

No Priming of the Immune Response in Newborn Brown Norway Rats Dosed with Ovalbumin in the Mouth

C. Madsen · K. Pilegaard

Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, Søborg, Denmark

Key Words

IgE · IgG · ELISA · Food allergy model · Oral · Neonatal rats

Abstract

Background: Other researchers have reported that the specific immune response to subsequent antigen challenge is primed in newborn mice or rats dosed orally by gavage. We wanted to investigate if priming of a subsequent specific IgE response could be achieved by dosing newborn rats orally with ovalbumin and if this method could be used in an animal model for food allergy. **Methods:** Newborn Brown Norway rats were dosed with ovalbumin in the mouth (100 µg or 6 mg). As young adults, the animals were dosed by gavage for 35 days with 1 mg ovalbumin/day or once intraperitoneally with 100 µg. Control groups were dosed by gavage or intraperitoneally but not as neonates. Additionally, young adult rats were dosed with 1 mg ovalbumin/day in the mouth for 35 days. Sera from individual animals were analysed for specific IgE and specific IgG. **Results:** In all experiments with neonatal rats the specific IgE and IgG responses were decreased compared to the control groups, however, not always reaching statistical significance. A statistically significant decrease in the specific immune response was found in young adult rats dosed in the mouth as compared to by gavage. **Conclusions:** Dosing

Brown Norway rats with ovalbumin in the mouth as neonates do not prime the specific immune response. The decrease in immune response found in our experiments when dosing newborn animals in the mouth in opposition to the priming seen by others when dosing by intragastric intubation may be explained by a dissimilar antigen presentation when dosing includes both oral mucosa and gut.

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Introduction

Our knowledge of the allergenicity of ordinary foods comes from the human clinic. The need to conduct risk assessments on new proteins (e.g. introduced via novel foods) not previously eaten by the human population has created a demand for methods to detect potential allergenicity. Animal models with the capacity to predict allergenicity of food proteins are therefore needed [1]. At present, no validated model exists.

An animal model should identify the same proteins that trigger allergy in humans. Studies have demonstrated that Brown Norway rats and humans identify the same protein and protein structures in milk and egg [2, 3]. IgE from C3H/HeJ mice recognizes the same peanut allergen isoforms and epitopes as IgE from human peanut-allergic patients [4]. The ideal animal model for food allergy

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Correspondence to: Dr. Charlotte Madsen
Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration
Mørkhøj Bygade 19
DK-2860 Søborg (Denmark)
Tel. +45 33 95 65 63, Fax +45 33 95 60 01, E-Mail cm@fdir.dk

should be able to predict if a protein is an allergen when eaten and to rank allergens into groups, e.g. strong, medium or weak.

One of the problems when developing animal models for food allergy is that induction of oral tolerance is the normal physiological response to ingestion of soluble foreign protein. Cholera toxin [5] or $\text{Al}(\text{OH})_3$ [6] have been used as adjuvant to help induce a specific IgE response. Other investigators have used the intraperitoneal route with or without adjuvant [7, 8]. Using adjuvant may introduce a qualitatively different immune response, thus making interpretation of results difficult. Because digestibility is a factor in food allergy, a reliable animal model for food allergy probably will have to include the oral route of sensitization. A model using intraperitoneal administration as the only route may give false-positive results.

When studying protein allergenicity, it is important that the animals have been on a diet free from the protein under investigation for at least two generations [2]. Knippels et al. [9] have developed a model using Brown Norway rats without the use of adjuvant. In one experiment, young male rats were dosed by gavage with ovalbumin (OVA) 1 mg/rat/day for 6 weeks. Serum samples were analysed for specific IgG and IgE using ELISA. In this experiment, 7/8 animals developed both specific IgG and IgE. Zhang and Crevel [10] have used Knippels' protocol to sensitize Brown Norway rats using several dose levels. At 1 mg OVA/rat/day, 4/10 animals produced IgG. No data on IgE were reported. Our experience with Knippels' protocol using OVA is that the IgE titres in our studies are comparable to the ones found by Knippels but that only up to 60% of the animals produce specific IgE [unpubl. data]. As OVA is a strong allergen, the method is not sufficiently sensitive.

In our attempt to develop an oral model for food allergy, we wanted to enhance the specific IgE response without using adjuvant. Hanson [11] and later Strobel and Ferguson [12] have shown that when newborn mice are dosed with OVA by intragastric intubation, the subsequent specific immune response after immunization is primed. Similar results have been obtained in Lewis rats with myelin basic protein [13]. The objective of the present study was to investigate if priming of a subsequent specific IgE response could be achieved by dosing newborn Brown Norway rats orally with OVA and if this method could be used in an animal model for food allergy. For ethical reasons, we did not want to dose newborn animals by gavage but chose to dose the newborn animals in the mouth. It was not expected that changing the dosing in

newborns from intragastric intubation to deposition of OVA in the oral cavity would influence the outcome. To further compare the gavage route with dosing in the oral cavity, we studied whether the specific IgE response was similarly affected in young adult animals.

Materials and Methods

Animals

Brown Norway rats purchased from Iffa Credo, France, were bred in our own facilities. The animals were kept on a diet free from OVA (Rat and Mouse No. 3 Breeding Diet (E), SDS, Witham, Essex, UK) for at least three generations. The mean weight of newborn pups was 6 g. The rats were weaned at 21 days of age. After weaning the rats were housed 2 per cage in wire cages with a 12-hour day:night cycle with light from fluorescent tubes from 9 a.m. to 9 p.m. The room temperature was $22 \pm 1^\circ\text{C}$ and the relative humidity $55 \pm 5\%$. The rats were given diet and acidified tap water ad libitum. The animals were observed twice a day and body weight was recorded once a week.

Ovalbumin

Animals were dosed with OVA grade II (A 5253 Sigma, Lot 47H0966, Crude, Dried Egg White) or with organic, pasteurized frozen egg white purchased in a local food store. Solutions for dosing were made at the beginning of the study, divided in aliquots and frozen at -20°C . Fresh aliquots were used every day. The OVA content of OVA grade II and of fresh egg white is approximately 50% [14]. Positive control sera were produced by immunizing Brown Norway rats intraperitoneally with OVA grade V (A 5503 Sigma, Lot 78H7007, minimum purity 98%).

Dosing

Newborn rat pups were dosed after being suckled but before 24 h of age. The pups were dosed in the mouth using a 0.9×38 mm disposable animal feeding needle (B&K Universal, Albertslund, Denmark) on a 1-ml syringe and not by gavage. Young adult animals, 4–6 weeks of age, were dosed by gavage or in the mouth using a 1.8×78 mm disposable animal feeding needle (B&K Universal). Intraperitoneal administration was done with 0.5 ml solution using a 23-gauge needle. Each group consisted of 16 rats with equal number of males and females, and included animals from 6 to 10 different litters, i.e. most groups had 1–2 animals from the same litter. In a few cases a group had 3 animals from the same litter.

Experimental Design

Dosing Newborn Animals in the Mouth

An overview of timing and route of administration used in the experimental designs is shown in table 1.

Low-Dose Egg White. Newborn rat pups were dosed in the mouth with 100 μg (approx. 17 $\mu\text{g/g}$ b.w.) OVA grade II in 20 μl water sweetened with 4% glucose within 24 h after birth. Another group of animals was dosed within 24 h after birth and once daily for the next 4 days. With carefulness, it was possible to make the pups swallow the solution. When 4–6 weeks old, the rats were dosed daily by gavage with 1 mg OVA (grade II) in 0.5 ml tap water for 35 days. The first low-dose experiment was repeated. The dose of 100 μg egg white was

Table 1. OVA was administered to newborn Brown Norway rats: the different designs are shown

	In the mouth age < 24 h	In the mouth age 2–5 days	Gavage for 35 days from age 4–6 weeks	I.p. once age 4–6 weeks	In the mouth for 35 days age 4–6 weeks
Low dose 1 ×	×		×		
Low dose 5 ×	×	×	×		
High dose + gavage	×		×		
High dose + i.p.	×			×	
Control (gavage)			×		
Control (i.p.)				×	
In the mouth					×

comparable to the dose of myelin basic protein ($2 \times 50 \mu\text{g}$) used in Lewis rats [13].

High-Dose Egg White. Newborn rat pups were dosed in the mouth with 60 μl pasteurized egg white containing 6 mg protein (approx. 1 mg/g b.w.) sweetened with 8% sugar. The palatability of pasteurized egg white was better than dried egg white. Therefore, pasteurized egg white was used in the high-dose groups. The dose was comparable to the dose used by Hanson [11] and Strobel and Ferguson [12]. When 4–6 weeks old, one group of rats was dosed daily by gavage with 1 mg OVA (grade II) in 0.5 ml tap water for 35 days. Another group was dosed once intraperitoneally with 100 μg OVA (grade II).

Dosing Young Adult Animals in the Mouth

Animals, 4–6 weeks of age, were dosed daily in the mouth with 1 mg OVA (grade II) in 0.2 ml water sweetened with 4% glucose. The experiment was repeated.

Control Groups

Two groups of animals dosed daily from age 4–6 weeks by gavage with 1 mg OVA (grade II) in tap water for 35 days served as the control group for the gavage studies. One group of animals dosed once intraperitoneally with 100 μg OVA grade II served as the control group for the intraperitoneal study.

Sampling of Serum. All animals: blood was drawn from the orbital plexus under anaesthesia 28, 35 and 42 days after commencement of dosing by gavage, or 21, 28 and 35 days after intraperitoneal dosing. The rats were anaesthetized subcutaneously with 0.15 ml/100 g of a solution consisting of 1 part of Hypnorm (Janssen Pharmaceutica) (5 mg/ml), 1 part of Dormicum (Roche) (5 mg/ml) and 2 parts of sterile water.

Enzyme-Linked Immunosorbent Assay (ELISA). Serum samples were analysed for specific IgE (capture ELISA) and specific IgG (direct ELISA). In each ELISA plate a positive and a negative control serum pool was included to check the reproducibility of test results. The positive sera were from animals immunized intraperitoneally with OVA (grade V) in 3% $\text{Al}(\text{OH})_3$. The negative sera were from undosed animals. All incubations were done at room temperature on a rotating table if not specified otherwise. Washing procedures between each step were done with PBS containing 0.05% Tween 20.

The test for specific OVA-IgE was modified after Knippels et al. [9]. In short, 96-well microtitre plates were coated with 100 μl well of a monoclonal mouse anti-rat IgE (HD-MAS 314p, Oxford Biotechnology Ltd, UK) 0.5 $\mu\text{g}/\text{ml}$. Serial dilutions of serum (starting at 1:4)

(50 $\mu\text{l}/\text{well}$) in PBS-T was added and incubated 1 h. Digoxigenin (DIG)-coupled OVA (grade V) 1 $\mu\text{g}/\text{ml}$ was added to each well and incubated for 1 h. 50 $\mu\text{l}/\text{well}$ anti-DIG (anti-DIG POD, Roche) diluted 1:1,000 was added and incubated 1 h. The reaction was visualized using TMB-One. The average mean extinction in negative control wells (pooled negative control sera diluted 1:4–1:512 in duplicate) plus $3 \times \text{SD}$ was used as reference value. The reciprocal of the furthest serum dilution with extinction higher or equal to the reference value was read as the titre if in addition a titration-dependent decrease in values could be identified. Analysis of individual test sera was done once. Specific serum IgE analysed by this test method correlates well with rat mast cell protease II in serum after challenge [15].

For the detection of specific OVA-IgG, half of a 96-well microtitre plate was coated with 100 $\mu\text{l}/\text{well}$ OVA (grade V) 1 $\mu\text{g}/\text{ml}$ overnight at 4°C or 2 h at 37°C . The other half was coated with rat albumin. Serial dilutions of serum (starting at 1:8) (50 μl) in PBS-T was added and incubated for 1 h. 50 $\mu\text{l}/\text{well}$ peroxidase-labelled goat anti-rat IgG [peroxidase-conjugated $\text{F}(\text{ab}')_2$ fragments] was added and incubated for 1 h. The reaction was visualized using TMB-One. Because of problems with unspecific background staining varying in intensity between individual animals, positive control sera as well as test sera were added to the OVA side and the rat albumin side of the plate in an identical manner. The readings in the OVA half of the plate was compared to the readings in the rat albumin side of the plate. The reciprocal of the furthest serum dilution in the OVA wells with extinction at least 1.6 times higher than the value in the corresponding albumin well was read as the titre. In addition, a titration-dependent decrease in values should be identified. Analysis of individual test sera was done once.

Statistics

IgE and IgG titres in males and females, in the two low-dose experiments in newborn animals, in the two experiments where adult animals were dosed in the mouth and in the two gavage control groups, were compared using the Mann-Whitney test. Since no statistically significant differences between male and female rats and between the repeated experiments were observed, the data were pooled.

Specific IgE and IgG titres from the experimental groups were compared to the control groups using the Mann-Whitney test. Differences between groups were considered statistically significant at $p < 0.05$. Data are given as single animal values. Horizontal lines indicate medians.

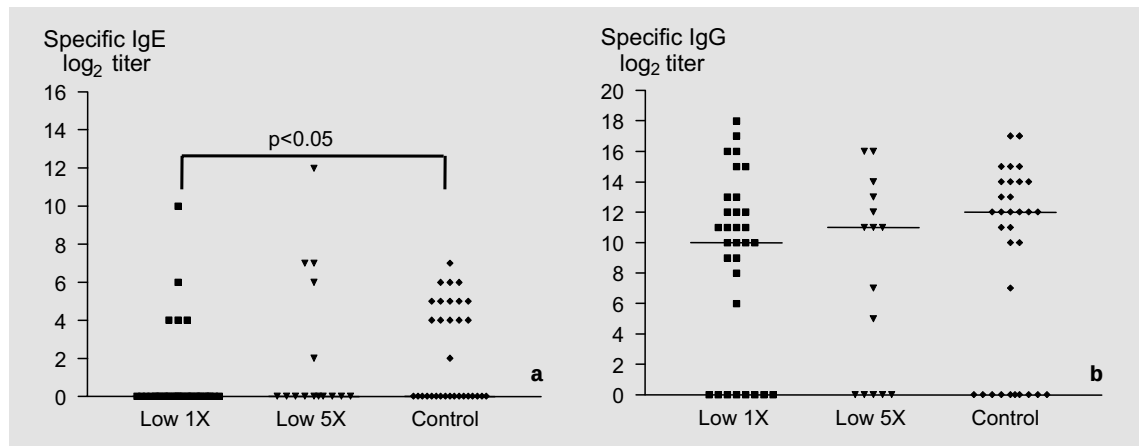


Fig. 1. Specific IgE (a) and IgG (b) titres to OVA in Brown Norway rats dosed in the mouth within 24 h after birth (Low 1 ×) with 100 µg OVA grade II (n = 32), or for the first 5 days of life (Low 5 ×) with 100 µg OVA each day (n = 16) or not dosed as neonates (control n = 32). From 4 to 6 weeks of age the animals were dosed by gavage with 1 mg OVA (grade II)/animal/day for 35 days. Serum samples were from the last day of gavage dosing. Data are given as single animal values. Horizontal lines indicate medians.

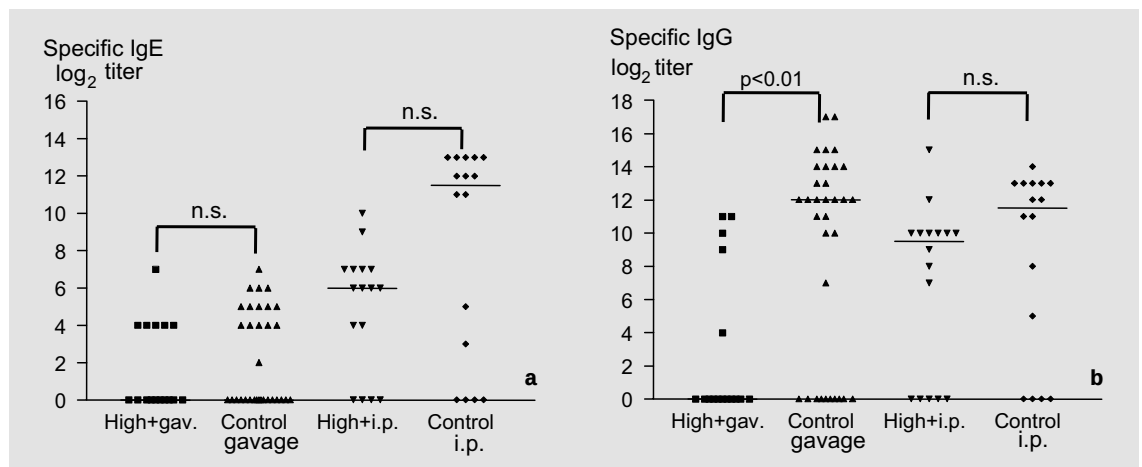


Fig. 2. Specific IgE (a) and IgG (b) titres to OVA in Brown Norway rats dosed in the mouth within 24 h after birth with 60 µl pasteurized egg white (approx. 6 mg egg white protein). From 4 to 6 weeks of age the animals were dosed either by gavage with 1 mg OVA (grade II)/animal/day for 35 days (High + gav. n = 16) or by a single intraperitoneal injection with 100 µg OVA (High + i.p. n = 16). The control groups were not dosed as neonates (gavage n = 32) or (i.p. n = 16). Serum samples were from the last day of gavage dosing or 35 days after i.p. dosing. Data are given as single animal values. Horizontal lines indicate medians.

Results

There were no effects of dosing on body weight gain or clinical features. In the low-dose experiment where newborn animals were dosed once in the oral cavity with OVA and subsequently by gavage as young adults, the specific IgE titre was statistically significantly decreased compared to the control group. Dosing newborn animals the

first 5 days of life with a low dose of OVA did not change the specific IgE response significantly as compared to the control group (fig. 1a). No effect on specific IgG responses was found by low or repeated low dosing (fig. 1b). When newborn animals were dosed in the mouth with a high-dose OVA followed by gavage as young adults, the specific IgE titre was lower compared to the control group, however not reaching statistical significance (fig. 2a). For spe-

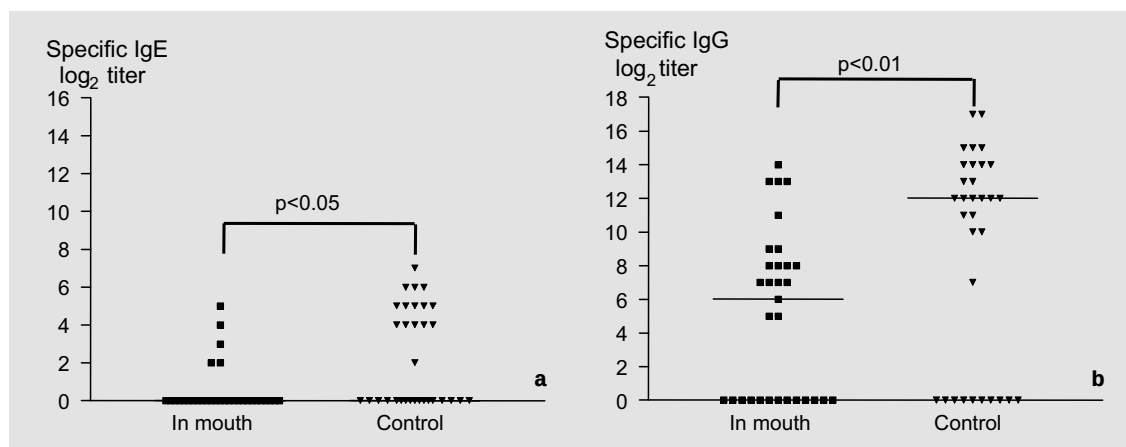


Fig. 3. Specific IgE (a) and IgG (b) titres to OVA in Brown Norway rats dosed from 4 to 6 weeks of age in the mouth ($n = 32$) or by gavage (control $n = 32$) with 1 mg OVA (grade II)/animal/day for 35 days. Serum samples were from the last day of dosing. Data are given as single animal values. Horizontal lines indicate medians.

cific IgG the reduction was statistically significant (fig. 2b). Dosing with high-dose OVA as neonates followed by subsequent intraperitoneal administration, resulted in a decrease in specific IgE (fig. 2a) and a slight decrease in specific IgG responses compared to the control group but without statistical significance (fig. 2b).

When adult animals dosed in the mouth were compared to animals dosed by gavage, both specific IgE and IgG titres were statistically significantly decreased (fig. 3).

Discussion

In the present study we have found that feeding newborn Brown Norway rats with OVA in the mouth results in comparable or decreased specific IgE and IgG responses compared to animals not dosed as neonates. This has been shown feeding the neonates a single low or high dose or five low doses. It has been shown using subsequent dosing by gavage or by intraperitoneal injection. Dried egg white (OVA grade II) or pasteurized egg white was used in the experiments with low or high concentration, respectively. It was possible to make the newborn rat pups swallow a solution of dried egg white in the low concentration but not in the high concentration. Fresh pasteurized egg white is more palatable than dried egg white and was therefore used in the high-dose groups. In our experience, gavage dosing with dried egg white or fresh egg white give comparable results on specific OVA IgE and IgG titres [unpubl. data]. In contrast to our findings,

earlier studies have shown that administration of antigen orally to neonates resulted in priming of the subsequent specific immune response [11, 12]. The studies were performed in BDF₁ [11, 12], CBA and BALBc [12] mice with OVA (1 mg/g b.w.) and in Lewis rats with myelin basic protein ($2 \times 50 \mu\text{g/pup}$) [13]. The dose used in the newborn Lewis rat was comparable to our low dose of OVA and the dose used in the newborn mice was comparable to our high dose.

Subsequent immunization was done with OVA in Al(OH)₃ intraperitoneally [11], OVA in complete Freund's adjuvant in the footpad [12] or with myelin basic protein intraperitoneally [13]. The priming of the immune system was seen measuring specific immunoglobulin (Farr assay) and specific IgE by passive cutaneous anaphylaxis [11], specific immunoglobulin (passive haemagglutination or ELISA) and cell-mediated immunity (delayed-type hypersensitivity) [12] or cell-mediated immunity (proliferation assay) and symptoms of induced experimental autoimmune encephalomyelitis [13]. Despite the differences in animal species and strains, antigens, dose and analytical methods used, these investigators all found that oral antigen dosing within the first 24 h of life induced priming of the immune system. Common to these three studies were that the neonates were dosed by gavage.

It has been shown in mice that dendritic cells from the oral mucosa can act as antigen presenting cells [16, 17]. Results indicate that these cells may acquire antigen in the buccal epithelium and migrate to the draining lymph nodes where they present processed antigen to MHC class

II-restricted T cells [17]. It has been suggested that dendritic cells not only provide T cells with stimulatory and co-stimulatory signals, but also with a polarizing signal 3 that determine the type 1/type 2 ratio in Th cells. The type of polarizing signal 3 sent by the dendritic cell is determined by the maturation of the dendritic cell that again is dependent of the character of a 'danger signal', reflecting the pathogen type and the type of tissue [18, 19]. Based on this theory it is possible that dendritic cells that acquire antigen in the buccal epithelium and present it to T cells in the local lymph node provide the Th cells with another type of stimulatory signals than dendritic cells acquiring antigen in the ventricle and intestine. If these T cells migrate to the intestinal mucosa it could have consequences for the immunologic process in the gut and the subsequent immune response. This may explain why dosing neonates in the oral cavity results in a different outcome than dosing by gavage.

Our results in the young adult rats showed that repeated dosing in the mouth gave a statistically significant lower specific IgE and IgG response compared to dosing by gavage. This is in line with the results in the neonatal rats and supports the conclusion that dosing in the oral cavity influences the immune response differently than dosing by gavage. This finding is also in accordance with a study by Holt et al. [20], where rats from various strains were dosed with OVA sublingually under anaesthesia for 10 days followed by OVA intraperitoneally. In Brown Norway rats the subsequent specific IgE but not IgG response was suppressed. In WF rats, both specific IgE and IgG responses were suppressed. The design in our study and in the study by Holt et al. [20] differ which makes comparison difficult, but the general conclusion is that involving the oral mucosa modifies the immune response differently than when the antigen is given by gavage. When young adult Brown Norway rats were dosed with OVA in the drinking water (2 or 20 mg/ml) daily for 6 weeks, the animals produced specific IgG but no IgE whereas when animals were dosed by gavage (1 mg/day) for the same period of time both specific IgE and IgG could be found [9]. Strobel studied the immune response in suckling pups after feeding the lactating dams bovine serum albumin. The study was not published but referred to by Strobel [21]. Strobel concludes that there is a priming of the cellular immune response when the pups receive bovine serum albumin in the milk on days 1–3, and further that there is no difference in results between gavage dosing of newborns and dosing via the milk. The figure illustrating the text shows that there is no difference in cellular immune response between the controls not dosed

as neonates and the animals dosed on days 1–3, thus based on the figure there is no priming.

We have found that dosing young adult animals in the mouth gave a statistically significantly lower immune response (IgE and IgG) as compared to dosing by gavage. These results supported the findings of Knippels et al. [9] and Holt et al. [20]. The results open a possibility for creating an animal model for sublingual immunotherapy. In such a model a stable and high specific IgE response is needed, e.g. induced by intraperitoneal injection followed by oral administration of the antigen. Such a model would be helpful in studying the mechanisms of sublingual immunotherapy.

In conclusion, dosing Brown Norway rats with OVA in the mouth as neonates does not prime the specific immune response to subsequent dosing either by gavage or intraperitoneally. On the contrary, in all experiments where rats were dosed as neonates, the specific immune response was decreased compared to the control groups. In some of the experiments the decrease was statistically significant. When young adult animals are dosed in the mouth for 35 days as compared to by gavage for 35 days, less specific IgE and IgG is produced. When investigating the immune response after oral administration, it is of importance to further specify whether dosing has been done directly in the oral cavity or via milk or drinking water, or by gavage since the way of antigen administration seems to modulate the specific IgE and IgG response.

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