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Denaturation of Ovalbumin Abrogates Oral Induction of Airway Hyperreactivity and IgG1, IgG2 Antibody Responses in Guinea Pigs

Abstract

Background: The effects of denaturation of ovalbumin (OVA) on the induction of oral sensitization in guinea pigs were examined. **Methods:** Guinea pig antibody and airway responses were assessed after 10 feedings of chemically or heat-denatured OVA or egg white (EW). **Results:** Their specific IgG, IgG1 and IgG2 antibody responses were orally sensitized by OVA or EW, but not by chemically or heat-denatured OVA or EW. When further exposed to 0.1% OVA or conalbumin aerosol, those fed OVA or EW, but not denatured OVA or EW, had increased pulmonary resistance and decreased tidal volume. On the other hand, in those fed denatured OVA, boiled EW or saline only, a second sensitization with 1% OVA aerosol generated antibody responses and airway hyperreactivity. Using a sandwich ELISA, guinea pig serum OVA was detected after feeding EW, but not chemically denatured or boiled EW. **Conclusions:** It is likely that guinea pig gut absorption of OVA may result in oral sensitization. Chemical or heat denaturation of proteins may minimize their intestinal uptake and thus abrogates the induction of oral sensitization in guinea pigs.

Key Words

Denaturation
Ovalbumin
Oral sensitization
Airway hyperreactivity
Guinea pigs

Introduction

Oral tolerance is the state of systemic immune hyporesponsiveness induced after feeding soluble proteins to unsensitized mice and rats [1, 2]. It is a physiological defense mechanism to maintain the homeostasis between the body and a great variety of food antigens. In contrast, rabbits, calves and guinea pigs generate antibody responses to dietary proteins [3–7]. They have been implicated in the pathogenesis of rheumatoid-like lesions [3], food allergy [4–7] and the sudden infant death syndrome [6, 7]. As guinea pigs develop fatal anaphylaxis to food proteins, they are widely used as an animal model for food allergy [5–7].

Foods are usually prepared in heat-denatured forms, but the effects of ingestion of denatured proteins are still un-

clear. We have recently shown that chemical or heat denaturation of ovalbumin (OVA) can abrogate the induction of oral tolerance in mice [8–10]. Whereas some reports revealed that piglets and calves fed heated soybean proteins developed severe intestinal hypersensitivity and antibody responses [11, 12], other reports showed that heat treatment of cow's milk could minimize oral anaphylactic sensitization in guinea pigs [13–16]. Such discrepancies may be due to different species of experimental animals. Recently, both specific IgG1 antibody responses and airway sensitization have been described in OVA-fed guinea pigs [17], but IgG2 antibody responses to oral OVA have not been studied. The orally sensitizing capacity of heated proteins has been evaluated in guinea pigs by fatal anaphylaxis only [13–16], but antibody and airway responses are much easier to evaluate

the sensitizing capacity of oral antigens in guinea pigs. Therefore, the effects of ingestion of chemically or heat-denatured proteins on specific IgG, IgG1 and IgG2 antibody responses and bronchial reactivity will be studied in guinea pigs for the first time. In addition, whether oral administration of denatured proteins to guinea pigs can induce tolerance or not will also be examined.

Materials and Methods

Animals. Male Dunkin-Hartley guinea pigs (200–250 g) were purchased from the Animal Center, National Taiwan University (Taipei, Taiwan). They were maintained on a chick-egg-free diet (Lab diet; PMI Feeds, St. Louis, Mo., USA) in the animal house of the Veterans General Hospital-Taipei. Groups of 6 guinea pigs were first used at 300–350 g.

Protein Antigens. OVA (grade V), ovomucoid (grade IV), conalbumin (type I) and lysozyme were obtained from Sigma (St. Louis, Mo., USA). Fresh chicken eggs were purchased from the supermarket.

Denaturation of Protein Antigens. Chemical denaturation of OVA and chicken egg white (EW) was performed as described previously [8]. Briefly, urea-denatured OVA and EW (UD-OVA and UD-EW) were prepared by reduction of disulfide bonds after stirring overnight under vacuum suction with 8.0 M urea and 0.2 M 2-mercaptoethanol (Merck, Darmstadt, Germany). Carboxy-methylated UD-OVA and UD-EW (CM-OVA and CM-EW) were further prepared by alkylation of the sulfhydryl groups in UD-OVA and UD-EW after stirring for 3 h in the dark with 0.3 M sodium iodoacetate (Sigma) at pH 8.0. The crude preparations of UD-OVA, CM-OVA and CM-EW were extensively dialyzed against distilled water and then lyophilized. Heat denaturation of EW was prepared by boiling of fresh chicken eggs at 100 °C for 60 min. Both raw EW and boiled EW were lyophilized.

Oral Administration of Protein Antigens. Lyophilized OVA, UD-OVA and CM-OVA were ground and prepared in 0.15 M saline at a concentration of 50 mg/ml. Lyophilized EW, CM-EW and EW boiled for 60 min were also ground and suspended in saline at a concentration of 100 mg/ml. Each guinea pig was daily fed 2 ml of protein solutions or saline only for 10 days (5 days per week).

Bronchial Sensitization. Guinea pigs were exposed to 0.1 or 1% OVA or conalbumin aerosol for 10 min. They were placed in a 20-liter plastic chamber connected to a Mefar MB3 dosimeter (Mefar Ele, Brescia, Italy) with driving pressure (1.65 kg/cm, flow rate 70 l/min, particle size of aerosol 4.0 µm).

Measurement of Pulmonary Function. After exposure to 2% OVA or conalbumin aerosol, guinea pigs were examined by measuring pulmonary resistance, dynamic lung compliance and tidal volume. They were first injected with pyrilamine (10 mg each) and ketamine (30 mg/kg), and placed in a supine position. Both pharynx and epiglottis were swabbed with 1% lidocaine to prevent gagging. A water-filled CH50 feeding tube was put in the esophagus to measure the intrathoracic pressure. Lung volume was measured with a LAB pulmonary evaluation and diagnostic system (Hatfield, Pa., USA).

Assessment of Specific IgG, IgG1 and IgG2 Antibody Responses. Specific IgG, IgG1 and IgG2 antibody responses were assayed by ELISA methods. Briefly, 96-well polystyrene plates (Immulon 2; Dynatech, Chantilly, Va., USA) were coated with 100 µg/ml OVA, con-

albumin, ovomucoid or lysozyme in 0.05 M carbonate buffer, pH 9.6 (100 µl/well) overnight at 4 °C. Free sites were blocked with 3% skimmed milk in 0.9% saline-Tween 20 for 3 h. After washes, serum samples (1/10) were incubated in duplicate for 5 h at room temperature. After washes, 100 µl of horseradish peroxidase-conjugated goat anti-guinea pig IgG, IgG₁ or IgG₂ (diluted 1/10,000, 10,000 or 3,000, respectively, in 3% skimmed milk; Bethyl, Montgomery, Tex., USA) were added and the plates incubated for 6 h at 4 °C. After washes, orthophenylenediamine (0.5 mg/ml; Sigma) in citrate-carbonate buffer containing 0.015% H₂O₂ was added and the plates were incubated in the dark. Color development was stopped by 4 N H₂SO₄. The absorbance at 492 nm was read using a reader (SPECTRAMax 250, Molecular Devices, Sunnyvale, Calif., USA).

Assessment of Serum Immunoreactive OVA. Serum OVA was assessed by sandwich ELISAs described previously [8, 10]. Briefly, plates were coated with 5 µg/ml rabbit IgG specific for OVA or CM-OVA overnight at 4 °C. After blocking with 1% normal rabbit serum for 2 h, serum samples (1/5) and standards (0–100 ng/ml OVA or CM-OVA) were incubated 6 h at room temperature. After washes, biotinylated rabbit IgG specific for OVA or CM-OVA, respectively, was added and the plates incubated overnight at 4 °C. After washes, streptavidin-conjugated HRP (1/1,000; Amersham, Amersham, UK) was added for 1 h. Color development was performed and read as above.

Statistical Analysis. Specific IgG, IgG1 and IgG2 antibody responses and changes in airway reactivity were presented as means ± SEM. All group comparisons were made using two-tailed Mann-Whitney U tests.

Results

Chemical Denaturation of OVA Prevents Oral Sensitization of Airway Hyperreactivity and IgG1, IgG2 Antibody Responses in Guinea Pigs

As previous described [18], our preliminary studies showed that guinea pigs were sensitized by twice weekly exposure to 1% OVA aerosol, but not by single exposure to 0.1% OVA aerosol. Whether ingestion of chemically denatured OVA could sensitize guinea pigs or not was first studied. Groups of 6 guinea pigs were fed 100 mg OVA, UD-OVA or CM-OVA daily for 10 days. The controls were fed saline only. Their antibody responses were assessed 1 week after the last feeding (fig. 1). Compared with the controls, those fed UD-OVA and CM-OVA showed no sensitization of IgG, IgG1 and IgG2 antibody responses to OVA. In contrast, the group fed OVA had oral sensitization of IgG, IgG1 and IgG2 antibody responses to OVA ($p < 0.005$, respectively). Two weeks after the last feeding, they were exposed to 0.1% OVA aerosol for 10 min. Their airway responses to 2% OVA aerosol were measured 1 week later (fig. 2). Significant increase in pulmonary resistance and decrease in tidal volume ($p < 0.005$, respectively) were found in the group fed OVA, but not in those fed saline or chemically denatured OVA.

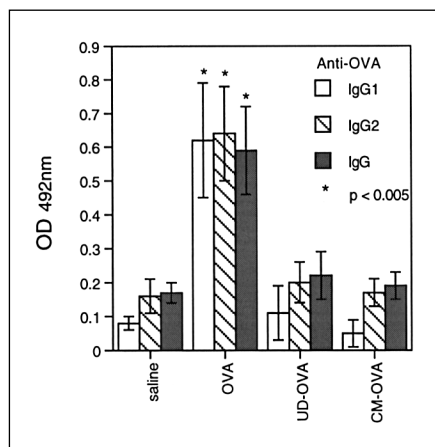


Fig. 1. Guinea pigs were fed 100 mg OVA, UD-OVA, CM-OVA or saline for 10 days. Their serum IgG, IgG1 and IgG2 antibody responses to OVA were measured 1 week after the last feeding.

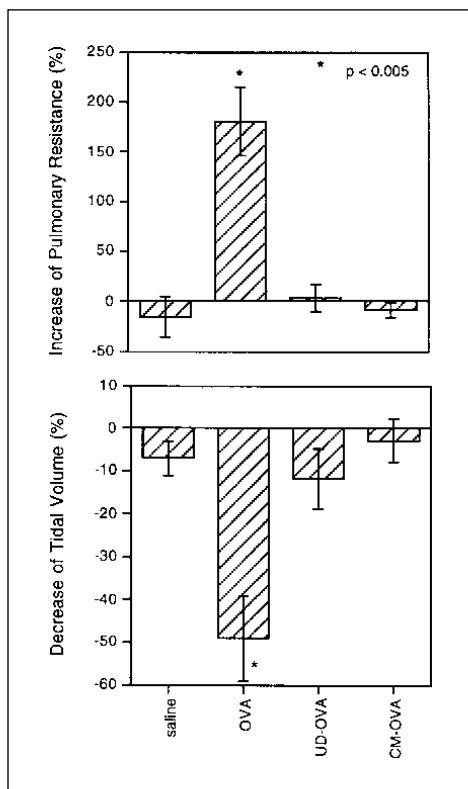


Fig. 2. Guinea pigs were fed 100 mg OVA, UD-OVA, CM-OVA or saline for 10 days. Two weeks later, they were exposed to 0.1% OVA aerosol for 10 min. Their airway responses to OVA were assessed 1 week after bronchial sensitization.

Ingestion of Heat-Denatured EW Fails to Sensitize Airway Hyperreactivity and IgG1, IgG2 Antibody Responses in Guinea Pigs

Whether ingestion of heat-denatured proteins could sensitize guinea pigs or not was further studied. Groups of 6 guinea pigs were fed 200 mg EW, CM-EW or EW boiled 60 min, respectively, for 10 days. The controls were fed saline only. Their antibody responses were tested 1 week after the last feeding (fig. 3). Compared with the controls, those fed CM-EW or boiled EW did not have increased IgG, IgG1 and IgG2 antibody responses to OVA, conalbumin, ovomucoid or lysozyme. In contrast, those fed EW showed oral sensitization of specific IgG, IgG1 and IgG2 antibody responses to OVA, conalbumin, ovomucoid or lysozyme ($p < 0.005$, respectively).

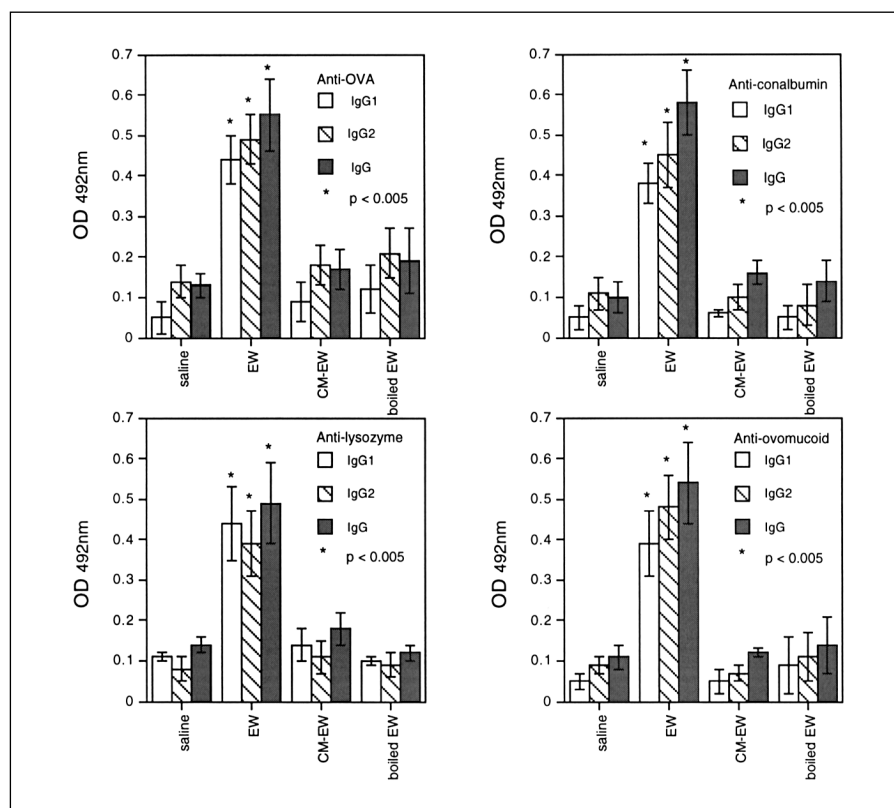
Two weeks after the last feeding, guinea pigs were exposed to 0.1% OVA aerosol for 10 min. Their airway responses to 2% OVA aerosol were measured 1 week later (fig. 4). Whereas the groups fed saline only, CM-EW or boiled EW showed no significant change in pulmonary resistance and tidal volume, those fed EW showed increased pulmonary resistance ($p < 0.005$) and decreased tidal volume ($p < 0.005$). In a repeated study, guinea pigs were similarly fed and then exposed 2 weeks after the last feeding to 0.1% conalbumin aerosol for 10 min. Their airway responses to 2% conalbumin aerosol were measured 1 week later (fig. 5). Whereas those fed saline, CM-EW or boiled EW showed no significant change in pulmonary resistance and tidal volume, those fed EW had increased pulmonary resistance ($p = 0.01$) and decreased tidal volume ($p = 0.005$).

Ingestion of Chemically or Heat-Denatured OVA Fails to Suppress Airway Hyperreactivity and IgG1, IgG2 Antibody Responses in Guinea Pigs

Whether ingestion of denatured OVA could induce tolerance or not was further studied. Groups of 6 guinea pigs were fed 100 mg OVA, CM-OVA or 200 mg EW boiled 60 min, respectively, for 10 days. The controls were fed saline only. One week after the last feeding, they were twice weekly sensitized with 1% OVA or saline aerosol for 10 min. Their antibody titers and airway responses to 2% OVA aerosol were tested 1 week after the last sensitization.

Consistent with the results of figures 2 and 4 that OVA-fed guinea pigs had airway hyperreactivity after single sensitization with 0.1% OVA aerosol, OVA-fed guinea pigs also had severe airway hyperreactivity after single sensitization with 1% OVA aerosol. In our preliminary study, all of them died of anaphylaxis during the second sensitization with 1% OVA aerosol. To avoid fatal anaphylaxis, in the subsequent studies, all guinea pigs received an

Fig. 3. Guinea pigs were fed 200 mg EW, CM-EW, boiled EW or saline for 10 days. Their serum specific IgG, IgG1 and IgG2 antibody responses were measured 1 week after the last feeding.



intraperitoneal injection of 10 mg pyrilamine 30 min before the second sensitization and before challenge with 2% OVA aerosol.

After the second sensitization with 1% OVA aerosol, all guinea pigs had airway hyperreactivity. There is no significant difference in airway hyperreactivity between saline-fed guinea pigs and those fed OVA, CM-OVA or boiled EW (fig. 6). Consistent with the data in figures 1 and 3, OVA-fed guinea pigs had increased specific IgG, IgG1 and IgG2 antibody responses (data not shown).

OVA Absorption after Feeding EW, But Not CM-EW or Boiled EW

Guinea pig serum concentrations of immunoreactive OVA or CM-OVA were measured 60 min after feeding 200 mg EW, CM-EW or EW boiled 60 min (6/group) by ELISA. Immunoreactive OVA was detected after feeding EW (134.5 ± 79.7 ng/ml), but not after feeding CM-EW or EW boiled 60 min. On the other hand, CM-OVA was not detected after feeding EW, CM-EW or EW boiled 60 min (table 1).

Discussion

We have shown that oral administration of chemically or heat-denatured OVA cannot induce oral tolerance of Th1 and Th2 immune responses in mice [8–10]. Furthermore, the guinea pig IgG, IgG1 and IgG2 antibody and airway responses were orally sensitized by OVA or EW, but not by chemically or heat-denatured OVA or EW. It was found that guinea pigs generated antibody responses to 1% OVA aerosol, but not to 0.1% OVA aerosol [18]. We revealed here that guinea pigs developed airway hyperreactivity to two sensitizations with 1% OVA aerosol, but not to a single exposure to 0.1% OVA aerosol. In contrast to a report stating that OVA-fed guinea pigs had bronchial hyperreactivity after exposure to 0.5, 1, 2, 4 and 8% OVA aerosol for 5 days [17], our data found that the airway responses in orally sensitized guinea pigs could be strongly amplified by a single exposure to 0.1% OVA aerosol. We also found that oral administration of chemically or heat-denatured OVA failed to induce oral tolerance in guinea pigs. The present report is consistent with studies reporting that extensive heat treatment of cow's milk could minimize oral anaphylactic sensitization in guinea pigs [13–16]. Taken together, the effects of oral presentation of proteins are dependent on the species

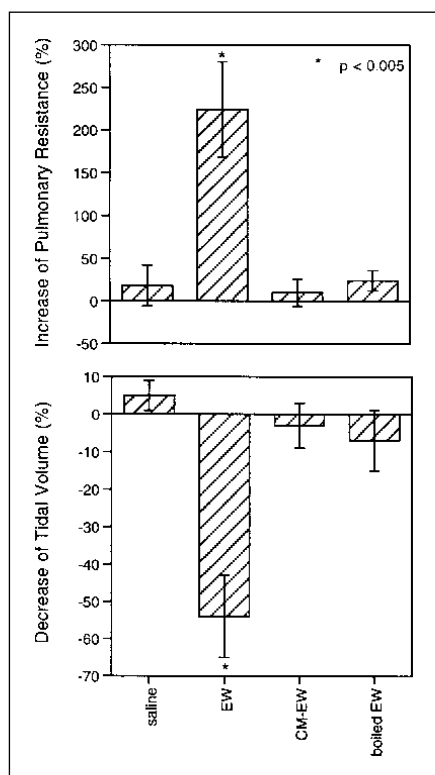


Fig. 4. Guinea pigs were fed 200 mg EW, CM-EW, boiled EW or saline for 10 days. Two weeks later, they were exposed to 0.1% OVA aerosol for 10 min. Their airway responses to OVA were assessed 1 week after bronchial sensitization.

Table 1. Immunoreactive OVA (ng/ml) in guinea pig serum collected 60 min after feeding of 200 mg EW, CM-EW or EW boiled 60 min, respectively

Feed	Raw EW	CM-EW	EW boiled 60 min
ELISA for OVA	134.5±79.7	ND	ND
ELISA for CM-OVA	ND	ND	ND

ELISA for OVA was assessed using rabbit anti-OVA IgG adsorbed by CM-OVA. ELISA for CM-OVA was assessed using rabbit anti-CM-OVA IgG adsorbed by OVA. ND = Not detected.

Table 2. The kinetics of immune responses to oral antigens are determined by the nature of oral antigens and the age and species of experimental animals

Species	Age	Oral antigens	Systemic immunity	Reference
Mice, rats	adult	native proteins	oral tolerance	1, 2
Mice	adult	denatured proteins	no oral tolerance	8–10
Guinea pigs	adult	native proteins	oral sensitization	5–7, 17
Guinea pigs	adult	denatured proteins	no oral sensitization	13–16
Mice	neonate	native proteins	no oral tolerance	29
Guinea pigs	neonate	native proteins	oral tolerance	27

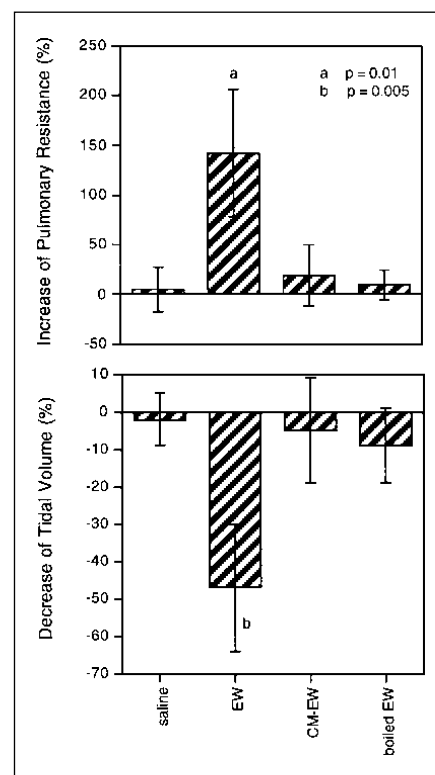


Fig. 5. Guinea pigs were fed 200 mg EW, CM-EW, boiled EW or saline for 10 days. Two weeks later, they were exposed to 0.1% conalbumin aerosol for 10 min. Their airway responses to conalbumin were assessed 1 week after bronchial sensitization.

of experimental animals and the physicochemical structure of oral antigens.

Chemical denaturation of proteins may disrupt the tertiary structure and lead to random-coiled aggregation and decreased solubility [19]. Both UD-OVA and CM-OVA were so much aggregated at the pH near the isoelectric point of OVA that they could be purified by centrifugation at pH 4.7 [9]. Indeed, the residual native OVA in the crude preparations of denatured OVA was removed in this study by centrifugation. In contrast to the report that CM-OVA induced only a 20% decrease in allergenicity [20], we and others

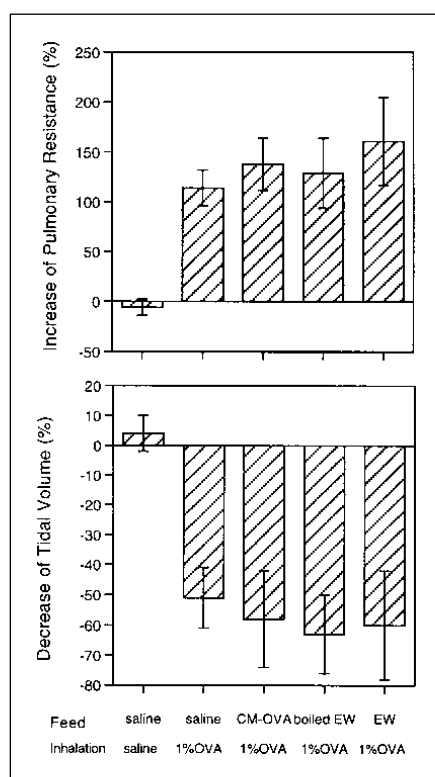


Fig. 6. Guinea pigs were fed saline only, 100 mg OVA, CM-OVA, or 200 mg EW boiled for 10 days. One week later, they were weekly sensitized twice with 1% OVA or saline aerosol for 10 min. Their airway responses to OVA were tested 1 week after the last sensitization.

found that CM-OVA had severely decreased allergenicity [9, 21]. It was reported that guinea pig IgG1 antibody responses could be orally sensitized by OVA [17]; we found that their specific IgG2 antibody responses were also sensitized. This report further revealed that guinea pig IgG1, IgG2 antibody and airway responses were not orally sensitized by chemically denatured OVA or EW. Oral sensitization to food proteins may lead to food allergy and atopic diseases [1, 2]. Thus, chemical denaturation of proteins may produce hypoallergenic foods for guinea pigs.

In contrast to the report that OVA heated at 80°C for 10 min had little change in allergenicity [20], we found that OVA boiled for 60 min had much decreased allergenicity in passive cutaneous anaphylaxis tests [10]. Foods are usually prepared in heat-denatured forms. Owing to the reports that boiled cow's milk has minimal oral anaphylactic sensitization in guinea pigs [13–16], heat treatment has been recognized as a simple way to reduce allergenicity. The present data further showed that guinea pig IgG, IgG1 and IgG2 antibody responses and airway reactions were orally sensitized by raw EW, but not by EW boiled 60 min. Indeed, heat

denaturation of proteins can minimize their oral sensitizing capacity in guinea pigs. On the other hand, we further found that ingestion of boiled EW failed to induce tolerance in guinea pigs. Our data suggest that there is no correlation between hypoallergenicity and oral tolerogenicity.

Absorption of minute amounts of fed proteins has been repeatedly described in mice [8, 10]. It has been postulated that the induction of oral tolerance in mice is related to the gut permeation of monomeric forms of fed antigens [2]. It is likely that the induction of oral sensitization in guinea pigs is also due to intestinal uptake of oral antigens. The present data in guinea pigs are similar to our reports in mice showing that serum OVA is not detected after feeding chemically or heat-denatured OVA [8, 10]. It is possible that poor uptake of denatured antigens may be due to rapid and extensive degradation by intestinal enzymes [2, 8].

Oral tolerance is readily induced after feeding native proteins to mice and rats [1, 2]. This natural route of tolerance induction has been applied in clinical trials in allergic and autoimmune disorders [22–24], but its clinical efficacy remains inconclusive. Whereas some volunteers showed oral tolerance to keyhole limpet hemocyanin [25], other reports found that IgG antibodies specific for food proteins are common in healthy individuals [26, 27]. Therefore, it is uncertain who can develop oral tolerance to food proteins. In fact, the species of animals are very important in the induction of oral tolerance (table 2). Whereas food proteins induced oral tolerance in adult mice and rats [1, 2], they induced oral sensitization in adult guinea pigs and rabbits [3, 5–7]. Whereas ingestion of proteins induced tolerance in neonatal guinea pigs and rabbits [28, 29], it failed to induce oral tolerance in newborn mice [30]. The difference between studies in mice [8–10] and in guinea pigs must be due to different animal species. Theoretically, in some humans oral tolerance may be induced as well as in inbred mice, but other subjects may be orally sensitized as worse as guinea pigs. As an animal model for food allergy, all the studies using guinea pigs may have implications for those subjects whose immune responses to food proteins are similar to guinea pigs, but not for the others whose immune responses to food proteins are similar to those of mice. Modern technologies may allow the food industry to develop denatured hypoallergenic products, but we must keep in mind that studies in mice and guinea pigs fail to induce oral tolerance to fed denatured proteins.

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