

Heat Denaturation of Egg-White Proteins Abrogates the Induction of Oral Tolerance of Specific Th2 Immune Responses in Mice

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Human foods are usually prepared by cooking. Boiling of chicken egg-white (EW) led to decreased allergenicity, and abrogated intestinal uptake of immunoreactive ovalbumin (OVA) when fed to mice. Therefore, the effects of oral administration of boiled EW were examined further in BALB/c mice. Specific IgE, IgG₁ and IgG antibody responses were suppressed by raw EW, but not by EW boiled for 5 or 60 min, fed prior to sensitization with 10 µg OVA or 1 µg DNP–OVA in alum. Similar results were obtained when mice were sensitized with 10 µg conalbumin, ovomucoid or lysozyme in alum. BALB/c spleen cell proliferation and secretion of Th2 cytokines IL-4 and IL-5 during *in vitro* stimulation with OVA were also suppressed by feeding raw EW, but not by boiled EW. Although heat denaturation of proteins can minimize allergenicity, the present results suggest that over-cooking of proteins may affect their intestinal antigen processing and thus prevent the induction of oral tolerance.

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INTRODUCTION

Oral tolerance is the state of systemic immune hyporesponsiveness induced after feeding soluble protein antigens to unsensitized animals and humans [1–3]. It is a physiological defence mechanism that maintains the homeostasis between the body and a great variety of food antigens. Early studies have shown repeatedly that the breakdown of oral tolerance may lead to hypersensitivity reactions to dietary protein antigens [4, 5]. Although the pathogenesis of food allergy is unclear, it is possible that factors abrogating the induction of oral tolerance may predispose to food allergy. Dietary proteins are usually prepared in heat-denatured and aggregated forms. Owing to different heat treatments, there are physico-chemical discrepancies in the structure of heated proteins [6, 7]. Because of the reports that boiled and evaporated cow's milk have a much decreased oral-sensitizing capacity in guinea-pigs [8, 9], heat treatment has been recognized as a simple way of reducing allergenicity [9, 10]. However, severe food hypersensitivity reactions are also described in piglets and calves fed heated soybean proteins [11, 12].

Mice are most commonly used in studies of oral tolerance, but

previous murine studies of the oral tolerogenicity of heated proteins are few and controversial. The oral tolerogenicity of heated proteins was demonstrated after prolonged feeding of whey proteins prepared at 90°C for 1 h [13], but was questioned by two other reports [14, 15]. One reported that collagen-induced arthritis could be suppressed by feeding soluble type II collagen, but not type II collagen heated at 56°C for 45 min [14]. The other reported that deaggregated human r-globulin was better than human r-globulin heat aggregated at 63°C for 25 min in the induction of oral tolerance [15]. Such discrepancies may be due to different protein antigens and their varying susceptibility to different heat treatments. Chicken eggs are the most common food and contain several food allergens such as ovalbumin (OVA), ovomucoid, conalbumin and lysozyme [16]. We have recently shown that chemical denaturation of OVA can prevent the induction of oral tolerance of Th1 and Th2 immune responses in mice [17, 18]. Therefore, the effects of heat denaturation of egg-white (EW) proteins on the induction of oral tolerance are investigated further.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased from the Animal Center,

National Cheng-Kung University (Tainan, Taiwan). Male Sprague–Dawley rats (350–450 g) were purchased from the Animal Center, 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 (Taiwan). Groups of three or six mice were first used at 7–9 weeks of age.

Protein antigens. Ovalbumin (OVA) and ovomucoid were obtained from Calbiochem (San Diego, CA, USA). Conalbumin (type I), lysozyme, human serum albumin (HSA) and dinitrophenyl HSA (DNP₃₅-HSA) were obtained from Sigma (St. Louis, MO, USA). Dinitrophenyl OVA (DNP₅-OVA) was prepared by overnight reaction of 100 mg OVA in 10 ml of 0.15 M potassium carbonate with 20 mg dinitrobenzene sulfonate (Aldrich Chemical Co, Milwaukee, WI, USA) at 4°C and dialysed against phosphate-buffered saline (PBS). Chicken eggs were purchased from the supermarket.

Oral administration of EW proteins. Mice were fed 0.5 ml of distilled water or 50 mg lyophilized EW, or EW boiled for 5 or 60 min, lyophilized and suspended, respectively. As EW contains ≈ 51% OVA, 12% conalbumin, 11% ovomucoid and 3.5% lysozyme [16], each mouse ingested ≈ 25 mg OVA, 6 mg conalbumin, 5.5 mg ovomucoid and 1.8 mg lysozyme. In some experiments, mice were fed 20 mg of native or boiled (60 min) OVA, ovomucoid or lysozyme suspended in 0.5 ml of distilled water.

Assessment of serum immunoreactive OVA. Mice were bled 1 h after feeding 50 mg raw EW or EW boiled for 5 or 60 min. Serum levels of immunoreactive OVA were examined by a sandwich ELISA as described previously [17]. Briefly, 96-well vinyl plates were coated with 5 µg/ml rabbit anti-OVA IgG at 4°C. After blocking and washing, serum samples (1/5) and standards (0–100 ng/ml OVA) were incubated for 8 h at room temperature. After washing, biotinylated rabbit anti-OVA IgG was added and the plates were incubated overnight at room temperature. After washing, horseradish-peroxidase (HRP)-conjugated streptavidin (1/1000; Amersham, Buckinghamshire, UK) was added for 1 h. After washing, orthophenylenediamine (0.5 mg/ml) was added and the plates were incubated in the dark. Colour development was stopped by 4 N H₂SO₄. Absorbance at 492 nm was read using a reader (SPEC-TRAmx 250; Molecular Devices, Sunnyvale, CA, USA) and the unknowns were interpolated.

Sensitization. Mice were injected i.p. with 1 µg DNP-OVA or 10 µg OVA, conalbumin, ovomucoid, lysozyme or HSA adsorbed onto 4 mg alum, respectively, and suspended in 0.5 ml of PBS. The day of sensitization is referred to as day 0 and the days of treatment are given relative to it.

Assessment of specific IgG and IgG₁ antibody responses. Mice were bled on day 14 and serum-specific IgG and IgG₁ antibody responses were assayed by ELISA as described previously [17, 18]. Briefly, 96-well vinyl plates were coated with 100 µg/ml OVA, DNP-HSA, lysozyme, conalbumin or ovomucoid in 0.05 M carbonate buffer, pH 9.6 (100 µl/well) overnight at 4°C. After blocking, serum samples (1/400 and 1/1000) and standards (1/100–1/12800 diluted mouse sera collected 1 week after monthly sensitization with 10 µg OVA, DNP-OVA, conalbumin, lysozyme or ovomucoid in alum, respectively, for 3 months) were incubated in duplicate for 5 h at room temperature. After washing, HRP-conjugated goat anti-mouse IgG or IgG₁ (1/4000; SBA, Birmingham, AL, USA) was added and the plates were incubated overnight at 4°C. Colour development was read as above. Samples were interpolated as arbitrary units.

Passive cutaneous anaphylaxis (PCA). Specific IgE antibody responses were assessed in triplicate by PCA tests in male Sprague–Dawley rats. First, 100 µl of twofold diluted pooled mouse serum

samples (1/2.5–1/1280) were injected i.d. into rats. They were challenged 48 h later by an i.v. injection of 1 ml of PBS containing 2 mg antigens and 5 mg Evans Blue. The reaction was read 30 min after the challenge. The PCA titre was the highest dilution giving a positive reaction of at least 5 mm in diameter. Results are expressed as mean titres ± 1 SEM.

Spleen cell proliferative responses. Two weeks after sensitization with 10 µg OVA in alum, BALB/c spleen cells were prepared and cultured (4×10^5 cells/well) in triplicate in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2-ME, sodium pyruvate, glutamine, nonessential amino acids, hepes and antibiotics) with or without OVA (100 µg/ml) in 96-well flat-bottomed microtitre plates (0.2 ml/well; Costar). Spleen cell cultures were then pulsed with 1 µCi/well of tritiated thymidine (Amersham) on day 4 for further 16 h. The cells were harvested and the total amount of thymidine incorporated was assessed using a β counter.

Spleen cell production of IL-4 and IL-5 in vitro. BALB/c spleen cells collected as above were cultured (5×10^6 cells/well) with or without OVA (100 µg/ml) in 24-well flat-bottomed microtitre plates (1 ml/well; Costar). The supernatants were harvested after 1–5 days of culture, separated from cells by centrifugation at 7200 g for 5 min and stored at –20°C until required. The production of IL-4 and IL-5 was assayed in duplicate using sandwich ELISA kits (Endogen, Cambridge, MA, USA).

Statistical analysis. Serum concentrations of OVA and specific IgG₁ and IgG antibody titres were expressed as means ± 1 SD. All group comparisons for specific IgG and IgG₁ antibody responses were made using two-tailed Mann–Whitney U-tests.

RESULTS

Hypoallergenicity of boiled egg-white proteins

The highest dilution of sequentially twofold-diluted sensitized mouse serum generating a positive PCA reaction to unheated EW proteins or EW proteins boiled for 5 or 60 min was evaluated in triplicate (Table 1). Compared with unheated EW proteins, boiled EW proteins showed a much-decreased allergenicity, especially after prolonged boiling for up to 60 min.

OVA absorption after ingestion of boiled EW

BALB/c serum concentrations of immunoreactive OVA measured 60 min after feeding 50 mg EW or EW boiled for 5 or

Table 1. Allergenicity of native and heat-treated EW proteins

PCA reactions to EW proteins:	Native	Boiled for 5 min	Boiled for 60 min
OVA	1280 ± 0	80 ± 0	< 3
conalbumin	853 ± 284	40 ± 0	13 ± 4
ovomucoid	533 ± 142	27 ± 9	12 ± 6
lysozyme	640 ± 0	20 ± 0	7 ± 2

The PCA titres of sensitized mouse serum challenged in quadruplicate with either native EW proteins or EW proteins boiled for 5 or 60 min, respectively.

Table 2. Immunoreactive OVA (ng/ml) in mouse serum collected 60 min after feeding 50 mg EW or EW boiled for 5 or 60 min

Feed EW	Not heated	Boiled for 5 min	Boiled for 60 min
ELISA for native OVA	151.2 ± 108.7	1.5 ± 2.1	ND

ND, not detected.

60 min (six/group) were assessed by ELISA. Immunoreactive OVA was detected after feeding raw EW (151.2 ± 108.7 ng/ml) and EW boiled for 5 min (1.5 ± 2.1 ng/ml), but not after feeding EW boiled for 60 min (Table 2).

Ingestion of boiled EW failed to induce oral tolerance of specific reagenic antibody responses to OVA or DNP

Whether ingestion of boiled EW could induce oral tolerance to OVA was studied first. Groups of six BALB/c mice were fed 50 mg EW, or EW boiled for 5 or 60 min, respectively. The controls were fed distilled water. Seven days later, they were sensitized with 10 µg OVA in alum or 1 µg DNP-OVA in alum, respectively. Their serum-specific reagenic antibody responses were tested 14 days after sensitization (Table 3). Compared with controls, those mice fed EW boiled for 60 min failed to show oral tolerance of specific IgG, IgG₁ and IgE antibody responses to OVA or DNP. In contrast, the group fed EW showed profound suppression of IgG, IgG₁ and IgE antibody responses to OVA or DNP ($P < 0.005$). Those fed EW boiled for 5 min showed slightly decreased IgE antibody responses to OVA or DNP, but did not

show significant suppression of IgG and IgG₁ antibody responses to OVA or DNP.

Ingestion of boiled EW failed to induce oral tolerance of spleen cell proliferation and Th2 cytokine production

The effects of ingestion of boiled EW on lymphocyte proliferation and Th2 cytokine secretion *in vitro* were examined further. Groups of three BALB/c mice were fed distilled water only, 50 mg EW or EW boiled for 5 or 60 min, respectively. They were sensitized with 10 µg OVA in alum 1 week later. Two weeks after sensitization, their spleen cells were collected and cultured with or without OVA *in vitro*.

Compared with spleen cells collected from mice fed boiled EW or distilled water only, spleen cells collected from mice fed raw EW showed weak proliferative responses when stimulated with OVA *in vitro* (Fig. 1). However, the proliferative response was not observed when spleen cells were cultured without OVA.

When spleen cells were stimulated with OVA *in vitro*, the production of IL-4 and IL-5 was much decreased in the group fed EW, but not in those fed distilled water only or EW boiled for 60 min (Fig. 2). The production of IL-4 and IL-5 in the group fed EW boiled for 5 min was lower than that in the group fed distilled water only or EW boiled for 60 min, but higher than that in the group fed EW. IL-4 and IL-5 were not detected when spleen cells were cultured without OVA (data not shown).

Feeding of boiled EW failed to induce tolerance of mouse reagenic antibody responses to ovomucoid, conalbumin and lysozyme

Whether feeding boiled EW could induce tolerance of reagenic antibody responses to other EW proteins was also studied.

Table 3. Ingestion of boiled egg-white (EW) failed to induce oral tolerance of reagenic antibody responses to OVA and DNP-OVA

Feed (d-7)	Immunization (d0)	IgG (d14)	IgG ₁ (d14)	IgE (d14)
Expt 1	OVA/alum	Anti-OVA antibody responses		
Water		64.8 ± 27.3	49.5 ± 23.6	1067 ± 356
EW		7.3 ± 2.6*	6.4 ± 2.1*	10 ± 0
EW boiled for 5 min		45.7 ± 26.2	38.6 ± 20.4	67 ± 18
EW boiled for 60 min		58.4 ± 41.3	56.1 ± 39.4	1280 ± 0
Expt 2	DNP-OVA/alum	Anti-DNP antibody responses		
Water		311.4 ± 130.1	364.7 ± 176.1	640 ± 0
EW		37.6 ± 12.4*	43.6 ± 24.4*	5 ± 0
EW boiled for 5 min		193.8 ± 120.5	254.7 ± 131.1	267 ± 71
EW boiled for 60 min		358.7 ± 196.2	429.4 ± 183.6	853 ± 284

Groups of six mice fed 0.5 ml of distilled water, 50 mg EW, or EW boiled for 5 or 60 min, were sensitized with 10 µg OVA/alum or 1 µg DNP-OVA/alum 1 week later. Two weeks after sensitization, their serum-specific antibody responses were assessed and expressed as mean titres ± 1 SD.

* $P < 0.005$.

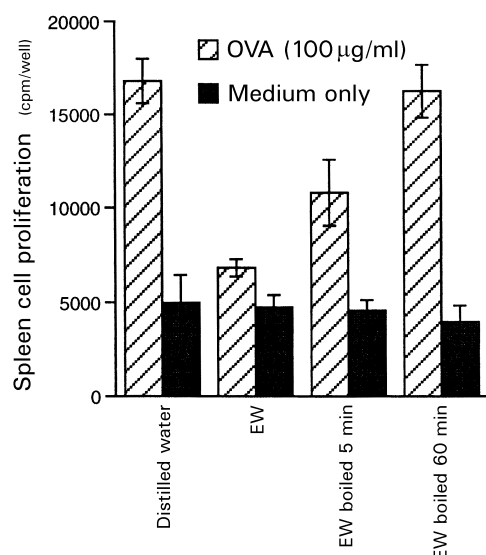


Fig. 1. Ingestion of boiled EW failed to tolerize spleen cell proliferative responses to OVA. Groups of three mice fed distilled water only, 50 mg EW or EW boiled for 5 or 60 min, were sensitized with 10 µg OVA in alum 1 week later. Their splenic lymphocyte proliferative responses to OVA *in vitro* (100 µg/ml) were assessed 2 weeks after sensitization and are expressed as mean cpm ± SEM of triplicate cultures.

Groups of six BALB/c mice were fed 50 mg EW or EW boiled for 5 or 60 min, respectively. The controls were fed distilled water only. One week later, they were sensitized with 10 µg ovomucoid, conalbumin or lysozyme in alum, respectively. Their antibody responses were tested 2 weeks after sensitization.

Compared with controls, those fed EW showed oral tolerance of specific IgG ($P = 0.02$, $P < 0.005$, $P < 0.005$, respectively), IgG₁ ($P = 0.005$, $P < 0.005$, $P < 0.005$, respectively) and IgE antibody responses (Table 4). Those fed EW boiled for 5 min showed a decrease in specific IgE antibody responses to ovomucoid, those fed EW boiled for 5 or 60 min showed no suppression of specific IgG, IgG₁ and IgE antibody responses to conalbumin, ovomucoid or lysozyme, respectively.

Further studies revealed that oral tolerance of specific reagenic antibody responses was induced in mice fed 20 mg OVA, ovomucoid or lysozyme, but not in mice fed EW proteins boiled for 60 min (data not shown). In addition, groups of BALB/c mice fed 0.5 ml of distilled water or 50 mg lyophilized EW were sensitized with 10 µg OVA, ovomucoid, conalbumin, lysozyme or HSA in alum, respectively. Whereas oral tolerance to EW proteins was found in EW-fed mice, oral tolerance to HSA was not observed in EW-fed mice (data not shown).

DISCUSSION

Consistent with our recent reports that the oral administration of chemically denatured OVA failed to suppress Th1 and Th2 immune responses [17, 18], we further show here that mouse reagenic antibody responses and Th2 cytokine production could

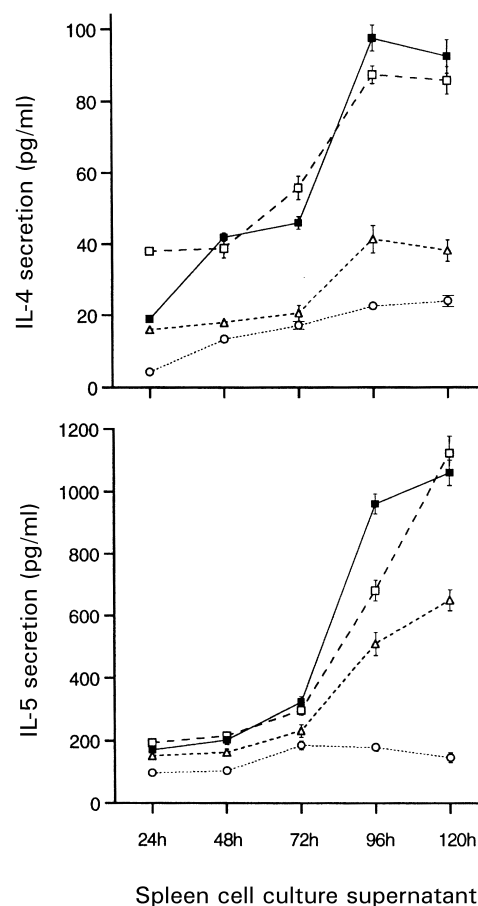


Fig. 2. Ingestion of boiled EW failed to tolerize Th2 cytokine production. Groups of three mice fed distilled water only, 50 mg EW or EW boiled for 5 or 60 min, were sensitized with 10 µg OVA in alum 1 week later. Two weeks after sensitization, their splenic lymphocyte secretion of IL-4 and IL-5 [after 1–5 days of culture in the presence OVA *in vitro* (100 µg/ml)] were determined by ELISA kits and are presented as mean cytokine concentrations ± SEM of supernatants. (■), saline; (○), EW; (Δ), EW boiled 5 min; (□), EW boiled 60 min.

be orally tolerized by raw EW proteins, but not by boiled EW proteins. This report suggests that heat denaturation of proteins may abrogate the induction of oral tolerance. Human foods are usually prepared by heat treatment. Since over-cooking is unfavourable for the induction of oral tolerance, it is likely that avoidance of extensive heat denaturation is needed to preserve the oral tolerogenicity of proteins when first encountered by the gut.

Heat denaturation of globular proteins may disrupt their tertiary structure and lead to random-coiled aggregation and insolubility [6]. Using gel filtration, we found that chemically denatured OVA were mainly fractionated over the void volume, but not over the same fractions of OVA [17]. Similarly, heat-denatured OVA were eluted at a position corresponding to high molecular weight polymers [7]. Whereas an early report showed that OVA denatured by heating at 80°C for 10 min had little

Table 4. Ingestion of boiled egg white failed to induce oral tolerance of reagenic antibody responses to conalbumin, ovomucoid and lysozyme

Feed	Immunization	IgG	IgG ₁	IgE
Expt 3	conalbumin/alum	anticonalbumin antibody responses		
Water		48.4 ± 13.4	43.3 ± 10.4	640 ± 0
EW		17.2 ± 11.8*	14.5 ± 9.2**	< 3
EW boiled for 5 min		30.9 ± 13.2	33.8 ± 13.2	320 ± 0
EW boiled for 60 min		43.5 ± 19.8	39.2 ± 17.9	533 ± 142
Expt 4	ovomucoid/alum	antiovomucoid antibody responses		
Water		492.9 ± 119.5	538.9 ± 152.0	533 ± 142
EW		29.8 ± 17.4***	31.9 ± 26.7***	8 ± 2
EW boiled for 5 min		426.2 ± 242.4	478.5 ± 171.9	67 ± 18
EW boiled for 60 min		564.5 ± 247.1	567.1 ± 201.0	427 ± 142
Expt 5	lysozyme/alum	antilysozyme antibody responses		
Water		434.1 ± 97.1	453.8 ± 118.5	640 ± 0
EW		86.2 ± 65.7***	108.6 ± 48.7***	4 ± 1
EW boiled for 5 min		333.7 ± 89.5	365.0 ± 145.6	267 ± 71
EW boiled for 60 min		391.9 ± 62.2	465.6 ± 103.0	640 ± 0

Groups of six mice fed 0.5 ml of distilled water, 50 mg EW, or EW boiled for 5 or 60 min were sensitized with 10 µg conalbumin, ovomucoid or lysozyme in alum 1 week later. Two weeks after sensitization, their serum antibody responses were assessed and expressed as mean titres ± 1 SD.

* $P = 0.02$; ** $P = 0.005$; *** $P < 0.005$

change in allergenicity [19], we found that the allergenicity of OVA and other EW proteins could be minimized after intensive heat denaturation. Although boiled EW proteins had reduced allergenicity, our data revealed that the oral tolerogenicity was also minimized.

The induction of oral tolerance has been extrapolated by mechanisms including active suppression [20–22], clonal anergy [22, 23] and deletion [24]. Whereas oral tolerance of Th1 cytokines has been shown in mice immunized with complete Freund's adjuvant [23], here oral tolerance of Th2 cytokines was shown when alum was used as adjuvant. Studies of conventional antigen processing showed that T-cell receptors recognized bimolecular ligands composed of processed peptides and self Ia molecules on the surface of antigen-presenting cells [25]. However, it is not known for the intestinal antigen processing underlying the induction of oral tolerance. A denatured protein may lose conformational epitopes [7, 26, 27], but its amino acid sequence is intact and can trigger T-cell proliferation [26, 27]. Whereas the denaturation of proteins may play a role in conventional antigen processing [28, 29], our studies have consistently shown that it prevents the induction of oral tolerance [17, 18].

Absorption of minute amounts of fed proteins has been described repeatedly [17, 30]. Although the effects of uptake of oral antigens are unclear, it has been postulated that the induction of oral tolerance is related to the intestinal permeation of monomeric forms of oral antigens from immuogenic aggregates [2, 15]. The intestinal antigen-presenting cells may process absorbed monomeric proteins and generate tolerogens [31].

Using gel filtration followed by ELISA, we have shown that both the charge characteristics and molecular weight of gut-absorbed OVA are indistinguishable from native OVA [30]. Using polyclonal antibodies specific for native or chemically denatured OVA, we have also shown that native OVA, but not chemically denatured OVA, is the unique molecular species in OVA-fed mouse serum [17]. Similarly, OVA was not detected in the mouse serum after ingestion of EW boiled for 60 min. On the one hand, it is possible that slow uptake of aggregated antigens may result in more extensive degradation by luminal enzymes and generation of tiny nontolerogenic fragments [2, 17]. On the other hand, if denatured proteins are really absorbed, it is also possible that the intestinal antigen processing between native and aggregated denatured antigens may be different.

The animal species used are important in oral tolerance studies. Feeding proteins usually induces tolerance in adult mice and rats [1, 2], but sensitizes adult guinea-pigs [8, 9]. There is no dispute between our studies and early reports that the oral-sensitizing capacity of cow's milk in guinea-pigs could be abrogated by heat treatment [8, 9]. Moreover, there is no report of the oral tolerogenicity of denatured proteins in guinea-pigs. Clinically, food allergy is most common in infancy [1]. A recent study has shown that adult volunteers can develop oral tolerance to keyhole limpet haemocyanin [3]. Contrary to adult mice which are prone to oral tolerance induction [17, 18, 20–24], feeding OVA to neonatal mice not only fails to induce oral tolerance, but may lead to sensitization [5]. Indeed, immaturity of the immune system may be responsible for the absence of oral tolerance in

neonatal mice and infants [5, 32, 33]. It is possible that abrogation of oral tolerance may predispose to food allergy. Therefore, early exposure of infants to extensively heat-denatured food proteins may be undesirable and needs further evaluation.

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