	4.0
OVA	UD-OVA	11428	10740	1.1
UD-OVA	UD-OVA	38734	7791	5.0
CM-OVA	UD-OVA	36574	9330	3.9
<i>Exp. 3</i>				
Saline	CM-OVA	29393	10265	2.9
OVA	CM-OVA	7760	9236	0.8
UD-OVA	CM-OVA	30991	8035	3.9
CM-OVA	CM-OVA	32111	8734	3.7

*0.1 mg/ml of OVA.

† *In vitro* stimulation of spleen cells on day 12.

random-coiled aggregation and decreased solubility [21]. As denatured OVA could be precipitated at the pH near its isoelectric point, it was separated from residual OVA by centrifugation in this study. In contrast to a report that crude preparations of OVA denatured by 8 M urea or heated at 80°C for 10 min had little change in allergenicity [22], the present study agreed with other reports that denatured OVA had decreased allergenicity compared with OVA [23, 24]. These studies confirmed that denatured proteins were hypoallergenic. We further showed that denatured OVA had decreased sensitizing capacity. Moreover, our result that CM-OVA-sensitized sera had positive PCA reactions to CM-OVA, but not to OVA, suggests that CM-OVA may gain new B-cell epitopes during chemical denaturation.

The induction of oral tolerance has been explained by active suppression [25–27], anergy [9, 27, 28] and deletion [29]. Whereas some studies demonstrated that oral tolerance was regulated by preferential activation of Th2 cells with subsequent inhibition of Th1 responses by Th2 cytokines [27, 28], other studies showed that both Th1 and Th2 cytokines were suppressed [30]. Using alum as the adjuvant, we showed here that the production of Th2 cytokines could be orally tolerized by OVA. Such discrepancies might be due to different adjuvants, strains of mice and/or experimental protocols. It has been reported that the reduction of disulphide bonds plays a role in antigen processing [31, 32] and proteolysis [20, 32]. We found that denaturation played no role in oral tolerance induction. Our finding was contrary to reports that *i.v.* administration of denatured OVA could tolerize reagenic antibody responses [23, 24]. It is possible that antigen processing and presentation between oral and parenteral administration of denatured proteins are different.

Since oral proteins can tolerize mice and rats [3–9] but

sensitize guinea-pigs [15, 16], there is no dispute between our data and early reports that the oral sensitizing capacity of cow's milk in guinea-pigs could be minimized by heat treatment [15, 16]. Although the pathogenesis of food allergy is unclear, it is likely that abrogation of oral tolerance may predispose to food allergy. Similar to our recent report [20], priming effects were occasionally induced by feeding denatured OVA. This finding can be explained by the hypothesis that aggregated antigen particles are preferentially taken up through Peyer's patches and result in sensitization rather than tolerance [1]. Our data suggest that a denatured protein is not the right choice for the first contact with the gut. The phenomenon of oral tolerance has been attempted for clinical trials in allergic and autoimmune disorders [10–13], but the results are still not convincing. Our data suggest that denatured allergens play no role in oral immunotherapy. On the other hand, if systemic immunity is aimed, denatured proteins may play a role in the development of oral vaccine.

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