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Stabilization of serum antibody responses triggered by initial mucosal contact with the antigen independently of oral tolerance induction

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Abstract

Initial contacts with a T-dependent antigen by mucosal routes may result in oral tolerance, defined as the inhibition of specific antibody formation after subsequent parenteral immunizations with the same antigen. We describe here an additional and permanent consequence of these initial contacts, namely, the blockade of secondary-type responsiveness to subsequent parenteral contacts with the antigen. When repeatedly boosted *ip* with small doses (3 µg) of ovalbumin (OVA) (or lysozyme), primed B6D2F1 mice showed progressively higher antibody responses. In contrast, mice primed after a single oral exposure to the antigen, although repeatedly boosted, maintained their secondary antibody titers on a level which was inversely proportional to the dose of antigen in the oral pretreatment. This phenomenon also occurred in situations in which oral tolerance was not induced. For example, senile 70-week-old B6D2F1 mice pretreated with a single gavage of 20 mg OVA did not become tolerant, i.e., they formed the same secondary levels of anti-OVA antibodies as non-pretreated mice. However, after 4 weekly challenges with 3 µg OVA *ip*, orally pretreated mice maintained the same anti-OVA serum levels, whereas the levels of control mice increased sequentially. This "stabilizing" effect of mucosal exposure was dose dependent, occurred with different proteins and was triggered by single or multiple oral or nasal exposures to the antigen.

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- Stability
- Memory

Introduction

Single or repeated contacts of naive animals with strong T-dependent immunogens by the oral route frequently result in partial inhibition of responses to subsequent parenteral immunizations with these antigens in adjuvants. This phenomenon, known as oral tolerance (1-3), has recently received a great deal of attention due to its potential use in the prophylaxis and therapy of human allergic (4) and autoimmune (5) diseases. Nasal exposure to antigens may have similar consequences (4). Conversely, intermittent oral exposures to these immunogens, especially under conditions that do not favor tolerance

induction - for example, in aged animals (6) or less sensitive antigen-strain combinations (7,8), may result in "oral immunization", expressed as the emergence of significantly high serum titers of specific antibodies, without any further parenteral injections of the antigen.

We have previously observed that, in contrast to animals primed by parenteral routes, animals showing serum antibodies resulting from "oral immunization" are refractory to boosting with further parenteral immunization (9). In the present study we confirm and expand these observations and show that this stability of specific responsiveness arising after an initial mucosal exposure to the antigen is a general consequence of antigenic contacts initiated by mucosal routes, regardless of whether or not tolerance is induced. These steady states of specific antibody formation obtained either exclusively by "oral immunization" or by an initial mucosal contact followed by parenteral immunization may be established at low, medium or high serum antibody levels.

Material and Methods

Mice

Groups of 5-8 young adult (8-10 weeks old) (C57BL/6J x DBA/2J) F1, or B6D2F1 mice raised in our colonies were used in most experiments. In some experiments senile (70 weeks old) B6D2F1 mice or mature (20 weeks old) Biozzi's high-responder selection-III mice (or H-III) were used. H-III breeding pairs were a gift from Dr. O.A. Sant'Anna, Instituto Butantan, São Paulo, SP, Brazil.

Immunogens

Grades III or V hen's egg albumin (ovalbumin, OVA) and hen's egg white lysozyme (HEL) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Oral treatments (gavage)

Mice were lightly anesthetized with ether and intubated with a curved metal cannula (Animal Intubation Curved Needles with Spherical Tip, Thomas Scientific, Swedesboro, NJ, USA) attached to a 1-ml syringe for gavage administration of 0.5 ml saline or saline containing grade III OVA or HEL directly into the stomach.

Nasal instillation

Unanesthetized mice were hand held and made to inhale a total of 20 µl of either saline or grade III OVA solutions slowly dispensed into their nostrils in microdrops from a precision micropipette (Eppendorf Standard Fixed-Volume Pipette, 20 µl; Brinkmann Instruments, Westbury, NY, USA).

Parenteral immunizations

For "priming" (primary immunization), mice were injected intraperitoneally (*ip*) with 0.2 ml of a saline suspension containing either 1 mg $Al(OH)_3$ gel (Aldrox) as an adjuvant, or the adjuvant plus 10 µg of either OVA (grade V) or HEL. Subsequent *ip* boosters with 0.2 ml of saline containing 3 µg of either OVA (grade V) or HEL without adjuvant were administered.

Bleedings

A 200-µl amount of blood collected by retro-orbital puncture with calibrated micropipettes (H.E. Pedersen, Copenhagen, Denmark) was centrifuged after clotting; the serum was diluted 1:10 in saline and frozen for subsequent ELISA. A blood sample was collected immediately before an *ip* booster injection and 7 days after the last booster.

ELISA

Anti-OVA and anti-HEL antibodies were

assayed by standard ELISA. High absorption plates (Nunc, Roskilde, Denmark) were incubated overnight at 4°C with 2 µg OVA or HEL per well. The plates were then washed twice with saline containing 0.05% Tween 20 (Sigma) and saturated with a 0.25% casein solution in PBS at room temperature for 2 h. Plates were washed twice and incubated with 6 serial dilutions of test and control sera starting at 1:100 and ending at 1:102,400 (6 serial 1:4 dilutions). The plates were then washed 6 times with saline-Tween and incubated at 37°C for 1 h with peroxidase-labeled goat anti-mouse Ig serum (Southern Biotechnology Associates, Birmingham, AL, USA). Plates were again washed 6 times with saline-Tween and incubated for 20 min at room temperature and in the dark with citrate buffer, pH 5.0, containing H_2O_2 and orthophenylenediamine as a chromogen. Reactions were ended by adding 20 µl of 1:20 H_2SO_4 and the absorbance at 492 nm was read in an ELISA reader (model 450 Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA). For each serum, the absorbances of the 6 serial dilutions were added and multiplied by 1000 and these coded values were called ELISA* (ELISA scores), as done in previous studies by our group (6,10). These ELISA scores were used for statistical analysis.

Statistical analysis

ELISA scores were treated by analysis of variance (ANOVA) considering the different treatment groups as nominal independent variables (groups) and ELISA scores as continuous dependent variables. Within each experiment, a factorial model ANOVA was used to compare the different groups after the first *ip* booster (secondary responses) to determine the occurrence of oral tolerance. The sets of ELISA scores were compared by repeated measurements model ANOVA to determine the patterns developed after repeated boosters. In all the ANOVA analyses, the F-value for the independent variable was greater than 1 ($F>1$) and $P<0.05$. After these analyses were performed, the mean ELISA scores for each experimental group were compared by the *post-hoc* Scheffé's test with $P<0.05$. In all figures, only the significance of the differences between the control group and the various experimental groups is shown.

Results

Orally immunized mice are refractory to booster injections

Three groups of mature (20 weeks old) H-III mice were given 3 gavages with 20 mg OVA on days 0, 7 and 28. A first control group received 3 gavages with saline, a second control group received 3 gavages with 20 mg OVA (orally immune), and for the third group (experimental group), only the third gavage (day 28) contained 20 mg OVA. One week later (day 35) all animals were test bled to assess the result of oral exposures to OVA. As shown in Figure 1, on this day, mice exposed to 3 gavages with OVA displayed significant (orally induced) anti-OVA titers in serum, while control mice orally exposed to saline on these three days and mice exposed to OVA only in the third gavage displayed very low anti-OVA titers, not different from those found in normal mice.

On this day (day 35), all animals were immunized *ip* with OVA + $Al(OH)_3$ and 14 days later a series of 5 weekly boosters with 3 µg soluble OVA was started. The results were clear cut. In the first group, the succession of boosters resulted in progressively higher antibody titers. In the orally immunized group and in the experimental group exposed to OVA only in the third gavage, the succession of boosters failed to modify the antibody titers significantly. Thus, the experimental group, after an initial increase from very low to high titers (days 35 to 56), showed no further increase (days 56 to 84).

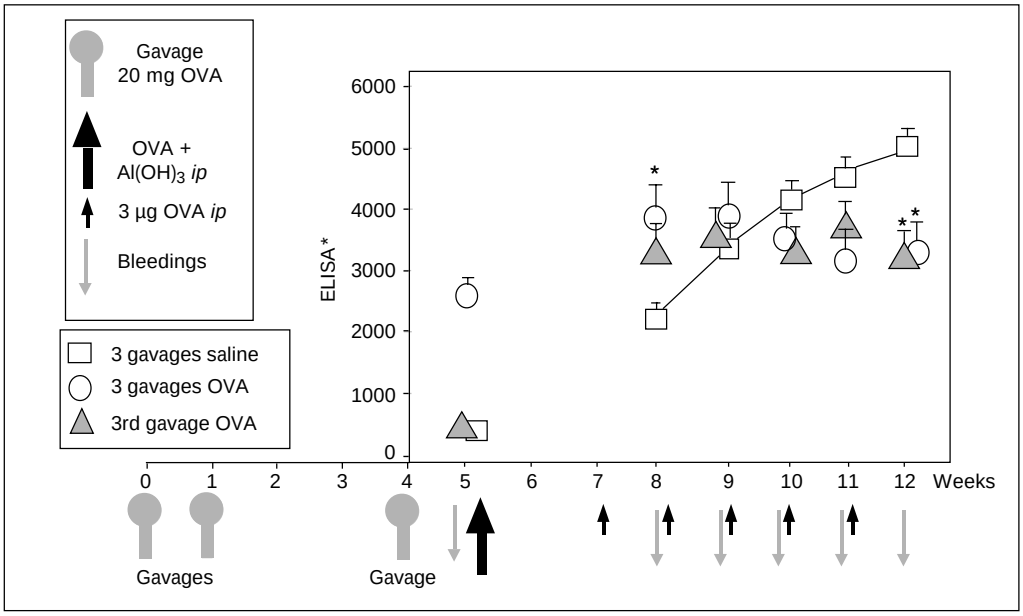

Figure 1 - Stabilization of anti-OVA antibody production in orally immunized mice. Control mice (squares) received 3 gavages with saline; orally immunized mice (circles) received 3 gavages with 20 mg OVA; experimental mice (triangles) received 2 gavages with saline and 20 mg OVA in the third gavage (4th week), one week before priming with 10 µg OVA + $Al(OH)_3$ *ip*. Starting two weeks after priming, all mice received 5 weekly *ip* boosters with 3 µg OVA. Asterisks indicate significant differences between control and experimental groups.

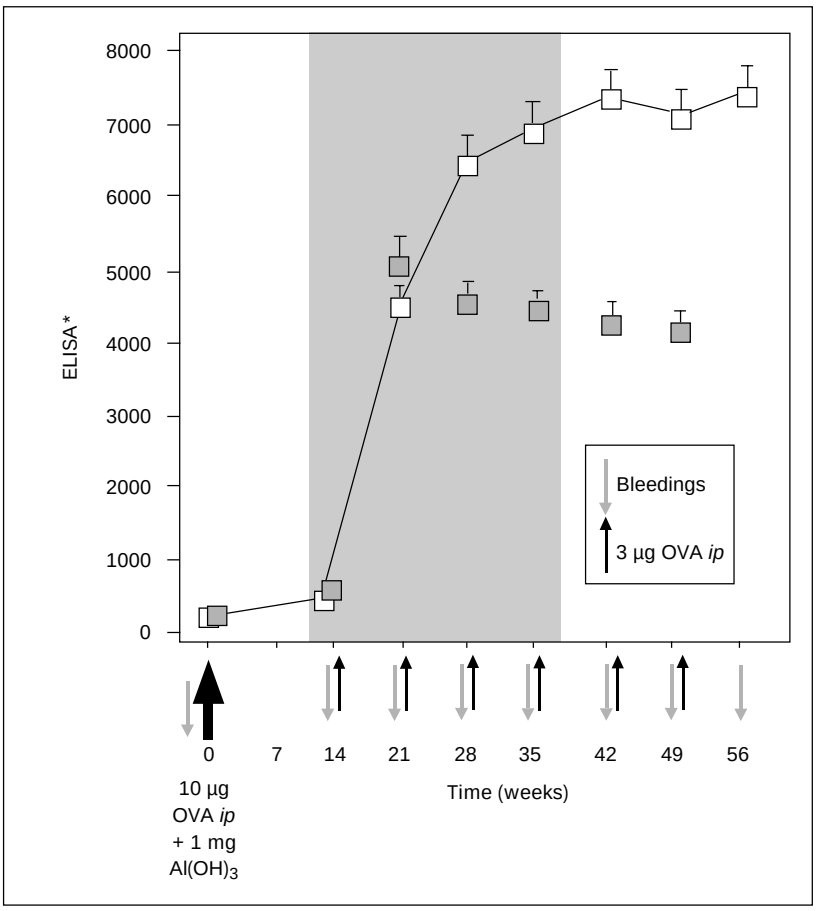

Figure 2 - Booster effect on the antibody response of parenterally immunized mice. After an initial bleeding to assess natural antibody levels, mice received 10 µg OVA + 1 mg $Al(OH)_3$ *ip*: starting 14 days later, a sequence of 6 weekly challenges with 3 µg OVA *ip* was performed (open squares); orbital bleedings were performed before each challenge. A control group of mice (filled squares) received only the first parenteral booster injection. The gray area displays the period during which the rising titers in sequentially boosted animals contrasted with the declining titers of the mice boosted only once.

A single initial gavage with 20 mg OVA, therefore, although unable to induce oral tolerance or oral immunization, conferred a strong refractoriness to further increases in antibody production on the animals.

This particular experiment was conducted with mature (20 weeks old) Biozzi's high-responder mice from selection-III (abbreviated H-III) (11) because their antibody formation during oral immunization to OVA is especially vigorous. As shown in the next experiments, however, similar results are obtained with other mouse strains and other antigens.

It might be argued that the inability to booster orally immunized mice by parenteral injection with small doses of antigen (10 µg) derives from neutralization by preformed antibodies. The results presented in Figure 2 show that this is unlikely. Mice primed by the parenteral route responded to subsequent sequential boosters with even smaller doses of antigen (3 µg). They displayed sequential increases in antibody formation (gray area in Figure 2), whereas a control group of mice boosted only once with 3 µg OVA *ip* on day 14 displayed slightly declining antibody titers. Control groups of mice injected *ip* with saline on the same days formed no anti-OVA antibodies throughout the experiment (data not shown).

Stabilization of serum antibody responses may be achieved at different levels, depending on the dose of antigen fed

Oral tolerance induction depends on several factors besides mouse strain, one of which is the dose of protein given orally (2,5,12-14). B6D2F1 mice are highly susceptible to oral tolerance induction when a single gavage of 20 mg OVA is used, but lower doses are not as efficient.

Figure 3 shows the result of oral pretreatment with different doses of either OVA or HEL before parenteral immunization. After priming with the specific antigens in $Al(OH)_3$ *ip*, a series of booster injections and orbital bleedings similar to those performed in the previous experiment were performed. The gray area in Figure 3 (day 28) highlights the secondary responses. These results are highlighted because they correspond to the values classically used to characterize the induction of oral tolerance. Upon the booster injections of 3 µg of antigen *ip*, the specific antibody titers of control mice orally pretreated with saline and serially boosted steadily increased. Oral pretreatment of mice with either 20 mg OVA or 10 and 30 mg HEL induced oral tolerance, as measured by the bleeding 7 days after the first booster (day 28). In this bleeding (day 28) the anti-

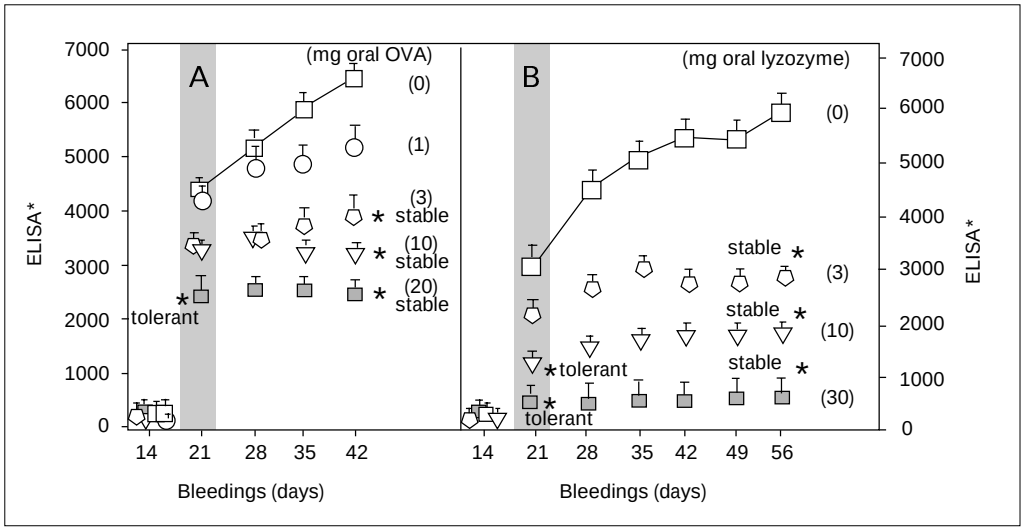

Figure 3 - Stabilization of serum antibody responses with different doses of fed antigen. *A*, Mice received a single gavage with saline (controls, open squares) or saline containing either 1 mg (circles), 3 mg (pentagons), 10 mg (triangles) or 20 mg OVA (filled squares), 7 days before primary *ip* immunization with 10 µg OVA + 1 mg $Al(OH)_3$ *ip*; 14 days later a series of 4 *ip* weekly challenges with 3 µg soluble OVA was initiated. The gray area highlights secondary responses to OVA. *B*, A similar experiment performed using lysozyme as the antigen. Asterisks indicate significant differences between control and experimental groups.

OVA and anti-HEL titers of mice orally pretreated with lower doses of antigen were not significantly different from those of saline-pretreated mice. However, in the subsequent bleedings, in spite of the repeated boosters, the anti-OVA titers of mice pretreated with 3 mg and 10 mg OVA or the anti-HEL titers of mice pretreated with 3 mg HEL remained stable; mice pretreated with 1 mg OVA were not significantly "stable" ($P = 0.56$).

These data suggest that, more subtly than oral tolerance, the stabilization of antibody production is an indicator of previous oral exposure to antigens.

Repeated oral exposures are more efficient than a single gavage in evoking both oral tolerance and the refractoriness to booster reactions

In the next experiment, B6D2F1 mice received gavages with saline alone or saline containing different doses of OVA on 3 consecutive days and were then submitted to the standard sequence of *ip* immunizations with OVA.

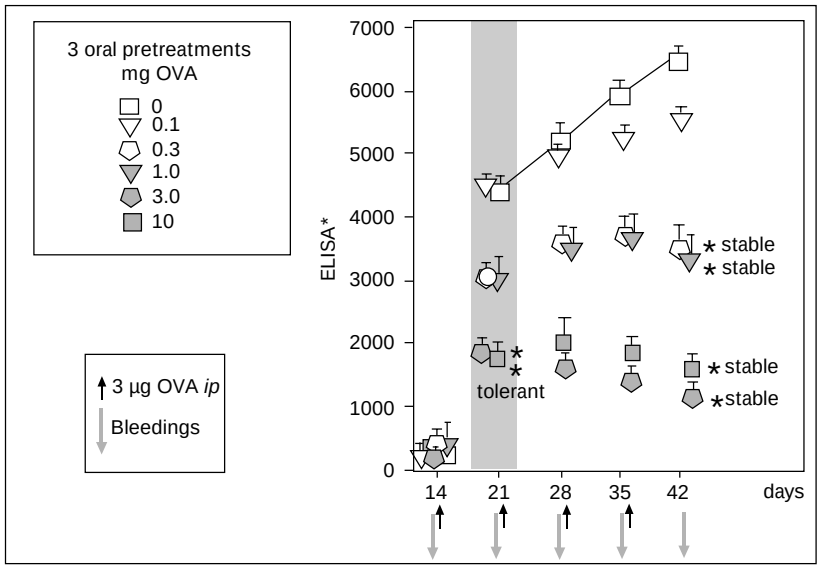

Figure 4 - Effect of repeated oral exposures to antigen on the stabilization of serum antibody responses. Mice received 3 gavages with saline (controls, open squares) or with saline containing either 0.1 mg (open triangles), 0.3 mg (open pentagons), 1.0 mg (filled triangles), 3.0 mg (filled pentagons) or 10 mg OVA (filled squares), 7 days before primary *ip* immunization with 10 µg OVA + 1 mg $Al(OH)_3$ *ip*; 14 days later a series of 4 *ip* weekly challenges with 3 µg soluble OVA was initiated. Immediately before each booster the mice were bled for measurement of antibodies by ELISA. Asterisks indicate significant differences between control and experimental groups.

Figure 4 shows that, as measured 7 days after the first *ip* booster, only the gavages with 3 mg or 10 mg OVA resulted in a significant reduction of anti-OVA antibody formation, i.e., induced oral tolerance ($P<0.001$). In the remaining experimental groups the secondary antibody titers were not significantly different from those of control animals. However, the mice in two of the remaining groups, those pretreated with 1 mg or 0.3 mg OVA, maintained stable antibody titers following the sequential boosters ($P<0.01$ and $P = 0.01$, respectively), a treatment which progressively increased antibody titers in control mice. Mice orally pretreated with 0.1 mg OVA were not significantly different from control mice ($P = 0.44$). A comparison between these last two experiments (Figures 3 and 4) shows that repeated oral exposures are more efficient than a single gavage in evoking both oral tolerance and the refractoriness to booster reactions.

Stabilization of serum antibody response following pretreatments by the nasal route

Similarly to oral contacts, nasal exposures to antigen have been shown to either inhibit or enhance subsequent responsiveness to parenteral immunizations (4). We repeatedly pretreated mice with OVA by the nasal route at doses ranging from 1 to 25 µg. None of the chosen doses of OVA was able to induce tolerance (Figure 5); actually, the responses of mice instilled with the largest dose of OVA (25 µg) were significantly higher than those of control mice instilled with saline ($P = 0.047$). Nevertheless, the animals showed a stable response. Mice pretreated with 5 or 10 µg OVA were significantly more stable ($P\leq0.03$) than control mice, whereas those instilled with 1 µg or 2.5 µg were not significantly different from controls ($P = 0.71$ and $P = 0.81$).

In this experiment, therefore, none of the experimental groups developed tolerance, but three out of these five groups became stable and the two remaining ones followed an intermediate path.

Stabilization of antibody responses in the absence of oral tolerance in senile B6D2F1

In the next two experiments we tested the effects of initial mucosal antigenic contacts in old mice which are refractory to oral tolerance induction (3,6,7). Seventy-week-old B6D2F1 mice were pretreated with either saline or saline containing 20 mg OVA. As expected, the OVA-pretreated mice did not (P = 0.27) become tolerant (Figure 6). Nevertheless, they did become refractory to booster challenges upon subsequent immunizations (P = 0.048). As observed in previous experiments (15), the control saline-pretreated senile mice were less able than young mice to develop progressively higher booster responses.

Discussion

Oral tolerance is classically demonstrable by the suppression of primary and secondary humoral and cell-mediated responses after parenteral immunization with antigens previously contacting the host by the oral route (2,5,12,14,16). In some situations, depending on the strain or dose of antigen used, tolerance induction may be only "partial" and when the magnitude of the responses is not significantly different from that of control animals it is simply assumed that nothing has happened. Another possible outcome of mucosal contacts with antigens is oral immunization which, in contrast with tolerance or parenteral immunization, results in significant serum titers of specific antibodies without further parenteral contacts with the antigen. There are thus three possible results of oral exposures: oral tolerance, partial oral tolerance (or even absence of tolerance) and oral immunization. Here we have shown that there is an additional consequence of mucosal contacts with antigens that can be clearly observed after a sequence of *ip* immunizations with low doses

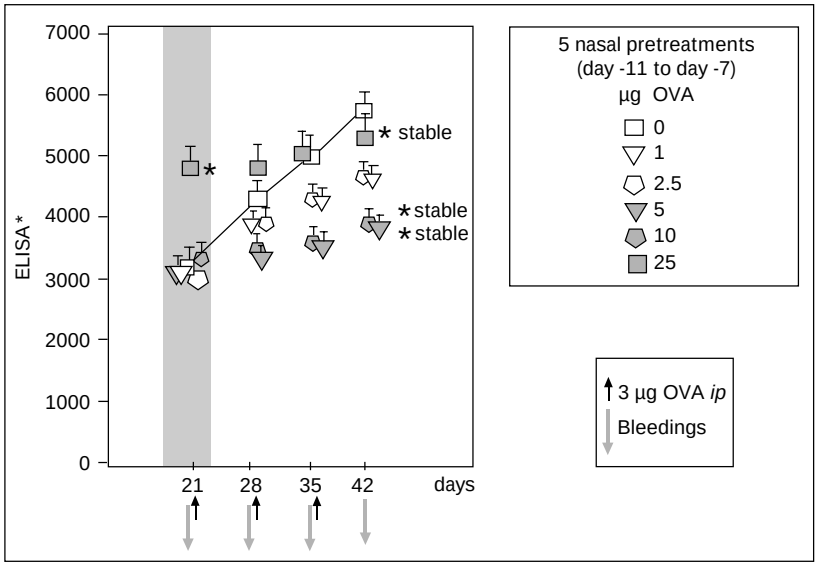

Figure 5 - Stabilization of serum antibody responses by nasal treatment with antigen. B6D2F1 mice were pretreated by nasal instillation for 5 consecutive days with either a microdrop (20 ml) of saline (open squares) or saline containing variable doses of OVA per exposure. Pretreatments ended 7 days before primary immunization with 10 µg OVA + 1 mg $Al(OH)_3$ *ip*; 14 days later, a series of 4 weekly challenges with 3 µg soluble OVA was initiated. Immediately before each booster the mice were bled for measurement of antibodies by ELISA. Asterisks indicate significant differences between control and experimental groups.

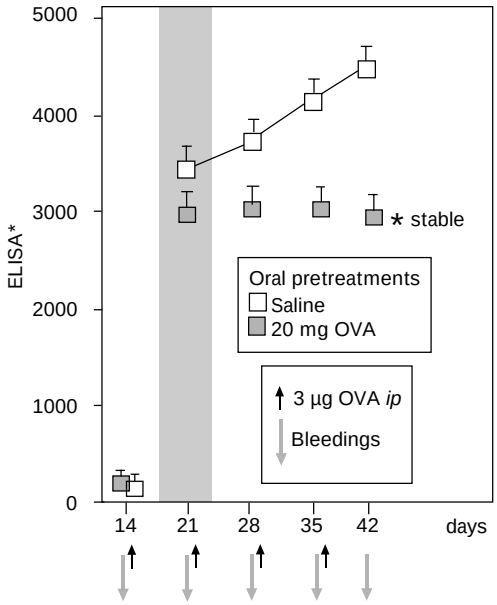

Figure 6 - Stabilization of serum antibody responses in the absence of oral tolerance in senile mice. Seventy-week-old B6D2F1 mice were pretreated with one gavage of either saline (open squares) or saline containing 20 mg OVA (filled squares) 7 days before primary immunization with 10 µg OVA + 1 mg $Al(OH)_3$ *ip*; 14 days later, a series of 4 weekly challenges with 3 µg soluble OVA was initiated. Immediately before each booster the mice were bled for measurement of antibodies by ELISA. Asterisks indicate significant differences between control and experimental groups.

of soluble antigens: the stabilization of serum antibody responses, i.e., a blockade of secondary-type responsiveness upon repeated exposures. This is a general consequence of oral exposures, common to oral tolerance, partial (or absence) of oral tolerance and oral immunization. The stabilization of specific antibody formation may occur at low, medium or high serum antibody levels.

The "stabilizing" effect was dose dependent (Figures 3 and 4), occurred with different proteins (Figure 3), was present in young and old mice (Figure 6) and was triggered by both the oral and nasal routes (Figure 5) even after a single initial oral exposure to the antigen (Figures 1, 3, and 6).

We suggest that oral tolerance or, more generally, mucosal tolerance, thus represents a particular instance of this stabilizing phenomenon, i.e., oral (mucosal) tolerance could be described as a situation in which antibody formation is stabilized at low levels. However, as we presently show, depending on the experimental conditions, specific responsiveness may also be stabilized at medium or high levels. In these situations, i.e., stabilization in the presence of ongoing high rates of antibody formation, we are neither dealing with deletion or anergy of responsive clones, nor with what is generally designated as "suppression" of immune responsiveness: we are facing high levels of stable responsiveness. In these situations specific immune responsiveness cannot be described as inhibited, or suppressed.

Nor can we say that the stabilization attained by initial mucosal contacts involves what is meant by immunological memory or secondary-type responsiveness: it is actually its operational reverse. Memory, or secondary-type responsiveness, is usually understood as promptness to respond, or the ability to undergo longer, higher and more rapid specific changes. This is what actually happens when the initial contact with the antigen occurs through the parenteral route. In the phenomenon we presently describe, the exact opposite occurs, i.e., the organism develops the ability to maintain a stable specific responsiveness upon repeated exposures to the antigen. The stability of the immune response triggered by initial mucosal contacts may be useful for exploiting vaccine development.

Immunoblotting methods allowing the analysis of the global reactivity of circulating immunoglobulins with complex antigen mixtures (17,18) have shown that the organism maintains a remarkable stability in its profiles of "natural" immunoglobulins throughout its life (19,20). Changes in natural immunoglobulin production, therefore, are highly restricted (21), although influenced by genetic and ontogenetic factors (e.g., maternal effects, aging, etc.) (17,18). On the other hand, specific immune responses to parenteral injections of antigen, which are usually considered to be the standard immunological phenomena, surprisingly may fail to trigger major changes in immunoblotting profiles (22). It is currently unknown whether or not the stabilization of responsiveness which we are presently describing is related to these stable profiles of natural immunoglobulins revealed by immunoblotting with complex antigenic mixtures. One of our major present goals is to analyze immunoblotting profiles of animals with stabilized responsiveness to external antigens following mucosal contacts. The understanding of both phenomena - those we are presently describing and the restrictions occurring in natural antibodies (21,23) - may require major modifications of our present understanding of the immune system.

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