

Long-term anergy in orally tolerized mice is linked to decreased B7.2 expression on B cells

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Abstract

Durable antigen (Ag)-specific T- and B-cell anergy induced by oral tolerance is an attractive strategy for immunotherapy of allergic diseases. Here, we address the lasting effect of oral tolerance induction in naïve or primed mice to ovalbumin (OVA) on antibody production. Single feeding with OVA prior to immunization or double feeding, before and after Ag priming, in A/Sn mice, induced a long-lasting suppression of IgE, IgG1 and IgG2a responses up to 8 months after immunization. In contrast, primed-fed mice had transient IgE inhibition. Naïve and double-treated mice showed marked Ag-specific unresponsiveness and scarce cytokines production. Inhibition of IL-2 and IFN- γ secretion in naïve-fed mice were restored in the presence of anti-CD28 mAb plus Ag stimulation. The durable inhibition of Ab production in OVA-fed mice was related to the persistent decrease of B7.2 expression on B cells. Ag feeding in naïve and primed status may be a prophylactic measure to avoid later Ag sensitization.

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Introduction

Oral tolerance refers to systemic antigen (Ag) hyporesponsiveness that occurs after oral Ag administration followed by a challenge with the same Ag (Mowat, 1987). Experimental models have shown that, depending on the Ag dose ingested, tolerance can be mediated through suppression, clonal deletion or anergy (Melamed et al., 1996; Van Houten and Blake, 1996; Weiner, 1997). Low and multiple Ag doses favor

immunosuppression (Kjaer and Frokiaer, 2002; Mucida et al., 2005) while high doses result in clonal deletion and T-cell anergy (Faria et al., 2003; Wakabayashi et al., 1999).

Several types of oral tolerance regimens, usually in naïve animals, have been addressed to obtain Ag specific unresponsiveness at T- and B-cell level. Although prevention of sensitization through Ag feeding has been demonstrated (Weiner, 1997), it seems difficult to revert existing immune responses through oral tolerance induction (Torseth and Gregerson, 1998; Leishman et al., 1998). Attempts with different regimens to induce oral tolerance are aimed at prophylaxis to Ag sensitization and/or therapeutic action on existing immune responses.

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Maintenance of T-cell tolerance is an ongoing process that is necessary for prevention of autoimmunity and allergic diseases. Anergy is characterized by failure of cellular proliferation and IL-2 secretion upon Ag receptor engagement (Van Houten and Blake, 1996; Omata et al., 2005) which could be related to the lack of costimulatory signal. Costimulatory molecules, including B7.1 (CD80) and B7.2 (CD86) – expressed on Ag-presenting cells – are specific for both the stimulatory receptor CD28 and the inhibitory receptor Cytotoxic T-Lymphocyte Associated Antigen (CTLA-4, CD152) (Fowler and Powrie, 2002). In vitro stimulation with anti-CD28 mAb can provide a costimulatory signal to naïve T CD4+ cells and T-cell clones blocking the induction of anergy (Chung et al., 2003). On the other hand, engagement of CTLA-4 limits the degree of T-cell activation (Chen et al., 2002). This inhibitory signaling seems to be active in oral tolerance since complete abrogation of T-cell tolerance induced by high dose Ag could be obtained by selective blockage of CTLA-4 (Samoilova et al., 1998).

The crucial role of B7 molecules on antibody production could be seen in mice deficient for B7.1 and B7.2 that failed to generate Ag-specific IgG1 and IgG2a responses and lacked germinal centers (Borriello et al., 1997). In another instance, B7 role was evidenced by lack of oral tolerance induction in CD40–/– mice (Kweon et al., 1999). Indeed CD40L-CD40 interaction up-regulates B7.1 and B7.2 expression (Sharp and Freeman, 2002).

Furthermore, an enhanced B7.2 expression on B cells has been observed in a murine model for atopic dermatitis to house dust mite (Santa et al., 2003). However, it is not known whether maintenance of anergy induced by high Ag feeding could be related to a long permanence of altered B7 expression since circulating Ag may disappear during this time.

In the present work, we sought to investigate the durability of the effects triggered by several regimens in oral tolerance to ovalbumin (OVA), in naïve or Ag primed mice, on antibody production and the costimulatory B7 molecules expressed on B cells. Moreover, the involvement of CTLA-4 expression in the anergic process induced by oral tolerance was further ascertained.

Materials and methods

Animals

Male A/Sn mice 8–10 weeks old were provided by the animal facility of the School of Medicine – University of São Paulo. Wistar Furth rats (RT1^{u,u}) of both sexes, 3–4 months old, bred in our own animal facility were used for passive cutaneous anaphylaxis (PCA) reactions.

Immunization protocol

Groups of mice were immunized intraperitoneally with 50 µg of OVA (grade V; Sigma, St. Louis, MO) in 0.1 mg of aluminium hydroxide [Al(OH)₃, alum]. Mice were boosted intraperitoneally on the 10th and 60th or 120th or 240th days after immunization with 50 µg of OVA in saline solution. Groups of mice were bled 7 days after each boost and serum samples were frozen at –70 °C until use.

Oral antigen administration protocol

Mice were intragastric (ig) administered with saline (ig-saline) or with 25 mg of OVA in 0.5 ml of saline by gavage under light ether anaesthesia within 3 (ig–3) or 7 (ig–7) days before priming (dbp) or 3 (ig+3) or 7 (ig+7) days after priming (dap). Groups were treated three dbp and three dap (ig–/+3) or seven dbp and seven dap (ig–/+7) or seven dbp and three dap (ig–7/+3) with a total of 50 mg of OVA. Some groups were fed with OVA 21 (ig–21) or 14 (ig–14) days before priming. Other groups were fed with 25 mg of OVA three dap and, 42 days after the first feeding, were re-treated with 100 mg of Ag (ig +3/+45). All groups were boosted on the 10th day after immunization.

Passive cutaneous anaphylaxis (PCA)

IgE antibodies were estimated by PCA reaction as described by Mota and Wong (1969). 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 OVA in 1.0 ml 0.5% Evans Blue by tail vein injection; 1 h 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 (Sato et al., 2002). Briefly, 96-well microplates (Costar, Cambridge, MA) were coated with 5 µg/ml of OVA 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-γ1 or anti-γ2a 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.

T-cell proliferation

Spleen aseptically collected from mice after 2 months of immunization was pressed through a stainless wire mesh in RPMI-1640 (Gibco BRL, Gaithersburg, MD), supplemented with 10% of fetal calf serum (FCS, Hyclone Lb. Inc., Logan, UT) and 40 mg/l gentamicin. The single cell suspension was centrifuged in a Ficoll-Hypaque gradient and washed, 4.0×10^5 cells/well were cultured in 96-well microplates (Costar, Cambridge, MA) in 0.2 ml of supplemented RPMI medium in the presence of Ag (OVA 200 µg/ml, Sigma) with or without hamster anti-mouse CD28 mAb (2 µg/ml, PharMingen) or only with hamster anti-mouse CD3 (2 µg/ml, PharMingen) at 37 °C in a humidified 5% CO₂ incubator. Thymidine incorporation was measured on day 4 of culture after 18 h pulse with 1 µCi [³H] thymidine (Amersham, Life Science). Cytokine production was measured from cultures containing 8.0×10^5 cells/well stimulated with OVA (200 µg/ml, Sigma) or OVA plus anti-CD28 mAb for 24, 48 and 72 h, whereupon cell-free supernatants were stored at –70 °C until cytokine assay.

Cytokine measurements by ELISA

IL-2, IL-4, IL-10, IFN- γ (OPTEia™ Pharmingen, San Diego, CA) 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 1 N sulfuric acid. Reading was performed in an ELISA assay 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 were 3.12 pg/ml, 3.9 pg/ml, 31.2 pg/ml, 15.6 pg/ml, respectively. Cytokine secretion values were given after subtracting the background reading from the respective cytokine's standard curve.

Flow cytometry

The expression of surface Ags was analyzed by flow cytometry. Splenic cells were stimulated by OVA

(200 µg/ml, Sigma), LPS (50 µg/ml, Sigma), anti-IL-10 (10 µg/ml, PharMingen), anti-TGF- β (10 µg/ml, PharMingen), anti-CTLA-4 at blocking concentration (100 µg/ml, PharMingen) or control antibodies (PharMingen) for 48 h at 37 °C in a humidified 5% CO₂ incubator. Cells were washed and Fc γ RII and Fc γ RIII were blocked with antibody against Fc γ RII/Fc γ RIII (anti-CD16/32, PharMingen) and incubated with the following antibodies: anti-B220 CyChrome (CyC, PharMingen), anti-IgM (Southern) FITC, anti-B7.1 (anti-CD80, PharMingen) PE, anti-B7.2 (anti-CD86, PharMingen) PE. All flow cytometry staining procedures were performed at 4 °C in PBS/1% BSA (Sigma). Cells were then washed in PBS/1% BSA and flow cytometry buffer before analysis of 10,000 gated events by Coulter Epics XL-MCL (Beckman-Coulter Electronics, Hialeah, FL) using System II software.

Statistical analysis

Difference between groups was considered statistically significant when $p \leq 0.05$, using the Mann–Whitney test.

Results

Kinetics of specific Ab isotypes production in naive or primed mice orally administered with OVA

To verify the effect of oral administration with OVA before and/or after Ag priming on the IgE, IgG1 and IgG2a specific Ab production at primary response and at 2, 4, 6 or 8 months after immunization, groups of A/Sn mice were ig administered with 25 mg of OVA or saline (ig-saline) following the regimens: (i) 3 days (ig–3) or 7 days (ig–7) before immunization; (ii) 3 days (ig+3) or 7 days (ig+7) after immunization; (iii) twice ig administered 3 days before and 3 days after immunization (ig–3/+3) or (iv) 7 days before and 7 days after immunization (ig–7/+7).

Oral Ag administration, 3 or 7 days before immunization, was able to decrease significantly IgE antibody primary response on the 17th day of immunization, compared to the ig-saline control group (Fig. 1). After 2 months of immunization, a 14-fold rise in the anti-OVA IgE Ab titers was detected in ig-saline mice. In contrast, anti-OVA IgE Ab titers in mice fed 3 days or 7 days prior to immunization increased four-fold or remained undetectable ($<1/5$), respectively (Fig. 1). Both naive fed mice groups remained with low anti-OVA IgE Ab levels until the end of the analyzed period (8 months), as compared with ig-saline mice.

On the 17th day of immunization, anti-OVA IgG1 and IgG2a Ab titers increased in the ig-3 mice group

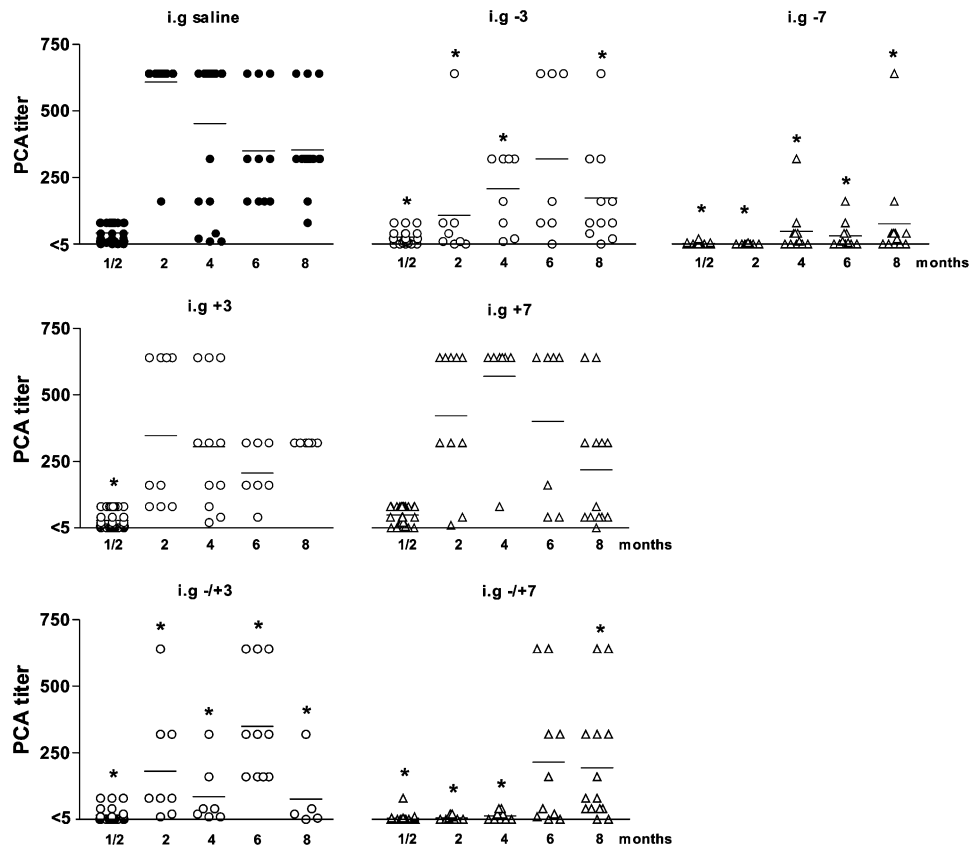


Fig. 1. Kinetics of anti-OVA IgE Ab production in different regimens of OVA feeding in mice. Groups of mice were fed with OVA 3 or 7 days before (ig -3, ig -7), 3 or 7 days after (ig +3, ig +7) or twice, 3 or 7 days before and after (ig -3/+3, ig -7/+7) priming. Control group was immunized with OVA (ig saline). Seven days after the antigen boost at 1/2, 2, 4, 6 or 8 months, sera were analyzed for specific IgE Ab by PCA reaction (mean of five different experiments, each one containing 5–7 mice). Each symbol represents an individual serum mouse. * $p \leq 0.05$ compared to ig saline group.

(Fig. 2). It is worth noting that OVA feeding at 1 week prior to immunization induced a marked inhibition on the IgG1 and IgG2a Ab levels, up to 8 months; whereas inhibition of IgG subclasses disappeared after 2 months of immunization, when Ag feeding was at 3 days before Ag-priming (Fig. 2).

Ag oral tolerance induction in naive mice is a well-established procedure to promote humoral and cellular peripheral unresponsiveness (Friedman and Weiner, 1994; Melamed et al., 1996). However, in primed mice fed with OVA after 3 days of immunization, a transient inhibition in the primary response of IgE only was observed (Fig. 1). Moreover, mice fed after immunization developed levels of IgG subclasses comparable to those of ig-saline mice (Fig. 2).

Fig. 1 shows a long lasting inhibitory effect on IgE Ab response in mice twice fed with OVA as in comparison to the ig-saline mice group. These mice groups showed a profile of IgE Ab production similar to that of naive OVA-fed mice. Besides the IgE inhibition in both naive fed mice groups, only those which received the first feeding 7 days before immunization (ig-7/+7)

showed decreased IgG1 and IgG2a Ab levels over up to 8 months of immunization (Fig. 2). These results demonstrate that down-modulation of Ab response in twice treated mice is due to mechanisms triggered by the first OVA feeding in naïve status, which are not interfered with by a second Ag feeding after priming.

Timing of OVA feeding in naive mice and Ag re-treatment in primed fed mice

To verify how long, before priming, OVA feeding is efficient to inhibit IgE response, we performed OVA administration at days 21 (ig-21) or 14 (ig-14) before priming. Fig. 3A shows that mice fed 14 days before Ag priming had significant decrease in the IgE Ab levels while those fed 21 days before had not.

As observed, OVA feeding after priming induced a transitory inhibition of IgE Ab primary response. Therefore, in order to maintain decreased IgE Ab production we sought to ascertain the effect of Ag

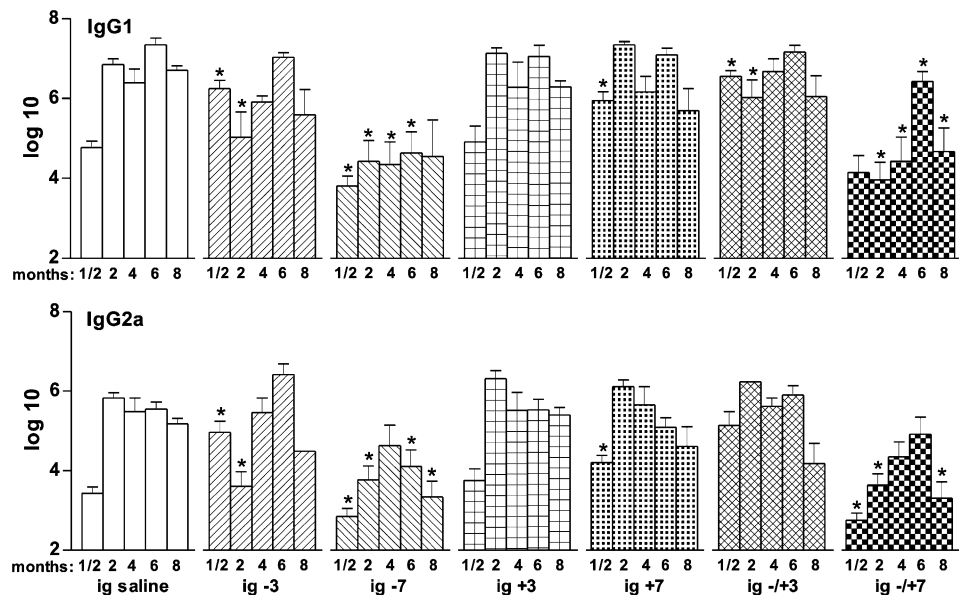


Fig. 2. Profile of anti-OVA IgG1 and IgG2a Ab production in different regimens of OVA feeding in mice. Groups of mice were fed with OVA 3 or 7 days before (ig -3, ig -7), 3 or 7 days after (ig +3, ig +7) or twice, 3 or 7 days before and after (ig -3/+3, ig -7/+7) priming. Control group was immunized with OVA (ig saline). Seven days after the antigen boost at 1/2, 2, 4, 6 or 8 months, sera were analyzed for anti-OVA IgG1 or IgG2a Ab by ELISA ($n = 8-17$ mice in each group per period, from 4–5 different experiments, mean $\pm$ SD). * $p \leq 0.05$, compared to ig saline group.

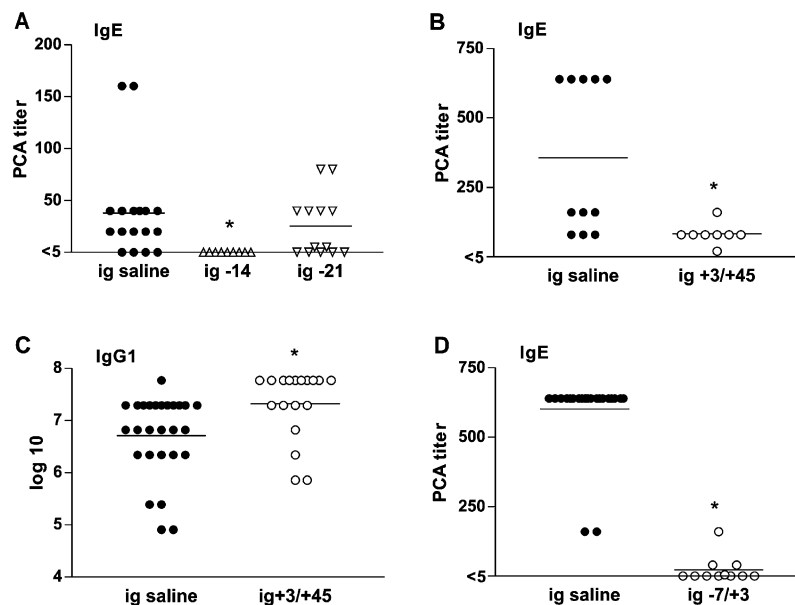


Fig. 3. Timing of OVA feeding in naive mice and Ag re-feeding in primed fed mice. Groups of mice were fed with OVA 14 days (ig -14) or 21 days (ig -21) prior to immunization and analyzed for anti-OVA IgE Ab by PCA reaction (A); mice fed at 3 days after priming were orally re-administered at 42 days after the first feeding (ig +3/+45) 7 days after Ag boost sera were analyzed for IgE Ab by PCA reaction (B) and anti-OVA IgG1 Ab by ELISA (C); anti-OVA IgE Ab production of mice fed at 7 days before and 3 days after priming (ig -7/+3) by PCA reaction (D). Control groups were mice immunized with OVA (ig saline). Each symbol represents an individual serum mouse. Data from three different experiments are shown. * $p < 0.05$ compared to ig saline group.

re-administration of primed fed mice. **Fig. 3B** shows that re-treatment using high dose of OVA (100 mg), 42 days after the first OVA feeding, led to a significant decrease in the IgE Ab levels as compared to ig-saline

mice. On the other hand, Ag re-exposure in primed fed mice enhanced IgG1 Ab production (**Fig. 3C**).

In addition, we addressed a schedule of twice Ag feeding, which was administered following the

appropriate number of days in order to down-modulate antibody production. The results shown in Fig. 3D demonstrate a marked inhibition of IgE Ab response when mice were fed 7 days before and 3 days after immunization (ig-7/+3).

In vitro activation of CD28 molecules restores proliferative response and IFN- γ production of naïve Ag fed mice

To address the Ag-induced proliferative response and cytokine production, mice groups orally administered with OVA 7 days before priming (ig-7) or 3 days after priming (ig+3) or double treated mice (ig-7/+3) were analyzed after 2 months of immunization.

Fig. 4A shows that both ig-7 and ig-7/+3 mice had a marked unresponsiveness to OVA in comparison to control ig-saline group. Mice administered with OVA

after priming (ig+3) showed a slightly decreased, but not significantly different, proliferative response. The proliferative response of naïve fed mice in presence of anti-CD28 mAb plus Ag induced responsiveness similar to that of ig-saline mice. In contrast to the Ag specific response, anti-CD3 stimulation induced similar response in ig-7 mice and significantly higher response in ig-7/+3 mice as compared to the ig-saline group (Fig. 4B).

In parallel to the inhibited Ag proliferative response, a low level of cytokines production was detected in the ig-7 and ig-7/+3 mice groups when compared to the ig-saline one. The results demonstrated that IL-2 and IFN- γ levels were inhibited in both OVA fed mice groups, whereas anti-CD28 mAb stimulation restored IL-2 secretion only in ig-7 mice and IFN- γ secretion in both OVA fed mice (Fig. 5). IL-4 and IL-10 Ag-induced secretion were barely detected in ig-7 and ig-7/+3 mice compared to the ig-saline group, which, in turn, did not alter after addition of anti-CD28 mAb to the cultures (Fig. 5).

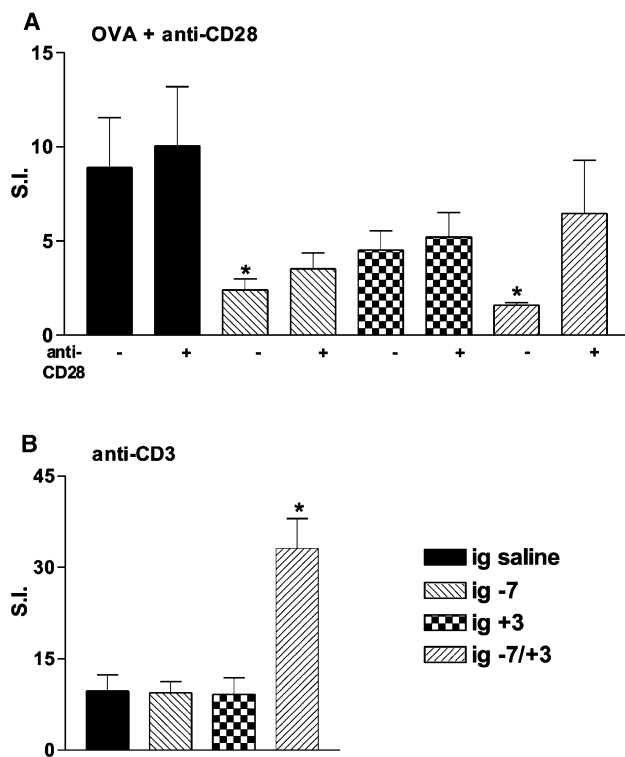


Fig. 4. Antigen specific T-cell anergy of naïve or double orally Ag administered mice. Two months after immunization spleen cells of naïve (ig-7), primed Ag fed mice (ig+3) and mice twice fed, before and after immunization (ig-7/+3), were cultured with OVA (200 μ g/ml) or OVA plus anti-CD28 mAb (2 μ g/ml) (A) or anti-CD3 mAb (2 μ g/ml) (B) for 96 h, and [^{3} H] thymidine uptake were measured [$n=8-17$ mice/per group assayed individually in 5 different experiments, results are represented by the mean $\pm$ SD of the stimulation index (SI) from the control group]. * $p \leq 0.05$ compared to ig saline group at similar stimulus.

Oral administration of OVA before immunization decreases the expression of costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on B cells

Altogether, the findings indicate that naïve Ag tolerized mice develop a durable anergy process as evidenced by the long lasting B-cell unresponsiveness.

To further verify the effect of oral tolerance, B7.1 and B7.2 expression on splenic B cells of naïve fed mice at different post-immunization periods was investigated by means of flow cytometry. The total number of B220+ / IgM+ splenic cells of ig-saline mice 2 months after immunization ($40.4 \pm 4.4 \times 10^6$ cells, mean $\pm$ SD) was similar to that found for ig-7 and ig-7/+3 mice groups ($64.1 \pm 17 \times 10^6$ cells, $42.0 \pm 4.1 \times 10^6$ cells, respectively).

After 2 months of immunization and upon in vitro stimulation with OVA, the percentage of B220+ B7.1+ cells in both ig-7 (2.84 ± 1.5 , mean $\pm$ SD) and ig-7/+3 OVA fed mice (5.6 ± 4.0) was not changed. However, a 2.3-fold increase in the percentage of B220+ B7.1+ cells in the control group was observed as compared to Ag non-stimulated B cells (Fig. 6). Stimulation with LPS induced a similar percentage (11.4%) of splenic B cells expressing B7.1 molecules in both analyzed groups, and an increase of 3.3-fold when compared with non-stimulated B cells. Fig. 6 shows one representative of such experiments.

In response to OVA stimulation, the percentage of B220+ IgM+ cells expressing B7.2 increased 2.1-fold ($45.6\% \pm 6.06$) in the ig-saline mice, compared to their baseline levels ($21\% \pm 4.91$), whereas for the ig-7 ($21.1\% \pm 2.86$) and ig-7/+3 ($20.6\% \pm 4.30$) mice groups, this percentage did not change. Diversely, up-regulation of B7.2 expression on B cells (2.5-fold) after

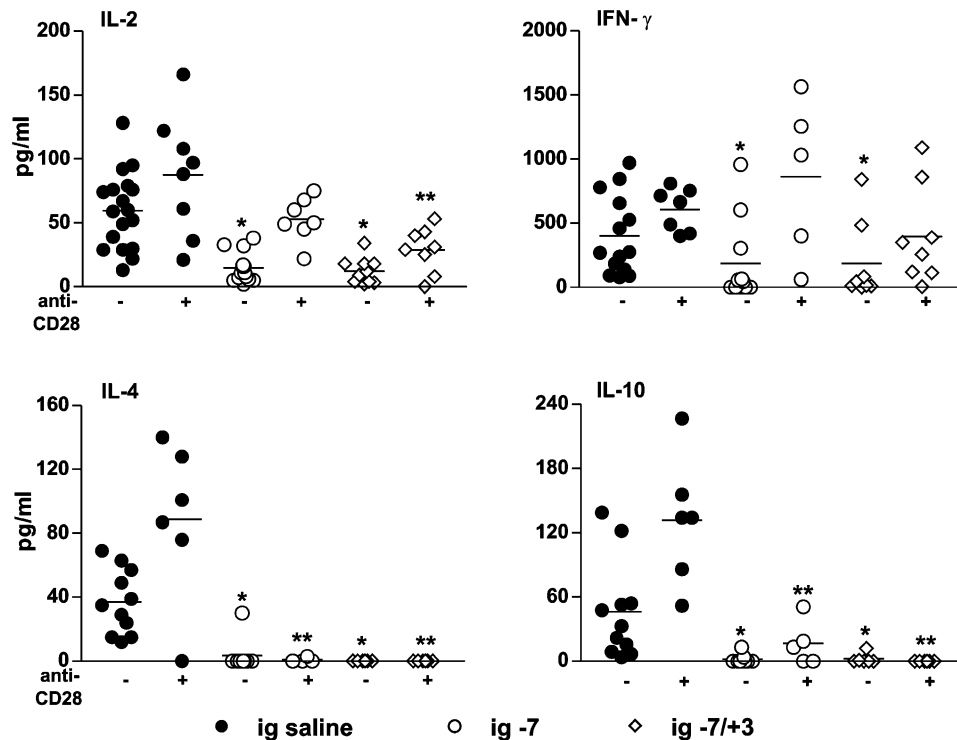


Fig. 5. Antigen-specific cytokines secretion of naïve and double treated mice orally administered with OVA. Supernatants of spleen cell cultures from naïve (ig -7) and twice fed mice (ig -7/+3) after 2 months of immunization were collected after 48 or 72 h of stimulation with OVA (200 μ g/ml) or OVA plus anti-CD28 mAb (2 μ g/ml). Measurements of IL-2, IFN- γ , IL-4, and IL-10 were performed by ELISA. Each symbol represents an individual mouse; data from four different experiments are shown. * $p \leq 0.05$ compared to ig saline group stimulated with OVA; ** $p \leq 0.05$ compared to ig saline group stimulated with OVA plus anti-CD28 mAb.

LPS stimulation was detected in all analyzed groups. In fact, the long lasting Ab inhibition in OVA-fed naïve mice was related to the down-modulation of B7.2 expression on B cells for up to 8 months of immunization (Fig. 6).

In the next step, the participation of IL-10, TGF- β or CTLA-4 molecules in the down-modulation of B7 expression on B cells of OVA-fed naïve mice at 2 months of immunization was assessed. Cultures were performed with splenic cells and OVA plus anti-IL-10, anti-TGF- β mAb and blocking concentration of anti-CTLA-4 mAb. The expression of B7.1 and B7.2 on B cells after Ag-stimulation in presence of anti-IL-10 and anti-TGF- β was not modified (data not shown). Despite the fact that anti-CTLA-4 mAb did not alter B7.1 expression, an increase in the percentage of B220+B7.2+ cells was observed in comparison with the isotypic mAb control (Fig. 6). These findings showed that rather than suppressive cytokines acting in the anergic process, altered costimulatory molecules appear to be important for the long-lasting inhibition of Ab response in mice Ag-fed at 7 days before priming, under single- or double-feeding schedule.

Discussion

Oral administration regimens with OVA in A/Sn mice show that single Ag feeding prior to immunization or twice Ag feeding before and after immunization were able to induce long lasting anergy on Ag specific B and T cells. The durable inhibition of Ab production in OVA-fed mice after feeding was simultaneous to decreased B7.2 expression on B cells. Also, a partial restoration of the expression of B7 molecules on B cells of naïve-fed mice was evidenced in vitro when blocking with anti-CTLA-4 mAb.

Oral tolerance induction in naïve status is a well-established phenomenon that depends on the type, timing and dose of Ag and the genetic background concerned (Faria and Weiner, 1999; Vaz et al., 2003).

We observed that a single oral administration of OVA prior to immunization or twice Ag administration at naïve and primed status inhibited IgE response until the end of the observation period (8 months). A similar profile of Ab response was observed with both regimens of Ag administration, showing that twice orally treated mice had established regulatory mechanisms after the

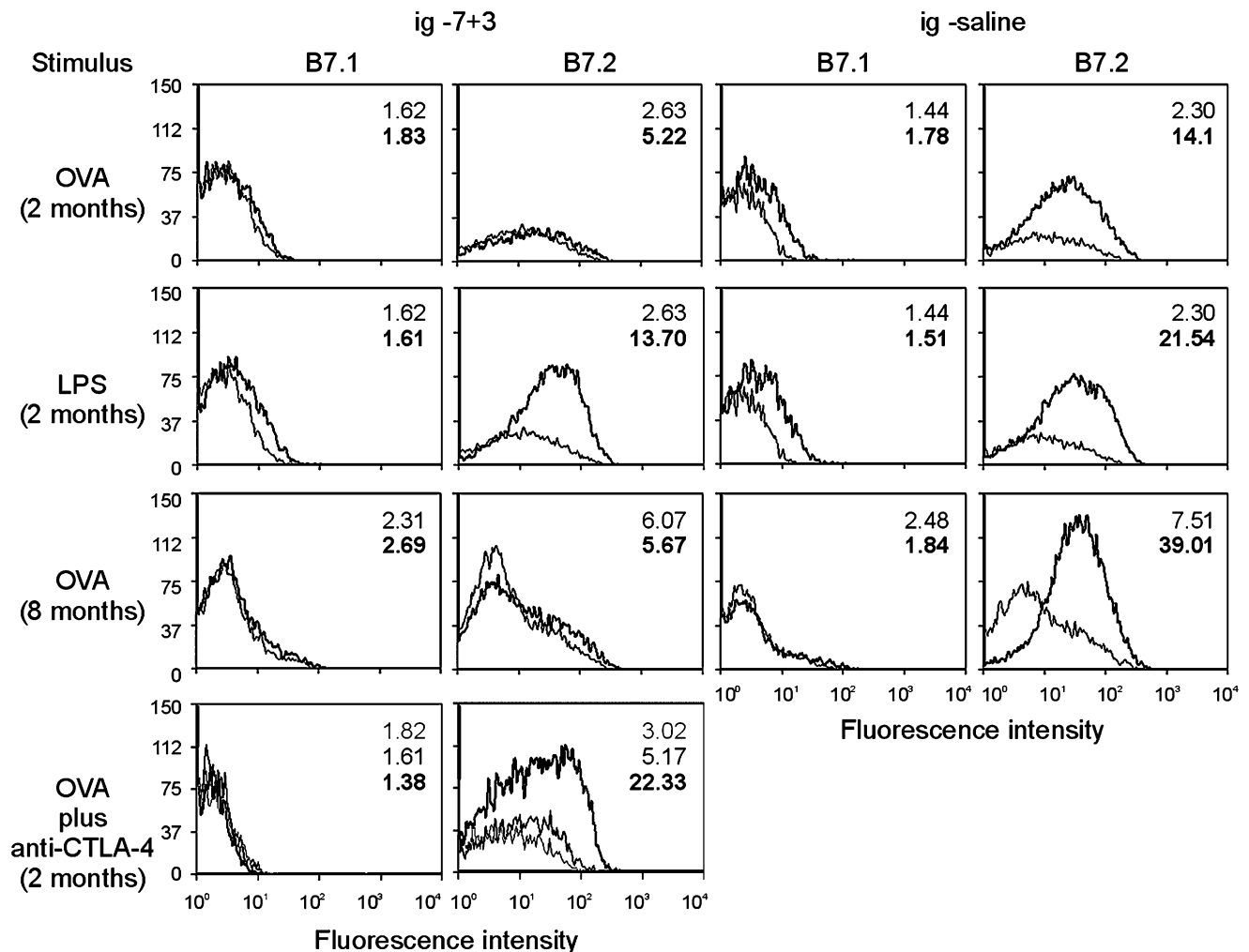


Fig. 6. B7.1 and B7.2 expression on B220+ cells. After 2 months of immunization spleen cells of double treated mice (ig -7/+3) and control immunized mice (ig saline) were cultured with OVA (200 µg/ml), LPS (50 µg/ml), OVA plus anti-CTLA-4 mAb (100 µg/ml) or OVA plus hamster control Ab (100 µg/ml). Single spleen cells suspension (individual mouse) of both mice groups were cultured with Ag, after 8 months of immunization. After 48 h of culture the cells were stained with anti-B220 (CyC), anti-IgM (FITC) and anti-CD80 (PE) or anti-CD86 (PE) and analyzed by flow cytometry. Histograms with light lines depict the non-stimulated cells and bold lines depict the cells stimulated with OVA. Numbers indicate the Mean Fluorescence Intensity (MFI) of non-stimulated cells (light number) and cells stimulated with OVA (bold number). Histograms of OVA plus anti-CTLA-4 mAb assay with gray lines depict the cells stimulated with OVA (gray number represent MFI), light line the cells stimulated with OVA plus control Ab (MFI, light number) and bold lines the cells stimulated with OVA plus anti-CTLA-4 mAb (MFI, bold number). Representative data from three independent experiments are shown.

first Ag encounter at a mucosal site, previous to Ag immunization.

It is worth noting that in naïve-fed mice the duration of IgG1 and IgG2a Ab inhibition was similar to that of IgE, whereas a more pronounced Ab inhibition was seen when Ag feeding occurred 7 days prior to immunization or upon double treatment – 7 days before and after immunization. Indeed, mice fed 3 days prior to immunization and those twice treated (3 days before and 3 days after immunization) lost the inhibitory effect on IgG1 and IgG2a response after 2 months of immunization. Our results show that the appropriate timing to

inhibit IgE response is up to 14 days before Ag priming. Other studies demonstrated that oral treatment prior to Ag immunization reduces Ag specific humoral responses (Simioni et al., 2004) for 11 months and inhibits delayed-type hypersensitivity response until 17 months (Strobel and Ferguson, 1987). Even though regulation of B-cell responsiveness appears to be more difficult in oral tolerance, a long-lasting suppression of specific Ab production is a desirable consequence in allergy immunotherapy. IgE, in its turn, is one exception, for it is relatively easily inhibited by oral tolerance (Vaz et al., 1977; Jarret, 1984; da Silva et al., 2004).

Primed mice could be tolerized by OVA feeding; however, the degree of tolerance and its effects on systemic immune responses were more limited than those in naïve animals (Torseth and Gregerson, 1998; Leishman et al., 2000). We observed that primed mice fed with OVA had decreased IgE Ab production only in the primary response, which disappeared 2 months after Ag feeding. Attempts at oral tolerance induction after Ag priming have demonstrated that high doses of Ag were more effective when given at 2–4 days after immunization (Leishman et al., 1998). Nevertheless, T and B unresponsiveness should be just a transitory effect in Ag primed mice, perhaps enhanced with Ag re-exposure. In fact, Ag re-treatment of primed OVA fed mice demonstrated an exacerbation of IgG1 Ab production. In addition, the type and dose of allergen are important factors to induce oral tolerance in primed mice. Indeed, in a previous work, we demonstrated that feeding mice with low dose (0.25 mg) of the mite *Dermatophagoides pteronyssinus* (Derp) at the 6th, 7th and 8th day of sensitization down-modulated IgE and Th2 response (Sato et al., 1998), probably due to stimulation of TGF- β secretion (Sato et al., 2001). Furthermore, the increase of Derp amounts (10 mg) in oral administration maintained the IgE inhibition but exacerbated IgG1 Ab response (Sato et al., 1998), an isotype which may exert an anaphylactic role in mice.

Oral administration with high doses of Ag can induce anergy – a consequence of the cell cycle arrest after Ag feeding (Sun et al., 2003). Anergic T cells do not proliferate or secrete IL-2 in response to Ag stimulation (Schwartz, 1990). In contrast to Ag primed fed mice, naive and double-treated mice showed a suppressed Ag-proliferative response that was paralleled by remarkable decreases in IL-2, IL-4, IL-10 and IFN- γ production. These findings indicate that T-cell anergy – induced by high dose of OVA feeding – affects both Th1 and Th2 responses. Moreover inhibition of IFN- γ secretion in naive fed mice was restored with in vitro anti-CD28 mAb stimulation. It has been shown in cultures that anti-CD28 mAb result in maximal proliferation of CD4+ T cells or T-cell clones that can block the induction of anergy (Chung et al., 2003).

Anergy can be induced in the absence of costimulatory signal, provided by soluble cytokines or by interactions between costimulatory molecules and the respective receptors on Ag presenting cells, including B cells. B7.1 and B7.2 molecules have dual specificity for two CD28 family members, CD28 and CTLA-4. Naive mice fed with high dose of OVA showed decreased expression of B7.2 molecules on B cells. This decreased expression in naive and double treated mice should lead to an inefficient CD28 interaction, resulting in T- and B-cell anergy.

There are considerable data to support a role for CTLA-4 in tolerance of both Th1 and Th2 responses

(Perez et al., 1997; Inobe and Schwartz, 2004; Chikuma et al., 2005), and in vivo CTLA-4 blockade accelerates Ag specific T-cell cycle progression following OVA feeding (Samoilova et al., 1998). We observed that blockade of CTLA-4 expression with high amounts of mAb in the cultures increased B7.2 expression on B cells from naive mice fed with high dose of OVA, whilst interference with suppressive cytokines by IL-10 and TGF- β mAb had no effect. This result suggests that the decreased expression of B7 molecules favored the binding with CTLA-4 and consequently the anergic response.

The data presented here demonstrates that feeding with OVA induces long-lasting anergy mediated by an impaired T–B-cell co-operation and Ab production, reflected by decreased B7.2 expression on B cells and negative signal delivered by CTLA-4.

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