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Oral tolerance induction with altered forms of ovalbumin

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Abstract

As a T cell-dependent phenomenon, oral tolerance is not expected to depend necessarily on native configuration of antigens. We investigated the induction of oral tolerance with modified ovalbumin (Ova). Oral administration of heat-denatured (HD-Ova) and cyanogen bromide-degraded ovalbumin was less effective than native Ova in inducing oral tolerance in B6D2F1 mice. HD-Ova was effective in suppressing delayed-type hypersensitivity (DTH) reactions but did not suppress specific antibody formation. Injection of Ova directly into the stomach, but not into the ileum or cecum, suppressed subsequent immunization to DTH reactions. Gavage with protease inhibitors (aprotinin or ovomucoid) before gavage with Ova was ineffective in blocking tolerance induction. Treatment with hydroxyurea to destroy cycling cells 24 h before gavage with Ova blocked oral tolerance induction and also the possibility to passively transfer tolerance to naive recipients with the serum of mice gavaged with Ova 1 h before. The implications of these findings about oral tolerance induction are discussed.

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- Mouse
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Introduction

Oral tolerance is defined as a marked and prolonged inhibition of immune responsiveness to T cell-dependent immunogens arising as a consequence of their ingestion as food components. The mechanisms responsible for its induction remain unknown (1,2). Oral tolerance may be installed very rapidly after antigen ingestion; only 24-48 h are sufficient to severely affect specific immunization (3,4). This period is too short to allow extensive clonal expansion, of suppressor cells, for example, to take place. Treatment with cyclophosphamide or hydroxyurea, that destroys cycling cells, is very effective in blocking tolerance induction if applied 24 h before antigen ingestion, but does not affect tolerance induction if applied 24-48 h after ingestion (5,6). Thus, oral tolerance induction probably depends on events that are already taking place before antigen ingestion.

Under natural conditions, oral tolerance takes place during normal feeding and there are scattered suggestions that the voluntary intake of proteins in solution or of grains of several seeds *in natura* by laboratory mice may be more effective for the induction of oral tolerance than the delivery of these antigens directly to the stomach by intubation (gavage) (7,8). There is also evidence that interference with digestive proteolysis hinders the induction of tolerance (9,10), suggesting that peptides, rather than the native proteins, are the actual inducers of oral tolerance.

Substantiating this hypothesis, serum collected from mice 1 h after gavage with ovalbumin (Ova) can transfer Ova-specific tolerance to delayed-type hypersensitivity (DTH) reactions to recipient mice, suggesting that Ova-derived peptides resulting from gut processing are the relevant tolerogens (11-14). On the other hand, tolerance may also be induced by antigen exposure through other mucosae, e.g., nasal, on which only a limited extent of proteolysis may take place (15).

In the present study, we describe experiments attempting to induce tolerance by gavage with ovalbumin degraded with cyanogen bromide (CNBr-Ova), thermally denatured Ova (HD-Ova) or by direct injection of Ova into different regions of the gastrointestinal tract. We also studied the effect of trypsin inhibition and pretreatment with hydroxyurea on oral tolerance induction and on the presence of tolerogenic materials in the serum of mice gavaged with Ova.

Material and Methods

Animals

B6D2F1 (C57BL/6 x DBA/2J) F1 mice of both sexes bred in our colonies were used. At the beginning of the experiments, the animals were 6-8 weeks old.

Antigens

Crystallized hen ovalbumin, Ova III (Sigma, Chemical Co., St. Louis, MO; Grade III), was used as antigen. In two experiments, thermally aggregated (2 min at 100°C) 4% Ova solutions (HD-Ova) were used. Cyanogen bromide-degraded ovalbumin (CNBr-Ova) was a gift from Dr. L.M. Lopes.

Protease inhibitors

Aprotinin, a serine-protease inhibitor, was dissolved in saline and 2 mg was given by gavage to mice 10 min before gavage with 20 mg Ova. Egg white ovomucoid, another trypsin inhibitor, was used at 50 mg/mouse by gavage.

Treatment with hydroxyurea

Hydroxyurea (HU) (15% in saline) was injected intraperitoneally (*ip*) at two doses of 1 mg/g body weight, separated by 7 h (1 cycle), a treatment sufficient to destroy the majority of cells in mitosis (5,16).

Oral tolerance induction

Mice were lightly anesthetized with ether and received a single dose of 20 mg Ova in 0.5 ml saline (0.15 M NaCl) by gavage. As immune controls, mice were gavaged with 0.5 ml saline. Gavages were given 7 days before primary immunization.

Administration of Ova in different gut regions

Normal mice were anesthetized with 50 µl Diempax® (5 mg/ml) *ip* followed 10 min later by 50 µl Nembutal® (14 mg/ml) and submitted to laparotomy. A volume of 0.2 ml 10% Ova (20 mg/mouse) was injected directly into the stomach, into the cecum or into the distal portion of the small intestine. Control mice were injected with 0.2 ml saline into the stomach.

Passive serum transfer of tolerance

Donor mice were bled by cardiac puncture under light ether anesthesia 1 h after gavage with 20 mg Ova in 0.5 ml saline. Control animals were bled after gavage with 0.5 ml saline. Serum pools of Ova-gavaged and saline-gavaged mice were formed and immediately transferred to recipient mice. Each recipient received 0.8-1.0 ml of serum *ip*.

Parenteral test immunizations for antibody formation

Mice were immunized *ip* with 10 µg Ova + 1 mg $Al(OH)_3$ in 0.2 ml saline. The animals were boosted *ip* with 10 µg Ova in 0.2 ml saline without adjuvant 14 days thereafter. Retroorbital bleedings for antibody assays were performed 7 days after the booster.

Antibody assays

Anti-Ova antibodies were titrated by ELISA, as previously described (17). The results (designated ELISA*) are reported as the mean ± SEM of the sums of absorbance values read between 1/100 and 1/12,800 serum dilutions. In our model, readings of positive (immune) sera fell in the most linear part of the absorbance curve. Extensive testing in our laboratory and consulting with statisticians confirmed that the results expressed by ELISA*, which were based on readings of 6 dilutions of each individual serum, are more reliable than evaluations based on a single serum dilution ("antibody titer" or those referred to as a standard antibody curve). Moreover, essentially the same results were obtained by evaluation based on a single serum dilution ("antibody titer"). Statistical significance ($P<0.05$) of differences between means was assessed by the Scheffé (ANOVA) test.

Parenteral test immunizations for DTH reactions

Mice were immunized subcutaneously (*sc*) at the base of the tail with 40 µl 2.5% Ova (100 µg/mouse) emulsified in Freund's complete adjuvant (CFA, Difco, Detroit, MI) 7 days after the tolerance-inducing treatments. Three weeks thereafter, the animals were injected *sc* into the left foot pad with 30 µl of 2% thermally aggregated Ova (600 µg/mouse; 2 min at 100°C) and into the right footpad with 30 µl saline. The thickness of the foot pads was measured with a caliper after 24 h.

Results

Mice were gavaged with 20 mg HD-Ova or CNBr-Ova and immunized 7 days later with Ova + CFA for DTH testing, or with Ova + $Al(OH)_3$ for antibody assays (see Methods). Pretreatment with either native Ova or HD-Ova significantly reduced DTH reactions to Ova. However, HD-Ova was not effective in blocking anti-Ova antibody formation (Figure 1), whereas CNBr-Ova blocked anti-Ova antibody formation (Figure 2). The ability of CNBr-Ova to block DTH reactions was not tested.

Mice injected with 20 mg native Ova directly into the stomach, the cecum or the ileum were immunized with Ova + CFA 7 days later and tested for DTH reactions 7

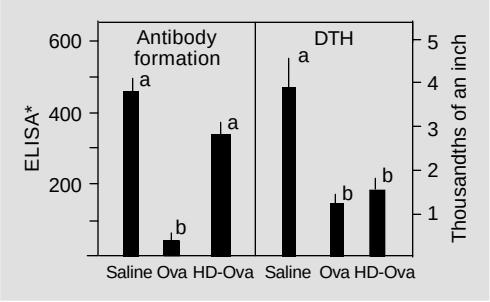

Figure 1 - Antibody formation and delayed-type hypersensitivity (DTH) in groups of 5-7 B6D2F1 mice pretreated (day -7) with 0.5 ml saline (controls), 20 mg native Ova or 20 mg heat-denatured ovalbumin (HD-Ova) by gavage. Immunization on day 0 for antibody formation with Ova + $Al(OH)_3$; immunization for DTH with Ova + CFA (see Methods). Bars with the same small letter indicate no statistically significant difference ($P<0.05$; ANOVA, Tukey test).

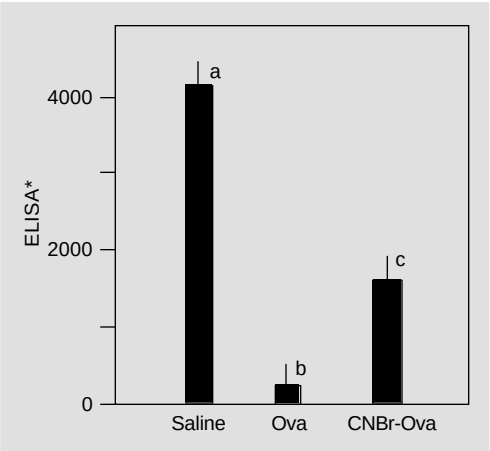

Figure 2 - Antibody formation in groups of 5-7 B6D2F1 mice pretreated (day -7) with 0.5 ml saline (controls), 20 mg native Ova or 20 mg cyanogen bromide-treated Ova (CNBr-Ova) by gavage. Immunization on day 0 for antibody formation with Ova + $Al(OH)_3$. The ELISAS* for the three groups were significantly different ($P<0.05$; ANOVA, Tukey test).

days thereafter. Mice injected into the stomach were significantly less reactive, whereas mice injected into the cecum or the ileum were only partially tolerant (Figure 3). None of these animals showed significant reduction in the anti-Ova antibody levels present in the circulation 20 days later as demonstrated by ELISA (data not shown).

Mice received 2 mg aprotinin or 50 mg ovomucoid (protease inhibitors) 10 min before gavage with 20 mg Ova or saline. As shown in Figure 4, these treatments failed to influence the induction of oral tolerance to Ova.

We established that one cycle of treatment with *ip* HU (see Methods) applied 24 h before gavage with 20 mg Ova blocked the induction of tolerance, as assessed by the inhibition of DTH reactions (data not shown). We then determined whether this same treatment with HU 24 h before gavage would block the passive transfer of tolerance to normal recipients with serum collected 1 h after gavage of the donors with 20 mg Ova, as measured by DTH reactions. Transfer of 1.0 ml of a serum pool from donors pretreated with HU and then gavaged with Ova resulted in no inhibition of DTH reactions; this transfer was equivalent to the transfer of serum from animals gavaged with saline; on the other hand, serum from positive control animals that received no pretreatment with HU and were gavaged with Ova inhibited DTH reactions (Figure 5).

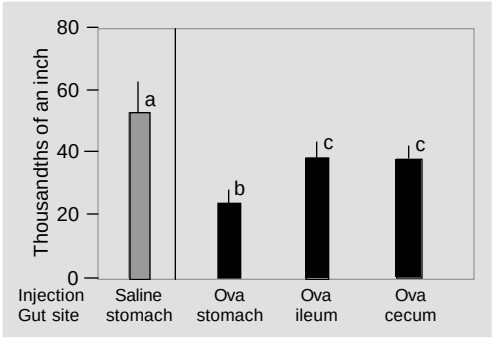

Figure 3 - Delayed-type hypersensitivity (DTH) in groups of 5-7 B6D2F1 mice pretreated (day -7) by the administration of 0.2 ml saline or 10 mg Ova in 0.2 ml saline directly into the stomach, the ileum or the cecum. Immunization on day 0 with Ova + $Al(OH)_3$. Bars with the same small letter are not significantly different ($P<0.05$; ANOVA, Tukey test).

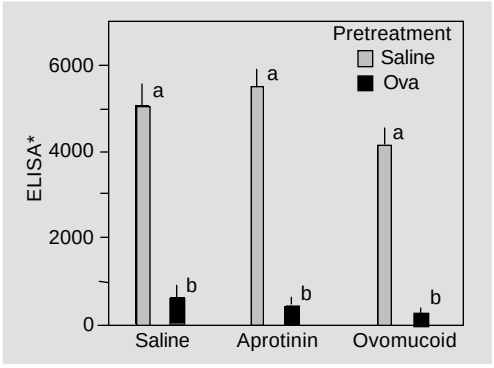

Figura 4 - Antibody formation in groups of 5-7 B6D2F1 mice pretreated (day -7) with 0.5 ml saline (controls) or 20 mg native Ova by gavage. On day -7, 20 min before gavage with saline or Ova, mice received gavage with saline, 2 mg aprotinin or 50 mg ovomucoid (protease inhibitors). Immunization on day 0 for antibody formation with Ova + $Al(OH)_3$. Bars with the same small letter are not significantly different ($P<0.05$; ANOVA, Tukey test).

Discussion

As a T cell-dependent phenomenon, oral tolerance is not expected to depend necessarily on the native configuration of antigens. Thus, it might be induced with thermally (HD-Ova) or chemically (CNBr-Ova) degraded Ova (18,19). As shown in Figure 1, gavage with HD-Ova resulted in a significant inhibition of DTH reactions, but there was no parallel reduction of anti-Ova antibody formation (Figure 2) which is known to occur in marginal states of oral tolerance (2). Gavage with CNBr-Ova induced a small but significant decline in specific antibody formation. Peng et al. (19) have recently shown that urea-denatured ovalbumin and carboxymethylated Ova (CM-Ova) did not induce oral tolerance to native Ova. In the same investigation, they showed that the absorption of CM-Ova is lower and its susceptibility to proteases is higher than that of native Ova. They suggested that the inefficacy of these materials in inducing oral tolerance to Ova may be ascribed to these properties. In our experiments with heat-denatured Ova there was a small but significant inhibition of tolerance induction. We used different conditions of denaturation and took no precaution to avoid renaturation of Ova.

The induction of oral tolerance with CNBr-Ova suggests the participation of peptides in the induction of oral tolerance. Hanson et al. (10), working with Ova in B6D2F1 mice, found that pretreatment with

aprotinin blocked oral tolerance. We were unable to duplicate these results either with aprotinin or with ovomucoid (Figure 3). Mowat (2) reported results similar to ours. Pretreatment with aprotinin before gavage increases the concentration of native Ova absorbed to the blood, and this increase may be involved in the inhibition of tolerance (9,10). Treatment with non-steroid anti-inflammatory drugs, such as indomethacin, increases the absorption of Ova from the gut and blocks the induction of oral tolerance (20). There is no consensus, therefore, concerning the role of proteolytic enzymes in the induction of oral tolerance. Our results with direct injection of Ova into different regions of the gut showed intragastric injection to be superior to injections directly into the ileum or the cecum (Figure 4). Whether this depends on the upstream position of the stomach and/or on the larger concentration of lymphoid elements in the upper small intestine or still other factors is as yet to be resolved. The number of lymphoid cells in the lamina propria of the gut falls gradually along the gut, being largest in the duodenum and lowest in the large intestine (21,22).

An important finding on oral tolerance induction was the possibility of passive transfer of tolerance with serum of animals that ingested the antigen 1 h before (11,12,23,24). As in other observations in oral tolerance induction, only DTH reactions, and not antibody formation, were affected. The transfer of serum of mice parenterally injected with Ova does not transfer tolerance (23) and, although the passage of the serum through affinity columns to remove Ova-specific material removes the tolerogenic moiety (22), the tolerogenic properties of the serum are not related to the concentration of intact Ova (12) and the tolerogen is not produced after gavage of immunodeficient (SCID) mice with Ova (13). The tolerogen is produced by BALB.B (H-2^b) mice, which are not susceptible to oral tolerance (25), and thus its production seems to depend on gut processing of the antigen. Recently, Furrie et al. (14) tentatively characterized the tolerogen as a 21-24-kDa Ova moiety which is still able to bind to anti-Ova antibodies.

Tolerance induced by passive transfer of serum is susceptible to treatment with cyclophosphamide (11), suggesting that it depends on cycling cells. Data from our laboratory have previously shown that treatment with HU, which destroys cycling cells (16), is able to block oral tolerance induction if applied 24 h before gavage with Ova (5). In mice pretreated with HU, the passive transfer of syngeneic lymphoid cells restored the susceptibility to oral tolerance, suggesting that the relevant effects of HU were not related to anti-mitotic effects on non-lymphoid tissues, such as the gut epithelium (5). In the present experiments, we confirmed that this treatment, as predictable, was able to block oral tolerance as assessed by DTH reactions (data not shown). We then showed that the treatment with HU 24 h before gavage blocked the emergence of the tolerogenic material in serum, which in turn blocked the transfer of oral tolerance (Figure 5). These results suggest that the generation of the tolerogen requires the activity of cycling cells. Whether these are lymphoid cells was not ascertained by our experiments, but experi-

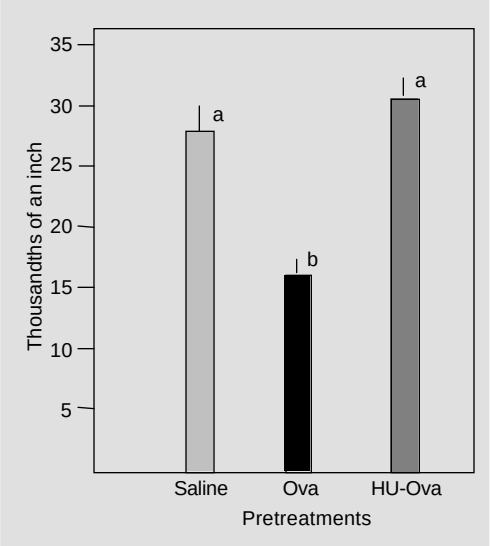

Figure 5 - Delayed-type hypersensitivity (DTH) in groups of 5-7 B6D2F1 mice pretreated (day -7) with 0.8 ml of serum pools collected from mice gavaged 1 h before with either saline or 20 mg Ova. Half of the mice gavaged with 20 mg Ova had been treated with one cycle of hydroxyurea (HU) 24 h before to destroy cycling cells. On day 0, mice were immunized with Ova + CFA and tested in the foot pad 7 days later. Bars with the same small letter are not significantly different ($P<0.05$; ANOVA, Tukey test).

ments with SCID mice (13) suggest they are.

Thus, the induction of oral tolerance depends on the way the antigen is initially encountered. The relative importance of extracellular (e.g., digestive proteolysis) or intracellular processing remains to be established.

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