

Induction of Oral Tolerance with Micro-Doses of Ovomucoid Depends on the Length of the Feeding Period

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Oral administration of antigen induces antigen-specific immunologic tolerance, which is known to be dose-dependent. We studied the influence of continuous oral administration of nanogram and microgram doses of antigen on oral tolerance induction. Mice were continuously exposed to varying doses (1 ng–1 mg/day) of ovomucoid (OM) for a minimum of 30 days and a maximum of 100 days. It was possible to induce oral tolerance measured as reduced proliferation and antibody production (immunoglobulin (Ig)G₁, IgG_{2a} and total Igs) when mice were fed 1 mg of OM/day for 40 or 50 days. It was not possible to induce oral tolerance with daily doses of antigen of 10 µg or less. Feeding of 100 µg OM/day for 40 and 50 days and 1 mg OM/day for 30 days generated tolerization of Th2-dependent responses, but retained an intact response of Th1-dependent antibodies, whereas feeding of 1 mg OM/day for 40 and 50 days resulted in tolerization of both Th1- and Th2-antibody responses. The results presented here suggest that there is a threshold of microgram-doses below which oral tolerance cannot be induced, and that selective suppression of Th2 responses can be achieved by continuous microdose feeding, while an extension of the feeding dose or feeding period tolerizes both Th1- and Th2-dependent responses.

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INTRODUCTION

Oral tolerance is a long-recognized method of inducing immune tolerance and refers to the observation that if a protein is fed, followed by immunization with the fed protein, a state of systemic hyporesponsiveness to the fed protein exists [1–3]. Oral tolerance is recognized as a part of the normal physiologic response, in particular to soluble antigens, whereas particles and multiplying micro-organisms in general induce an active immune response. The physiological significance of oral tolerance is not yet clear, but food allergy is believed to be owing to a breakdown of oral tolerance [4]. However, through a normal diet, antigens are usually not ingested in the high concentrations used in animal model systems of oral tolerance. Usually, oral tolerance is achieved by feeding one or a few large doses of protein, whereas ng–µg doses are believed to be below a threshold where oral tolerance can be induced [5].

Induction of oral tolerance most likely involves more than one immune mechanism, and many experimental factors, such as age, species, genetics, feeding dose and timing, antigen structure, and immunogenicity [3] may influence the induction of oral tolerance. Current evidence suggests that T lymphocytes are the major cell type involved in the induction of oral tolerance and that the mechanisms of oral tolerance are dose-dependent. High doses of antigen appear to promote deletion and anergy [6–9] and lower doses of antigen appear to activate a population of regulatory T lymphocytes that actively suppresses immune responses [7].

It has been demonstrated that it is easier to induce tolerance of Th1 lymphocytes than of Th2 lymphocytes [8, 10]. In other studies, reduction of both Th1 and Th2 cytokines after feeding high doses of antigens has been observed [11], indicating that both Th1 and Th2 cells are involved in oral tolerance induction. Oral tolerance was found not to require

Th2 cells [11] and can even be induced in the absence of functional Th1 and Th2 cells [12]. However, the amounts fed in the mentioned studies are relatively large, and low doses of antigen in these studies refer to an amount of milligrams fed per day for a few days. Continuous oral administration has been performed [13–15]; this was, however, done with high doses of antigen [14], in T cell receptor (TCR)-transgenic mice [13], or with high doses in TCR-transgenic mice [15]. The majority of oral tolerance experiments have been performed with oral administration of 1–5 mg doses of ovalbumin (or autoantigens). However, as the amount and type of antigen as well as the length of feeding period are factors known or suspected to be important for oral tolerance induction, studies with other antigens and feeding regimens are needed.

In the present study, we used ovomucoid (OM) to mimic a physiologically relevant situation and studied the long-term effect of a daily intake of very low doses of soluble antigen on oral tolerance induction.

MATERIALS AND METHODS

Antigen feeding, immunization and bleeding. Groups of BALB/c mice (M & B, Denmark) (or in some experiments (BALB/c × CF1) F1, Bomholtgård, Denmark) were fed OM (type II-0, Sigma Co. St. Louis, MO, USA) added to the drinking water in varying amounts for 30, 40, 50, 75 and 100 days. Concentrations in the drinking water varied from 0.2 ng/ml to 0.2 mg/ml for individual groups of mice. The average water consumption per mouse was 5 ml per day, resulting in a consumption of OM in the interval from 1 ng/day to 1 mg/day. For evaluation of the immune response, mice were immunized intraperitoneally with 10 µg of OM in Freund's complete and incomplete adjuvant, respectively, on day 1 and 14 after the last feeding day. Blood samples were collected before feeding, the day after the last feeding day, and 2 and 3 weeks after the last feeding day. Blood was drawn from retro-orbital venous plexus using calibrated 50 µl heparinized micropipettes. Blood was immediately diluted in 750 µl phosphate buffered saline (PBS) and stored at –20 °C. The OM-specific antibody response was measured by enzyme-linked immunosorbent assay (ELISA) on plasma and the cell-mediated immune response was evaluated 3 weeks after the last feeding day as [³H]-thymidine incorporation in spleen cells cultured with and without OM.

Measurement of OM-specific proliferation in vitro. Single-cell suspensions were prepared from spleens in DMEM (Gibco, Life Technologies, Scotland). Red blood cells were lysed with ammonium chloride lysis buffer (0.83% NH₄Cl). Cells were washed and resuspended in X-VIVO 10 (serum free medium, Bio Whittaker, USA) at a final concentration of 4 × 10⁶ cells/ml and placed in 200 µl/well in flat-bottom 96-well tissue culture plates (Nunc, Denmark). Cells were cultured with medium alone or with OM (50 µg/ml) in a total volume of 225 µl. For the last 24 h of the 4-day culture period, 0.25 µCi of [³H]-thymidine (Amersham, UK) was added. Cells were harvested onto glass fibre filters (Whatman GF/C (10.2 × 25.4 cm)) using an automated sample harvester (Dynex Technologies Autowash 2000 cell harvester). Cell proliferation was

determined by measuring [³H]thymidine incorporation with a Tri-Carb[®] Liquid Scintillation Analyzer using Ultima Gold (Packard Bioscience Company, The Netherlands) as scintillation liquid.

Assay for anti-OM antibodies. Presence of total Igs, IgG₁ and IgG_{2a} plasma antibodies specific for OM was tested by ELISA. In brief, microtiter plates (Nunc) were coated with OM overnight at 4 °C and plasma and controls were serially diluted in the washed plates. Bound OM-specific Igs were detected with peroxidase-conjugated rabbit antimouse Igs (DAKO, Denmark). OM-specific IgG₁ and IgG_{2a} were detected with rabbit antimouse IgG₁ and IgG_{2a} antibodies (Zymed, CA, USA) followed by peroxidase-conjugated swine antirabbit Igs (DAKO). Hydrogen peroxide and tetramethyl benzidine (TMB, Merck, Germany) were used as substrates and the reaction was stopped after 10 min by the addition of phosphorous acid (2 M). Plates were measured spectrophotometrically at 450 nm using 690 nm as reference on an ELISA-reader (Bio-kinetics reader, EL 340, Biotek instruments). Controls, monoclonal antibodies (MoAb) against OM (of different isotype) and a pool of plasma from OM-immunized animals, were included on each plate. The antibody titres were expressed as log₂ titres and defined as the interpolated dilution (4-parameter analysis, kineticale software, version 2.13, Biotek instruments) of a blood sample leading to an absorbance of 0.2 above background.

Statistical analysis. The statistical significance of differences between experimental groups was determined using Dunnett's multiple comparison tests, where fed groups were tested versus control groups. Statistical analysis was done with GraphPad Prism 3.0 software and statistical significance was accepted at *P* < 0.05.

RESULTS

Continuous oral exposure to OM induces a reduced specific T-cell response in vitro for either relatively large fed amounts of OM or extended feeding periods

To determine if oral tolerance could be induced with ng–µg doses of antigen, we exposed groups of mice to different concentrations of OM in their drinking water for different periods of time. Concentrations in the drinking water varied from 0.2 ng/ml to 0.2 mg/ml and exposure was continued for up to 100 days. Mice were immunized with OM in Freund's complete adjuvant one day after feeding and in Freund's incomplete adjuvant 2 weeks later. Control mice were given drinking water alone and were primed with OM in Freund's complete adjuvant and Freund's incomplete adjuvant similar to fed mice. Spleen cells were collected 1 week after the second immunization. Oral tolerance was measured as a significantly reduced antigen-specific immunological response after immunization compared with control mice. Significantly reduced OM-specific proliferation was observed when mice were fed 100 µg or 1 mg per day for 30, 40 and 50 days (Table 1). Feeding of 0.001, 0.01, 0.1, 1, and 10 µg per day resulted in OM-specific proliferation on the same level as control mice (Table 1, results not shown for feeding 0.001, 0.01, 0.1 and 1 µg for 30, 40, 50, 75 and 100 days), even when the feeding period was extended to 100 days.

Table 1. Effect of oral administration of microgram and milligram doses of ovomucoid (OM) on OM-specific proliferation (Δ cpm). Mean $\pm$ SE of OM-specific proliferation*

Days of OM feeding	Amount of OM fed per day (μ g)			
	Control	10	100	1000
30	6720 $\pm$ 1656	6826 $\pm$ 1384	2465 $\pm$ 772†	1216 $\pm$ 206†
40	9272 $\pm$ 1824	4710 $\pm$ 527	3732 $\pm$ 985†	1941 $\pm$ 682†
50	7340 $\pm$ 1760	5526 $\pm$ 594	1603 $\pm$ 408†	2589 $\pm$ 468†

*Groups of five mice were fed water (control) or OM in the indicated amount per day. Mice were immunized twice and spleen cells were collected 7 days after the last immunization. Proliferation was measured as incorporation of ^{3} H-labelled thymidine in spleen cells from individual mice stimulated with or without OM.

† $P < 0.05$ versus control.

Table 2. Ovomucoid (OM)-specific antibodies (titres) in plasma of OM-fed mice and control mice. Mean titres of a group of five mice $\pm$ SE*

Days of OM feeding	Amounts fed per day (μ g)			
	Control	10	100	1000
30	14.2 $\pm$ 0.9	15.5 $\pm$ 0.4	13.2 $\pm$ 0.2	9.1 $\pm$ 1.6†
40	15.3 $\pm$ 0.3	14.9 $\pm$ 0.3	12.4 $\pm$ 1.4	8.0 $\pm$ 1.0†
50	15.9 $\pm$ 0.5	15.0 $\pm$ 0.2	8.5 $\pm$ 1.8†	7.4 $\pm$ 1.0†

*Groups of five mice were fed water (control) or OM in the indicated amount per day. Mice were immunized twice and blood was collected 7 days after the last immunization.

† $P < 0.05$ versus control.

Total OM-specific antibodies in plasma are reduced for mice fed OM for extended periods of time or fed large amounts of antigen

To test an *in vivo* state of oral tolerance, plasma collected 1 week after the second immunization was analysed for OM-specific antibodies. ELISA was used to determine the total amounts of OM-specific antibodies (all classes and subclasses). Feeding of 100 μ g OM per day resulted in a reduced OM-specific antibody response, but only for mice fed for 50 days (Table 2), whereas specific tolerance measured as proliferation *in vitro* was observed also after 30 and 40 days of feeding 100 μ g per day. Oral exposure to 1 mg OM per day administered by the drinking water induced tolerance measured as titres for feeding for 30, 40 and 50 days (Table 2). It was not possible to induce tolerance with 0.001, 0.01, 0.1, 1, or 10 μ g OM fed per day, not even by extending the feeding period (Table 2, results not shown). Hence, either more OM per day or a longer feeding period is required to measure a reduced total OM-specific antibody response compared with a reduced *in vitro* proliferation response.

Mice continuously exposed to microdose OM do not secrete Th2-dependent antibodies in vivo but do secrete Th1-dependent antibodies

IgG₁ and IgG_{2a} isotype profiles can serve as indicators of murine Th subset activity *in vivo* [16, 17]. B-cell production of IgG_{2a} is under the control of the Th1 cytokine interferon (INF)- γ , while production of the IgG₁ subclass is directed by the Th2 cytokine interleukin (IL)-4 [18]. IgG_{2a} thus represents a Th1-mediated response, and IgG₁, a Th2-mediated response. Consequently, plasma was analysed for OM-specific IgG₁ and IgG_{2a} antibodies. IgG₁ antibodies were significantly reduced in plasma of mice fed 100 μ g OM/day for 40 and 50 days and fed 1 mg OM/day for 30, 40 and 50 days (Fig. 1). In contrast, IgG_{2a} antibodies were at the level of the controls for all groups except for mice fed 1 mg for 40 and 50 days (Fig. 1). Accordingly, inhibition of Th2-mediated responses is accomplished with lower amounts of antigen and with a shorter feeding period than inhibition of Th1-mediated responses. The requirements for tolerization of Th1-dependent antibodies are thus 1 mg OM/day for at least 40 days. Feeding

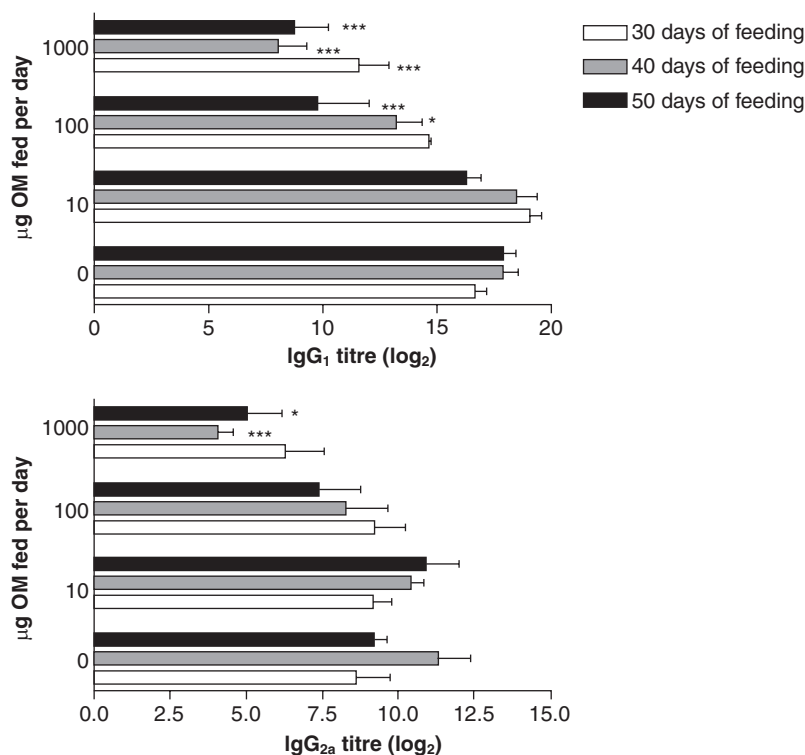


Fig. 1. Ovomucoid (OM)-specific immunoglobulin (Ig)G₁ and IgG_{2a} antibodies in plasma of OM-fed and control mice. Mice were fed OM in the drinking water with the indicated amount of OM per day for 30, 40 or 50 days. Control mice (0 µg OM) received water alone. All mice were immunized twice with OM (day 1 and 14 after the last feeding day), and blood was collected 21 days after the last feeding day. Result shown are mean titres of a group of five mice $\pm$ SE (** P < 0.001, * P < 0.05 tested versus relevant control group).

of amounts less than 100 µg of OM per day resulted in responses at the same level as control mice independent of the total number of feeding days.

The use of Freund's adjuvant may influence the T-cell balance. Antigen in alum promotes selective Th2-mediated antibody production and cannot be used to confirm Th1 tolerance. In contrast, antigen in Freund's complete adjuvant leads to antibodies of all antibody subclasses, indicating that Freund's complete adjuvant supports both Th1 and Th2 responses *in vivo* [19, 20, unpublished observations]. Here we have used Freund's complete adjuvant as a primary adjuvant and Freund's incomplete adjuvant as a secondary. However, as it is the primary adjuvant that guides the immune response [21], a second immunization with Freund's incomplete adjuvant will not influence the response obtained with immunization with Freund's complete adjuvant. Besides, antibody responses of blood samples collected 2 weeks after the first immunization showed the same tendency as blood samples collected after two immunizations (results not shown).

DISCUSSION

It is well established that feeding high doses of soluble antigen induces hyporesponsiveness visualized as a suppressed immune response upon subsequent priming, both at the serum antibody and at the cellular level. Only few investigations on feeding nanogram and microgram doses of antigen have been reported. Early reports indicated that low doses

of antigen primed the immune system [22, 23]. It is suspected that low doses of antigen at any frequency do not result in tolerance induction – a threshold exists [5]. This is however, very sparsely elucidated. Moreover, different antigens have different requirements in terms of dosing and timing, and most studies have used ovalbumin as model protein.

Using OM as model antigen, we show here that it is not possible to induce oral tolerance with nanogram doses of antigen even if the feeding period is extended to 100 days. Thus, based on the present results, it seems reasonable to define a threshold at microgram dose (at least 100 µg/day) for oral tolerance induction. We find that OM-specific proliferation and antibody responses are reduced when feeding is performed continuously for 50 days with 100 µg of OM per day, but not for 30 or 40 days. When 1 mg per day is fed, the immune responses are reduced after feeding for 30, 40 and 50 days. The period of antigen exposure is thus an important factor for oral tolerance induction. Others have also observed this, though they used only one feeding regimen with continuous low-dose feeding (5 mg daily) compared with short-term feeding [15]. Very recently Gonnella *et al.* [24] used oral doses of ovalbumin from 0.5 mg to 500 pg administered five times by gastric intubation. They found that oral administration of 500 µg and 250 ng of ovalbumin five times resulted in suppressed cellular proliferation in normal C57BL/6 mice, whereas 100, 10, 1 and 0.5 ng of ovalbumin five times did not result in suppression of cellular proliferation [24].

Obviously, different antigens have different dose-response curves. Nevertheless, in other studies where mice were fed standard rodent pills containing soybean trypsin inhibitor from the day they were born, we have found that the amount required for oral tolerance induction resembled the amount of OM necessary for oral tolerance induction. In these experiments, oral tolerance was induced when mice had 100 µg soybean trypsin inhibitor per day per mouse for 30–40 days, but was not induced when mice had 10 µg soybean trypsin inhibitor per day per mouse for 30–40 days (unpublished observations). Moreover, continuous feeding of 4 mg ovalbumin per day for 20 days has been found to result in tolerance [14] and this is in close proximity to the amounts of OM here shown to induce oral tolerance.

Priming of the immune response after feeding various low doses of antigen has been observed [22–24]. This was, however, seen after short-term exposure to oral antigen by gavage. Priming may precede induction of tolerance depending on the length of the feeding period. This is supported by the findings that oral tolerance has been found to be induced preceding an initial antigen-specific T-cell priming [25–27].

Many studies of oral tolerance have demonstrated T-cell tolerance with intact B-cell responsiveness [2, 28–30]. Likewise we find that B-cell tolerance measured as a reduction of total Ig production *in vivo* is induced after feeding 100 µg OM per day for 50 days or feeding 1 mg for 30, 40 or 50 days, whereas proliferation responses are reduced for all of the above feeding regimes and also for feeding of 100 µg OM per day for shorter periods (30 and 40 days).

We find that IgG₁ antibody responses are suppressed after fewer feeding days or with smaller amounts of fed antigen than are IgG_{2a} antibody responses. Both Th1 and Th2 responses are suppressed by high-dose ovalbumin feedings [11, 12]; however, it has been shown that oral tolerization of both Th1 and Th2-controlled responses requires relatively high doses of antigen and prolonged feeding [14]. These observations are in accordance with the results presented here, where both Th1 and Th2 responses (measured as OM-specific IgG_{2a} and IgG₁, respectively) were diminished when the highest dose of OM (1 mg/day) was fed. On the other hand, when using ovalbumin as a model protein, Melamed *et al.* [14] found that oral tolerization of Th1 lymphocytes required a shorter feeding period and that Th2 lymphocytes required an extended period of continuous exposure to high doses of antigen for tolerization. This is in contrast to what we find here. However, Melamed *et al.* used higher doses than in the present study, a shorter feeding period and a different antigen (20 days and 4 mg ovalbumin/day). In accordance with the results presented here, Wu *et al.* [13] found that feeding of 100 µg ovalbumin per day for 14 days gave a reduced IgG₁ response, whereas IgG_{2a} and total IgG was not affected, thus only the Th2 response was affected. Moreover, feeding of one high dose of ovalbumin

has been found to induce tolerance that was more pronounced and persistent in Th2-mediated immune responses than in Th1 [31].

Several reports indicate that Th1 cells are more susceptible to induction of tolerance than Th2 cells [6, 10, 32, 33]. However, IgE responses are readily tolerized [34] and nonatopic individuals have allergen-specific T cells in their blood, which produce high levels of IFN-γ [35]. Moreover, feeding antigen can induce an IFN-γ response in the gut-associated lymphoid tissue of mice [36–38] and humans [39]. Besides, oral tolerance can be induced in the absence of functional Th1 or Th2 cells [12]. Thus, the presence of Th2-biased responses in the gut after feeding antigens is not a universal finding [3, 40]. The conflicting findings may be owing to the induction of different immunological mechanisms associated with different cytokine induction because feeding has been performed with various doses, different frequencies and occasionally in different genetically modified animals. The length of the feeding period has been shown here to be important for the induction of tolerance.

Several mechanisms have been identified to induce oral tolerance; in the present study it is not known by which mechanism tolerance was induced. Clonal deletion as a mechanism for oral tolerance has mainly been demonstrated in transgenic mice fed very high doses and thus under non-physiological conditions [41, 42] and is therefore not likely to represent the mechanism under the present more physiological conditions. High doses of orally administered antigen may result in inactivation of antigen-specific cells by anergy, as demonstrated in various ways by different groups [7–9]. Anergy has, however, only been demonstrated after feeding of high doses of antigen and may possibly only be a mechanism after absorption of higher doses of antigen to the bloodstream or lymph. Prolonged oral administration or feeding with low doses of antigen could also selectively activate CD4⁺ or CD8⁺ T cells, which secrete suppressive cytokines, e.g. transforming growth factor (TGF)-β, IL-4 and IL-10 [1, 3]. In the present study, peripheral Th2 lymphocyte-dependent antibodies were downregulated, thus leaving TGF-β as a candidate for active suppression. Although speculative, induction of Tr1 cells secreting IL-10 [43] or Th3 cells secreting TGF-β [44] could also be a mechanism for the suppression of the Th2 antigen-specific response seen in the present study. Tr1 cells have been reported to inhibit Th2 responses *in vitro* [45] and continuous feeding of 5 mg ovalbumin daily in the drinking water to ovalbumin TCR-transgenic mice for 12 weeks was associated with production of IL-10 and TGF-β both systemically and locally [15].

By continuous feeding of varying concentrations of OM in the drinking water for long periods, we have demonstrated that it is not possible to induce systemic oral tolerance to OM with amounts less than 100 µg per day. The length of the feeding period is thus important for tolerance induction.

Moreover, tolerization of Th2-dependent responses are accomplished with smaller amounts of fed antigen and fewer feeding days than Th1-dependent responses.

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