

Bystander Effect in Synergy to Anergy in Oral Tolerance of *Blomia Tropicalis*/Ovalbumin Murine Co-Immunization Model

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Oral tolerance is an important approach in allergic diseases and murine model can provide useful information to improve its understanding and therapeutic measures. To address the influence of non-related allergen sensitization in immunized mice with the mite *Blomia tropicalis* (Bt) or ovalbumin (OVA) or with both Bt/OVA allergens. Furthermore, we sought to verify oral tolerance effect in the Bt/OVA co-immunization model. Mice sensitized with Bt and then exposed to OVA developed an enhanced IgE response to both allergens; contrariwise, this effect was not observed when OVA-sensitization was prior to Bt-sensitization. Co-injection of Bt and OVA led to a dominant IgE response towards OVA over Bt, which was not observed when co-immunization was performed with a 240-fold less amount of OVA. Induction of oral tolerance with OVA, prior to co-immunization, suppressed IgE response to both allergens, probably as a consequence of the increased levels of IFN- γ found in these animals. The results evidenced that, depending on allergenic potential, new allergen exposure may exert an adjuvant effect to the first allergen used in the sensitization. The bystander suppression to non-related allergens through oral tolerance should be a useful mechanism to control sensitization to new allergens.

KEY WORDS: *Blomia tropicalis*; ovalbumin; co-immunization; IgE; cytokines; oral tolerance.

INTRODUCTION

The prevalence of atopy, allergic rhinitis, asthma and eczema has increased in recent decades. House-dust mites

are the commonest cause of allergic sensitization and are considered to be one of the main factors for the increasing incidence in IgE-mediated sensitization among patients. The mite *Blomia tropicalis* (Bt) is the most important allergen for allergic diseases in tropical and subtropical regions (1, 2). However, despite the bulky work investigating mites, there are scarce data on type-I hypersensitivity to Bt in murine models (3, 4).

Besides genetic predisposition, exposure to multiple inhalant allergens in daily life of atopic individuals may contribute to polysensitization. It has been considered that house-dust mite sensitization and, at a lower degree, pollen sensitization, probably play a role in triggering the development of polysensitization in atopic asthmatic children (5). Currently, allergen immunotherapies that alter the natural course of disease can reduce symptoms and may prevent the evolution towards polysensitization (6, 7).

Oral tolerance is the physiologic mechanism that prevents hypersensitivity to food proteins and probably to comensal bacteria (8, 9). This ability, inherent to the mucosal immune system, has increased the interest in studying oral tolerance in autoimmune (10–12) and in allergic diseases (13–15). The phenomenon of oral tolerance, which leads to systemic immunologic unresponsiveness, involves at least three main mechanisms: anergy, clonal deletion and suppression (10, 11, 16–20). The antigen dose has been shown to be an important factor, as high oral dose leads to T cell clonal deletion or anergy and low antigen dose favors active suppression (17, 21). Some regulatory T cells, involved in oral tolerance model, have been related to active suppression *in vitro* and *in vivo* through antigen-specific and effector non-specific manners (10, 21, 22). Bystander suppression occurs when an immune response to one epitope suppresses the response to another one by secreting suppressive cytokines that act unspecifically. Considering that oral tolerance could mediate bystander suppression and exert suppression to a

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non-related antigen, it is an attractive strategy for therapy in allergic individuals.

In the present study, we addressed the influence of a non-related antigen exposure on the type-I hypersensitivity response to Bt or to OVA. Moreover, the effect on the IgE response when both allergens, Bt extract and OVA, were co-injected as well as oral tolerance induction to OVA in co-immunized mice were investigated.

MATERIAL AND METHODS

Animals

Female A/Sn mice 8–10 weeks old, bred in our own animal facility (São Paulo University Medical School), and Wistar Furth rats (RT1^{u,u}), 3–4 months old, were used for passive cutaneous anaphylaxis (PCA) reaction.

Immunization Protocol

Groups of mice were injected subcutaneously (s.c.) with 20 μ g of *Blomia tropicalis* extract (Bt, 37.650 units of biological equivalents/mL, kindly provided by the International Pharmaceutical Immunology-ASAC) in 6 mg Al(OH)₃. Mice were boosted intraperitoneally (i.p.) on the 14th and 21st day after immunization (d.a.i.) with 20 and 100 μ g Bt, respectively, without adjuvant. Mice were bled through the retro-orbital plexus on the 28th day after immunization, and sera were stored at -20°C until use. A similar procedure was carried out for the ovalbumin immunization (OVA, Grade V, Sigma, St. Louis, MO). To assess the influence of a new allergen exposure on the ongoing sensitization to the mite Bt or to OVA, (i) groups of mice immunized with Bt extract were, 2 weeks later, subjected to the OVA-immunization protocol and (ii) groups of mice immunized with OVA were submitted to the Bt-immunization protocol 2 weeks later. Mice were bled at the end of each allergen-immunization protocol. Co-immunization was achieved through s.c. injection of Bt (20 μ g) and OVA (20 μ g) in 6 mg Al(OH)₃, and boosting was carried out through i.p. injection on the 14th d.a.i. with Bt (20 μ g) and OVA (20 μ g) in saline and, once again, on the 21st d.a.i. with 100 μ g of each allergen, without adjuvant. Another group of co-immunized mice received fixed concentration of Bt extract (20 μ g/0.2 mL) with decreased amounts of OVA (0.5, 0.166, or 0.08 μ g) in the co-immunization. Control groups were immunized with equivalent concentrations of OVA.

Oral Antigen Administration Protocol

Mice were fed with a single 50.0 mg OVA dose in 0.5 mL of PBS or PBS alone by gavage under light anes-

thesia. The feeding was performed on the 5th day prior OVA-immunization or co-immunization with OVA and Bt extract. All groups were boosted on the 14th and on the 21st day after immunization.

Passive Cutaneous Anaphylaxis (PCA)

IgE antibodies were estimated by passive cutaneous anaphylaxis reaction as described by Mota and Wong (23). Briefly, 0.1 mL of serum dilutions from each mouse was inoculated intradermally on the shaved back of the rats. After 18 h, the rats received 0.5 mg of mite extract or OVA in 1.0 mL 0.5% Evans Blue by tail vein injection. One hour later, the rats were killed, and the reciprocal of the highest serum dilution that produced a spot larger than 5 mm in diameter was taken as the PCA titer.

Determination of Antibody Levels

An enzyme-linked immunosorbent assay (ELISA) was performed to detect IgG isotypes as previously described (3, 24). Briefly, 96-wells microplates (Costar, Cambridge, MA) were coated with 20 or 5 μ g/mL of Bt extract or OVA, respectively, in 0.1 M carbonate–bicarbonate buffer, pH = 9.5. The wells were blocked with PBS-0.5% bovine serum albumin (BSA Sigma, St. Louis, MO) before incubation of the serum samples for 1 h at 37°C and overnight at 4°C . Detection was performed with biotinylated anti- γ 1 or anti- γ 2a mouse chain antibodies (1:3000, Southern Biotechnology Ass, Birmingham, AL) and streptavidin-peroxidase (1:200,000, Sigma, St. Louis, MO) and TMB (3,3', 5,5'-tetramethyl-benzidine, Sigma). The reaction was stopped with sulphuric acid and read at 450 nm in an ELISA microplate reader (Bio-rad, USA). The results were expressed as antibody titers in reference to serial dilution of a titrated serum pool from immunized adult mice with high levels of specific antibodies.

Cytokine Measurements by ELISA

Spleens from mice were aseptically collected and pressed through a stainless steel wire mesh in RPMI 1640 culture medium (GIBCO BRL, Gaithersburg, MD), supplemented with 10% fetal calf serum (FCS, Hyclone III Lb. Inc., USA) and 10 mg/mL gentamicin. Single spleen cell suspensions were centrifuged on Ficoll–Hypaque gradient, washed and cultured in 96-wells microplate (Costar, Cambridge, MA) at 8.0×10^5 cells/well in 0.2 mL of supplemented RPMI medium, Bt extract (100 μ g/mL) or OVA (200 μ g/mL, Sigma) for 24 and 72 h at 37°C in a humidified 5% CO₂ incubator; after which cell-free supernatants were stored at -70°C until cytokine assay. IL-2, IL-4, IL-10, IFN- γ (OPTEIATM Pharmingen, San Diego, CA) and TGF- β 1 (Promega,

Madison, WI, USA) measurements were performed following the manufacturer recommendations. Briefly, 96-well microplates (High binding, Costar) were coated with an anti-cytokine monoclonal antibody overnight at 4°C. After the washes, samples were incubated for 2 h at room temperature. New washes were carried out before biotinylated mouse anti-cytokine antibodies were incubated followed by streptavidin-peroxidase addition. The reaction was developed after TMB addition and stopped with 1N sulfuric acid. Reading was performed in an ELISA microplate reader at 450 nm. OD units were correlated to the protein concentration using provided standard curves from provided standards. Detection limit levels of IL-2, IL-4, IL-10, IFN- γ and TGF- β 1 were 3.2, 7.8, 31.3, 31.3, and 32 pg/mL, respectively. Cytokines secretion values were given after subtracting the background reading from the respective cytokines standard curve.

Statistical Analysis

Difference between groups was considered statistically significant when p value ≤ 0.05 , using *Mann Whitney* test.

RESULTS

Influence of Non-Related Antigen Sensitization on Type-I Hypersensitivity Response to the Mite Blomia Tropicalis (Bt) and to Ovalbumin (OVA) in Mice

In order to investigate the influence of non-related allergen sensitization in immunized mice, groups of Bt-immunized animals were, 2 weeks later, submitted to OVA immunization; or, vice-versa, mice first immunized with OVA were later immunized with Bt or co-injected with both allergens, Bt and OVA.

Figure 1 shows that sensitization with OVA prior to Bt-immunization has significantly increased the IgE response to Bt and to OVA, but such response was not observed in the reverse experiment, when OVA-immunization was prior to Bt-immunization. However, when both the antigens, Bt and OVA, were co-injected, it was observed a significant decrease in the anti-Bt IgE Ab production and an enhancement of IgE response to OVA, as in comparison with mice immunized with Bt and OVA, separately. On the other hand, the opposite trend was observed for the IgG1 Ab; co-immunized mice showed decreased levels in anti-OVA IgG1 and increased response as anti-Bt IgG1 Ab (Fig. 1). Mice immunized with Bt prior to OVA-immunization had increased IgG1 Ab levels to Bt in comparison to control-immunized group.

Co-immunized mice had a significant decrease in anti-Bt IgG2a Ab levels, compared to Bt-immunized mice, but similar anti-OVA IgG2a antibody levels to those of the OVA-immunized group. Mice previously immunized with OVA demonstrated a significant decrease in the anti-OVA IgG2a antibody production compared to the respective control group (Fig. 1).

Induction of Oral Tolerance to OVA in Bt/OVA Co-Immunized Mice

Considering the predominant IgE response to OVA over Bt in co-immunized mice, we have evaluated the effect of oral administration with OVA five days prior the co-immunization.

Oral administration with OVA prior to OVA-immunization was able to significantly decrease IgE and IgG1 Ab levels compared to those observed in OVA-immunized mice (Fig. 2). Feeding with OVA prior to co-immunization led to a more efficient inhibition of antibody response, as shown by the decreased levels of anti-OVA and anti-Bt IgE, IgG1 and IgG2a Ab, as compared to the co-immunized group (Fig. 2).

Subsequently, we evaluated the cytokines secretion *in vitro* upon OVA or Bt stimulation. OVA-fed mice has significantly increased the IFN- γ level and decreased IL-2, IL-4 and IL-10 secretion induced by OVA-stimulation compared to OVA-immunized mice (Fig. 3). TGF- β 1 level, in turn, was barely detected in OVA-fed mice. Co-immunization has not changed cytokines secretion upon OVA-stimulation resulting in a profile of cytokines similar to that observed for OVA-immunized mice.

Mice fed with OVA and co-immunized afterwards revealed increased IFN- γ levels in parallel to decreased levels of TGF- β 1, as compared to the co-immunized group. In contrast, when cells from co-immunized and OVA-fed mice were stimulated with Bt, only secretion of IL-10 was significantly decreased as compared to Bt-immunized mice (Fig. 3).

Influence of the OVA Amount in Co-Immunization on the Type-I Hypersensitivity Response to Bt

To ascertain the influence of OVA amounts in co-immunized mice that showed a predominant IgE response to OVA and inhibited response towards Bt, decreasing concentrations of OVA were tested in the co-immunization protocol.

Figure 4 shows that decreasing 40 or 120-fold the OVA amounts for co-immunization, anti-Bt IgE Ab response remained inhibited in comparison with Bt-immunized mice. This inhibitory effect on anti-Bt IgE response has only disappeared upon 240-fold less OVA initial dose (0.08 μ g) in the co-immunization protocol (Fig. 4).

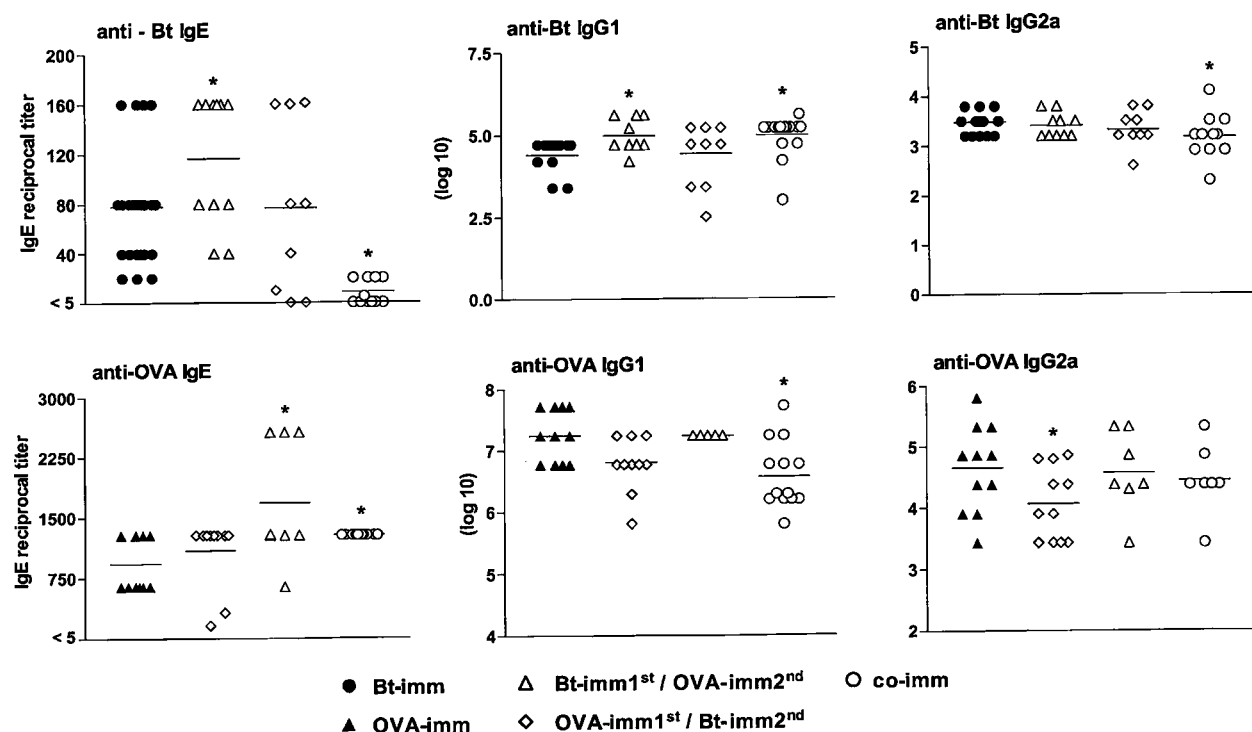


Fig. 1. Evaluation of new allergen sensitization during Bt or OVA immunization or co-immunization. Groups of mice were first immunized with Bt and, 2 weeks later, with OVA (Bt-imm^{1st}-OVA-imm^{2nd}) or received first immunization with OVA followed by Bt-immunization (OVA-imm^{1st}-Bt-imm^{2nd}) or were co-injected with both allergens, Bt and OVA (co-imm). Control groups were mice immunized with OVA (OVA-imm) and immunized with Bt (Bt-imm). Seven days after the last antigen boost, sera were analyzed for specific IgE Ab by PCA reaction or anti-Bt and anti-OVA IgG1 or IgG2a Ab by ELISA. * $p \leq 0.05$ compared to respective immunized-control group.

Oral Tolerance Induction in Mice Co-Immunized with Low-Dose OVA

Since similar IgE antibody titers to Bt and OVA were obtained when 240-fold less the OVA amount was used in the co-immunization protocol (Fig. 4), we assessed the effect of oral OVA administration in this protocol. Figure 5 shows that mice fed with OVA prior to co-immunization presented simultaneous decrease of anti-Bt and anti-OVA IgE Ab production. It was thus demonstrated that inhibition of IgE response to Bt is not related to OVA dominance in the co-immunization, but due to bystander effect of oral tolerance.

Curiously, anti-Bt IgG1 and IgG2a Ab production in co-immunized mice fed with OVA was similar to those of the co-immunized control group (Fig. 5). OVA feeding has not interfered with IgG response to Bt, which was highly decreased when feeding was prior to co-immunization with similar amounts of both allergens. Further, we assessed cytokines secretion in mice fed with OVA prior to co-immunization with low-dose OVA. IFN- γ secretion induced by OVA was detected at high levels associated with normal levels of IL-10 in co-immunized mice fed with OVA, as in comparison to co-immunized mice (Fig. 6).

No changes were observed in the level of other cytokines, such as IL-2, IL-4 and TGF- β 1, under OVA or Bt stimulation (data not shown).

DISCUSSION

We have observed an enhancement of type-I hypersensitivity to mite *Blomia tropicalis* (Bt) or to ovalbumin (OVA) in mice immunized with Bt followed by exposure to OVA. In contrast, co-injection with both allergens, Bt and OVA, led to a marked suppression in IgE response to Bt, while enhanced response to OVA. We further noticed that feeding co-immunized mice with a single high-dose OVA inhibited IgE and IgG antibody production to both allergens. Contrariwise, mice fed with OVA prior to co-immunization with low amounts of OVA presented simultaneous decrease in anti-Bt and anti-OVA IgE Ab production, but lacked the inhibitory effect in the IgG response. These findings show that a more pronounced inhibition in the production of specific and of non-related allergen Ab after OVA feeding is determined by the OVA amounts employed in the co-immunization.

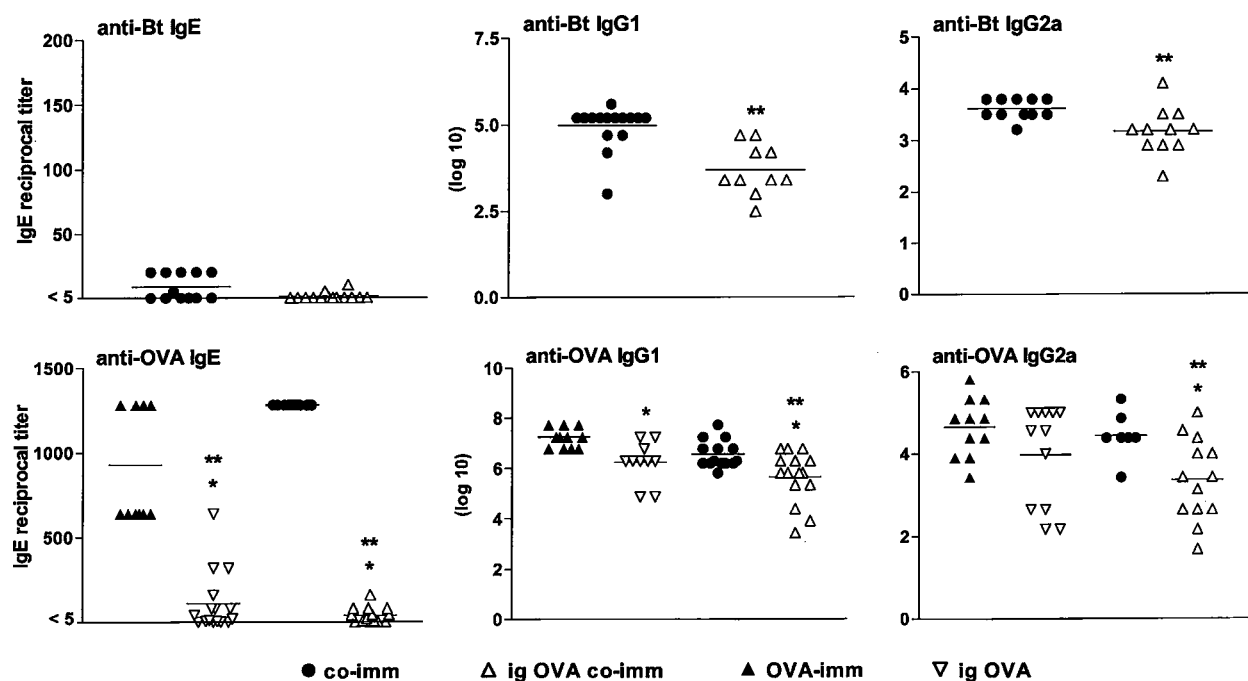


Fig. 2. Induction of oral tolerance to OVA in co-immunized mice. Mice were fed with OVA five days prior OVA-immunization (ig OVA) or co-immunization with OVA and Bt (ig OVA co-imm). Control groups were mice immunized with OVA (OVA-imm) and immunized with Bt (Bt-imm). Seven days after the last antigen boost, sera were analyzed for specific IgE Ab by PCA reaction or anti-Bt and anti-OVA IgG1 or IgG2a Ab by ELISA. * $p < 0.05$ compared to immunized group; ** $p \leq 0.05$ compared to co-immunized group.

Immunization with Bt prior to OVA immunization has induced IgE and IgG1 Ab production to both allergens. A different behavior in Ab response was seen when OVA-immunization was after the Bt-immunization; in which case, a reduction in anti-OVA IgG2a Ab levels occurred without interfering in the production of IgG Ab to Bt. This result indicates that sensitization with Bt down-regulates the Ab response in OVA-immunized mice, in contrast to the OVA-adjuvant effect on the IgE response to both allergens, when mice were firstly sensitized with Bt. In fact, it has been described that some specific class of aeroallergens could favor the development of polysensitization (5), probably as a reflection of the diverse predisposition to specific sensitization and/or antigen crossreactivity (25). Furthermore, it has been described that egg-allergic children have very high risk of subsequent sensitivity to perennial inhalant allergens (26).

Mice injected with both allergens, simultaneously, showed a marked predominance of IgE response to OVA over Bt. This competitive IgE response was antigen-dose dependent, in co-immunized mice, as revealed by the abrogation of the inhibitory effect on IgE response to Bt, when OVA concentration was 240-fold lower. It has been described that the co-injection of the major bee venom allergen, phospholipase A₂ (PLA₂), with high-dose OVA

immunization in mice generates a strong predominance of OVA over PLA₂ IgE response (27). Despite the inhibition on anti-Bt IgE Ab in co-immunized mice, we have observed an opposite trend for B-cell IgG1 Ab production, which was mostly towards Bt. This differential effect on Ab response might be due to a counterbalance mechanism of the immune system after simultaneous encounter with two relevant allergens. The contrasting effects of allergen-specific IgE and IgG1 Ab, both isotypes influenced by Th2 cytokines, suggest that these isotypes could be differentially regulated, as observed in intranasal tolerance inducing protocol (14).

Whether the profile of Ab response in co-immunized mice could be influenced by cytokines secretion or not—one cannot tell—as no remarkable changes in cytokines production were observed. However, spleen cells from co-immunized mice upon Bt stimulation secreted high amounts of IFN- γ concomitantly with decreased IL-10 levels induced by OVA. This probably contributes to the down-regulation of the anti-Bt IgE and IgG1 Ab response in the co-immunized mice, but does not correlate with the decrease in the anti-Bt IgG2a Ab levels.

Induction of oral tolerance is a procedure aimed to produce peripheral unresponsiveness by mechanisms that operate in antigenic dose-dependent manner (7, 8).

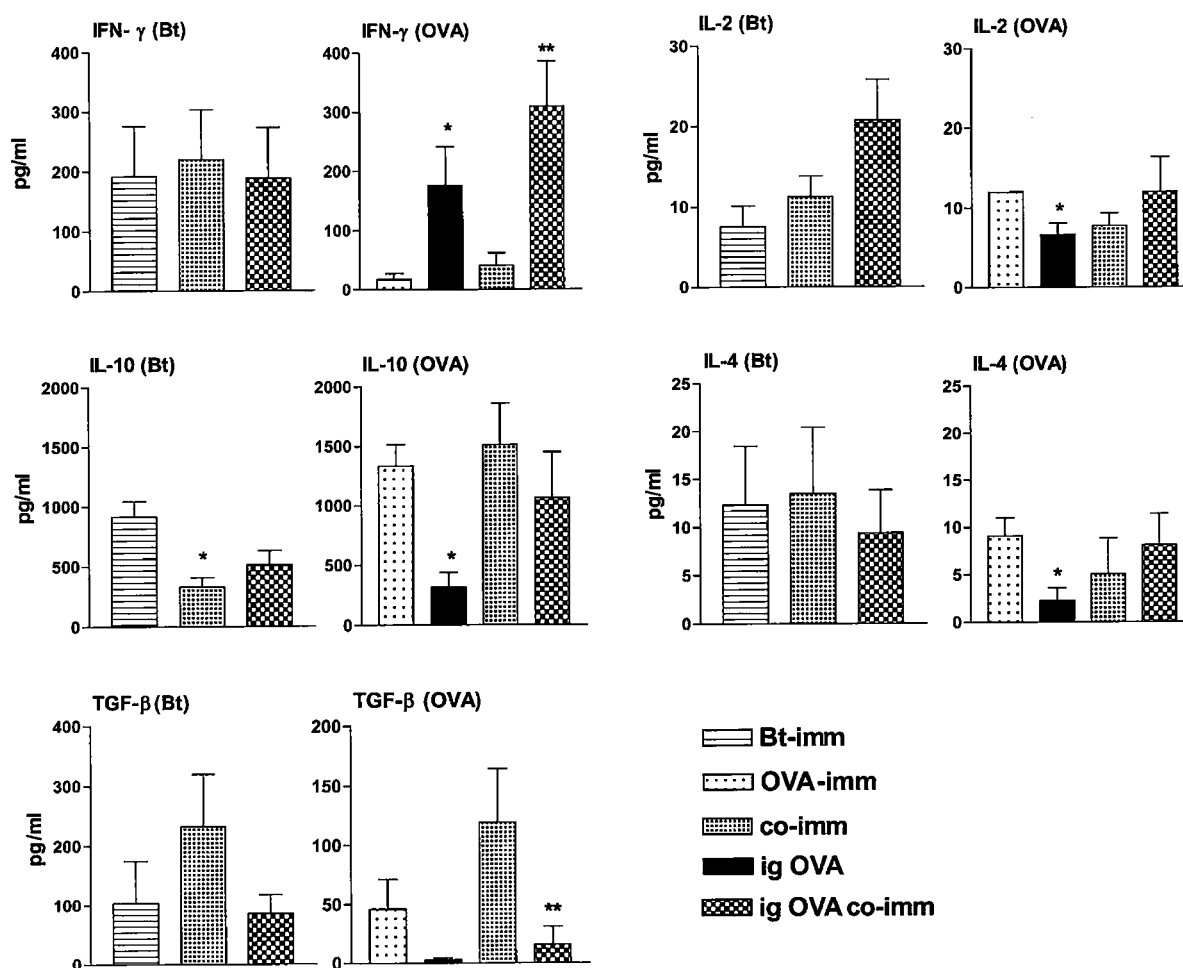


Fig. 3. Antigen-specific cytokines secretion in co-immunized mice orally administered with OVA. Supernatants of spleen cell cultures from OVA-immunized (ig OVA) or co-immunized mice fed with OVA (ig OVA co-imm) or from OVA-immunized (OVA-imm) and Bt-immunized mice (Bt-imm) were collected after 24 or 72 h of stimulation with Bt (100 μ g/mL) or OVA (200 μ g/mL). Measurements of IFN- γ , IL-2, IL-10, IL-4 and TGF- β 1 were performed by ELISA. Data presented are mean values of three independent experiments ($n = 10$). * $p \leq 0.05$ compared to immunized group; ** $p \leq 0.05$ compared to co-immunized group.

Different regimens have been described for oral antigen administration under different regulatory mechanisms. On one instance, high-antigen dose was able to anergize or delete both Th1 and Th2 cells (16, 18). On another, single dose or intermittent feeding regimens produce effective tolerization of Th1 response (28). We have found that OVA feeding prior to OVA-immunization induced marked inhibition of IgE and IgG1 Ab response and decrease in IL-4, IL-10 and IL-2 levels, whereas it enhanced IFN- γ secretion significantly. This result emphasizes that feeding with one single high dose is required to promote Th2-tolerization instead of Th1. Probably, IFN- γ levels in tolerized mice contribute to the anergy process induced by the high antigen dose. These findings reinforce that IFN- γ lead lymphocytes to anergic state and induce tolerance

(29, 30). The influence of IFN- γ in oral tolerance has also been shown in IFN-null mice that failed to become tolerized upon high dose orally-administered Ag (31).

Other factors are also crucial to T cell anergy, such as the nature of the antigen and property of the Ag-presenting cells (32, 33). In fact, we have been noticing, in a study in progress, that high-dose OVA leads to a long-lasting B-cell anergy—up to eight months after feeding—resulting in a marked alteration in the expression of co-stimulatory B7 molecules on B cells (unpublished results).

Bystander suppression is the phenomenon by which regulatory cells, generated by oral tolerization, are primed in an Ag-specific manner but act in the respective microenvironment in a non-Ag specific manner. It is worth noting that OVA feeding was more effective to inhibit Ab

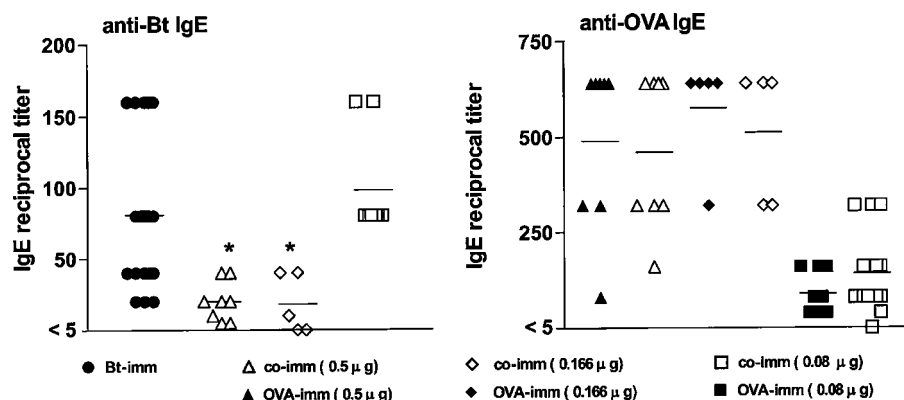


Fig. 4. Interference of OVA dose on the IgE response to Bt in co-immunized mice. Groups of mice were co-immunized (co-imm) with 20 μ g Bt plus 0.5 μ g, or 0.166 μ g or 0.08 μ g OVA. Control mice were immunized with the respective dose of OVA (OVA-imm) or Bt (Bt-imm). Seven days after the last antigen boost, sera were analyzed for anti-Bt or anti-OVA IgE Ab by PCA reaction. * $p \leq 0.05$ compared to immunized group.

production when it was previous to co-immunization than to single-OVA immunization. Co-immunized mice fed with OVA developed marked inhibition of Ab levels against OVA as well as against Bt. This inhibitory effect on antibody response to unrelated antigen of tolerated anti-

gen cannot be explained only by bystander suppression; since there are no changes in suppressive cytokines, such as, TGF- β and/or IL-10 (34–36), despite the increase in IFN- γ levels in co-immunized mice fed with OVA. In fact, the indirect effect of oral tolerance could be mediated by

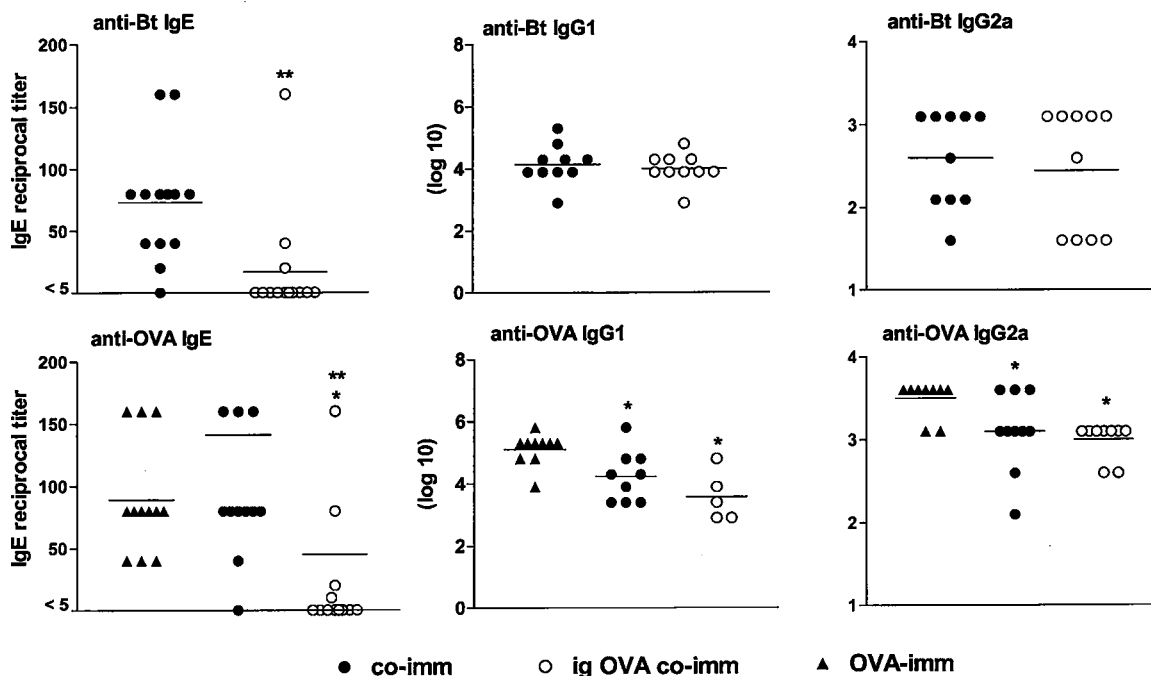


Fig. 5. Evaluation of Ab response in mice orally administered with OVA prior co-immunization with low-dose OVA. Groups of mice were fed with OVA five days prior co-immunization (ig co-imm) with Bt (20 μ g) and OVA (0.08 μ g). Control mice were co-immunized with equal amounts of Bt and OVA (co-imm), or immunized with respective dose of OVA (OVA-imm) or Bt (Bt-imm). Seven days after the last antigen boost, sera were analyzed for specific IgE Ab by PCA reaction or anti-Bt and anti-OVA IgG1 or IgG2a Ab by ELISA. * $p \leq 0.05$ compared to immunized group; ** $p \leq 0.05$ compared to co-immunized group.

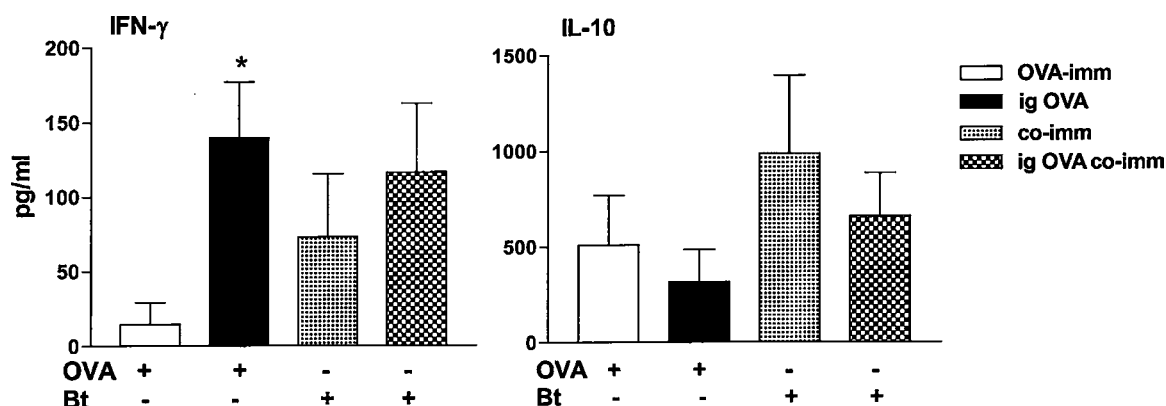


Fig. 6. Cytokines secretion in mice orally administered with OVA prior co-immunization with low-dose OVA. Supernatants of spleen cell cultures from co-immunized mice fed with saline (white bars) or OVA (black bars) were collected after 24 or 72 h of stimulation with Bt (100 μ g/mL) or OVA (200 μ g/mL). IFN- γ and IL-10 were measured by ELISA. Data presented are mean values of three independent experiments ($n = 10$). * $p \leq 0.05$ compared to immunized group.

active suppression and/or anergy process—a phenomenon induced with high dose Ag feeding (18). Nonetheless, overlapping mechanisms could be cooperative, also involving regulatory T cells in antigen fed mice. It has been described increased number of CD4 + CD25 + regulatory T cells when high dose of Ag was placed in the drinking water or gavaged to OVA TCR Tg mice (37). In addition, CD4 + CD25 + regulatory T cells acted as stable suppressors able to mediate bystander suppression upon TCR stimulation, but leaving recall responses unaffected in the absence of cognate Ag (38).

To verify whether the inhibition of IgE response in co-immunized OVA-fed mice was due to the immunization regimen, decreasing amounts of OVA were employed in the co-immunization until no predominance of IgE response to OVA over Bt was observed. The co-immunization regimen with 240-fold less the OVA dose induced equal IgE Ab levels to both allergens, and predominant IgG response to Bt over OVA. This finding revealed that Bt played an inhibitory effect on the Ab response to OVA in co-immunized mice, similarly to what was observed in mice first immunized with OVA followed by Bt-sensitization. Induction of oral tolerance in co-immunized mice with low-dose OVA maintained the suppression on IgE response to both allergens, whereas the down-modulation of IgG1 and IgG2a to Bt and to OVA disappeared. With this regimen of co-immunization, a prominent effect of oral tolerance was expected; however, inhibition of specific IgG response was abolished. These results provide the first evidence that oral tolerance induction with high Ag dose seems to be influenced by the immunization dose rather than by the Ag feeding dose. Furthermore, mice fed with single high-dose OVA prior to co-immunization with low-dose OVA inhibited IgE Ab

response, what was probably related to the increased levels of IFN- γ found in these animals.

Altogether, these findings showed that exposure to a non-related antigen could amplify the IgE and IgG1 responses of ongoing allergen sensitization, depending on their allergenic potential. Furthermore, oral antigen administration before *Blomia tropicalis*/ovalbumin co-immunization led to specific and bystander effect on Ab response, depending on these allergens' doses. The oral tolerance bystander effect to non-related antigen could be an interesting mechanism to control new allergens sensitization.

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