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Coupling of palmitate to ovalbumin inhibits the induction of oral tolerance

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Abstract

Oral tolerance is a phenomenon that may occur in animals exposed to protein antigens for the first time by the oral route. They become unable to produce immune responses at the levels normally observed when they are immunized parenterally with antigen in the presence of adjuvants. Lipids have been used as adjuvants for both parenteral and oral immunization. In the present study we coupled ovalbumin with palmitate residues by incubating the protein with the N-hydroxysuccinimide palmitate ester and tested the preparation for its ability to induce oral tolerance. This was performed by giving 20 mg of antigen to mice by the oral route 7 days prior to parenteral immunization in the presence of $Al(OH)_3$. Mice were bled one week after receiving a booster that was given 2 weeks after primary immunization. Specific antibodies were detected by ELISA. Despite the fact that the conjugates are as immunogenic as the unmodified protein when parenterally injected in mice, they failed to induce oral tolerance. This discrepancy could be explained by differences in the intestinal absorption of the two forms of the antigen. In fact, when compared to the non-conjugated ovalbumin, a fast and high absorption of the lipid-conjugated form of ovalbumin was observed by "sandwich" ELISA.

Key words
- Oral tolerance
- Lipid-protein conjugates
- Antigen absorption
- Gut mucosa
- Ovalbumin

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The gut mucosa represents the principal region through which most of the antigens penetrate the body. This is mainly due to its extensive area, as well as to the presence of different substances derived from food and of the intestinal flora. The absorption of such antigens is mainly due to the presence and activity of M cells which are in close association with the Peyer's patches (1,2). In the local lymphoid tissue, antigens may induce the production of secretory IgA, which also regulates their absorption, as well as a phenomenon called oral tolerance (3-5). In this case, animals that have been orally treated with antigen usually do not produce humoral or cellular immune responses when they are later parenterally immunized, even in the presence of adjuvants (6-8). Many factors can interfere with the occurrence of oral tolerance, such as the nature of the antigen as well as the dose, frequency and time interval between doses (9). There are also other host-related factors, such as its immunological history, age and genetic background, that interfere with the phenomenon (8-13).

In most of our studies on the induction of oral tolerance (5,8,10,13) we have chosen B6D2F1 mice (an abbreviation for (C57/Bl6

x DBA/2) F1 mice) as hosts and ovalbumin (Ova) as antigen. The possibility of coupling hydrophobic palmitate residues to proteins, which interested us when studying antigen processing and presentation by B cells carrying surrogate antigen receptors (14-17), opened new perspectives for the study of the mechanism of oral tolerance induction. Lipids have been used as adjuvants in many protocols for both parenteral and oral immunization.

The aim of the present study was to determine whether the coupling of palmityl residues to ovalbumin would affect oral tolerance induction in B6D2F1 mice of both sexes, obtained from our breeding unit at the Federal University of Minas Gerais, and used when approximately 8 to 12 weeks old. The lipid-ovalbumin conjugates (Ova-palm) were prepared according to a technique previously described by our group (17), although some small modifications were introduced. Briefly, 1 g of the N-hydroxysuccinimide palmitate ester (Sigma Chemical Co., St. Louis, MO) dissolved in 100 ml of absolute ethanol (55°C) was mixed with 4 g chicken egg albumin (ovalbumin) grade V (Sigma) dissolved in 800 ml of phosphate-buffered saline (PBS) with 0.1% $NaHCO_3$, pH 8, in the presence of 0.6% deoxycholate (DOC; Sigma). After overnight incubation at room temperature, the mixture was centrifuged and the precipitated conjugate (Ova-palm) was collected and washed in 50 mM $NaHCO_3$. After freezing in liquid nitrogen, the precipitate was lyophilized.

In order to test the tolerogenic properties of the conjugate, we performed the following protocol for the induction of oral tolerance (by gavage) which we had described previously for ovalbumin (5). Briefly, after being lightly anesthetized with ether, mice were intubated with a urethral polyvinyl catheter calibrated to reach the stomach, and given 20 mg of antigen (Ova or Ova-palm) in 0.3 ml of saline (0.15 M NaCl) intragastrically (*ig*). Gavages were performed 7 days prior to parenteral immunization, when mice were then injected intraperitoneally (*ip*) with 0.5 ml saline containing 10 µg Ova or Ova-palm mixed with 1 mg $Al(OH)_3$ as adjuvant. Two weeks later, mice were submitted to the same protocol as a booster, but without $Al(OH)_3$. Mice were bled one week after secondary immunization, and after clotting and centrifugation the serum samples were collected and stored at -20°C. The presence of anti-Ova antibodies in the samples was assayed by ELISA procedures. Briefly, polystyrene plates (Nunc, Copenhagen, Denmark) were coated overnight at 4°C with 2 µg Ova diluted in 100 µl coating buffer per well, washed with saline containing 0.05% (w/v) Tween-20, saturated with 0.25% (w/v) casein in PBS, washed again and then coated with serial dilution of mouse antiserum starting at 1:40. After 1 h at 37°C, plates were washed, incubated for 1 h at 37°C with goat anti-mouse globulin antiserum conjugated to peroxidase (Southern Biotechnology, Birmingham, AL), washed and developed by the addition of H_2O_2 and ortho-phenylene-diamine (OPD; Sigma); the reaction was interrupted at 20 min by the addition of H_2SO_4 at 1/20, and read at 492 nm in EIA-reader (Bio-Rad, Hercules, CA). The absorbance values obtained in the assays were reported as a score (ELISA*), which represents the mean ± SEM (N = 4-6) of the sums of the absorbance values of ELISA tests run with serum dilutions of 1/40 to 1/5120. This way of expressing the results was equivalent to reporting the area under the titration curves or electing absorbance at one particular serum dilution as representative. The significance of the difference between experimental and control groups was assessed by two-tailed Student *t*-tests. Positive and negative control samples were run on every plate to standardize the assays.

As shown in Figure 1, mice produced the same amount of anti-Ova antibodies after being orally treated with saline and immunized with either Ova-palm or unmodified

Ova. This happened despite the presence of hydrophobic residues in Ova-palm conjugates, which might modify the shape of the protein and induce the display of a different set of epitopes. This view is supported by the fact that the conjugates are not as soluble as ovalbumin. As expected, mice orally pretreated with Ova displayed significantly lower levels of anti-Ova antibodies after being parenterally immunized with either form of the antigen. On the other hand, oral pretreatment with Ova-palm did not induce tolerance. Both forms of the antigen were able to elicit the production of anti-Ova antibodies at the same level as controls.

Since Ova and Ova-palm conjugates exhibited similar immunogenic characteristics but different tolerogenic properties, we designed a protocol to evaluate the kinetics of their intestinal absorption. Mice were treated orally with both forms and, at different times, 100 µl of blood samples were collected from the retro-orbital plexus with calibrated micropipettes (H.E. Pedersen, Copenhagen, Denmark). Samples were immediately diluted in 0.4 µl saline and, after clotting and centrifugation, the supernatant was collected for detection of the antigen by "sandwich" ELISA. Briefly, rabbit anti-Ova antibodies were purified by affinity chromatography using sepharose-4B columns (Pharmacia, Uppsala, Sweden) coupled with Ova. These antibodies were used to coat polystyrene plates as described above. After blocking with PBS/casein, washing and addition of supernatants, plates were incubated for 1 h. The same rabbit anti-ova antibodies were biotinylated. Briefly, 2 mg of antibodies and 50 µg of biotinyl-N-hydroxysuccinimide ester (Sigma) were dissolved and mixed in 1.0 ml 0.1 M $NaHCO_3$. After a 4-h incubation at room temperature, the solution was dialyzed against PBS. The biotinylated antibodies were allowed to react with streptavidin coupled with peroxidase (Sigma) for 1 h and the reaction was completed as described above. A standard solution of ovalbumin was run in parallel for quantification of the antigen in the samples. As we can see in Figure 2, when compared to mice orally treated with Ova, the antigen was found earlier and at higher levels in the blood of mice that were orally treated with the same amount of Ova-palm.

In the present communication, we have shown that ovalbumin coupled with palmitate residues is unable to induce oral tolerance. On the other hand, its immunogenic property remains the same when administered parenterally. We also noticed that antibodies produced by mice immunized with both forms of the antigen can be equally detected by ELISA using plates coated with either unmodified or palmitate-conjugated ovalbumin (data not shown). These data indicate that the antigenic characteristics, as well as the immunogenic properties, remain the same.

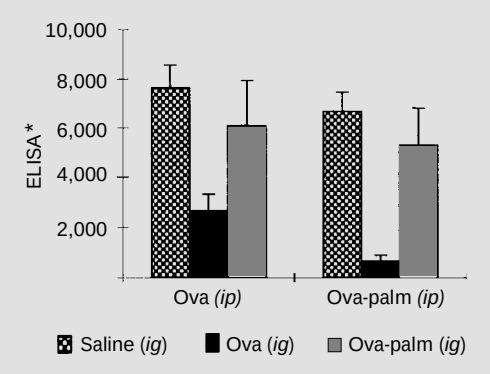

Figure 1 - Failure to induce oral tolerance with Ova-palmitate conjugates. B6D2F1 mice were pretreated with 20 mg of Ova, Ova-palm or saline (in 0.3 ml), *ig*, 1 week before being immunized with 10 µg of Ova or Ova-palm in $Al(OH)_3$, *ip*. Two weeks later, mice were submitted to the same protocol as a booster, but without $Al(OH)_3$. Mice were bled one week after secondary immunization. Anti-Ova antibodies were detected by ELISA. Data are reported as a score (ELISA*), which represents the mean ± SEM (N = 4-6) of the sums of absorbance values from serum dilutions of 1/40 to 1/5120.

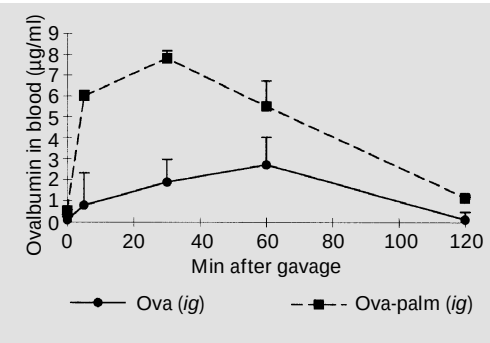

Figure 2 - Kinetics of antigen appearance in blood after intragastric administration. B6D2F1 mice were treated intragastrically with 20 mg of Ova or Ova-palm (in 0.3 ml saline). Animals were bled at several time intervals. Data detected by "sandwich" ELISA represent the mean ± SEM (N = 8-9) of antigen concentration in blood.

An explanation for the lack of tolerance induction by Ova-palm conjugates may be related to excess antigen. Antigen doses are crucial for oral tolerance induction (9). Since we observed high levels of intestinal absorption with the conjugates, we tried to induce oral tolerance with the conjugate using lower amounts of the antigen (10-100 times less), but again we were unable to induce oral tolerance (data not shown).

As mentioned earlier in this paper, although little is known concerning their mechanisms of action, lipids have been used as adjuvants for both parenteral and oral immunization. Their use includes the preparation of liposome vesicles, immunostimulating complexes (ISCOMs) and water-in-oil emulsions like Freund's complete and incomplete adjuvants (18-20). We suggest that ovalbumin coupled with lipid residues might induce immune responses instead of tolerance when administered by the oral route.

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