

Regulation of Antigen-Specific IgE, IgG1, and Mast Cell Responses to Ingested Allergen by Mucosal Tolerance Induction¹

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Mucosal administration of soluble protein Ags results in profound immunologic nonresponsiveness, characterized by reduced production of Th1 and Th2 cytokines and concomitant suppressed Ig production. It has been suggested that Th2 cells are required for the induction and maintenance of this tolerogenic state. In this study, we show that oral tolerance induction abrogates subsequent Th2-driven Ag-specific IgE and IgG1 responses, while intranasal tolerance induction only blocks the production of IgE, but not IgG1. Consistent with suppressed IgE serum levels, elevated IFN- γ production was observed in the spleens of tolerized mice. Moreover, both oral and intranasal tolerance induction were found to inhibit intestinal mast cell responses upon subsequent priming and intragastric provocation. Transfer of total splenocytes or purified CD4⁺, but not CD8⁺, T cells from intranasally tolerized mice clearly suppressed ongoing Ag-specific IgE, but not IgG1, responses in primed recipients. In addition, coadministration of IFN- γ -neutralizing Abs completely blocked the transfer of suppression to primed recipients. These results show that Th2 cells can be subjected to tolerance induction, by inducing cross-regulatory, IFN- γ -producing CD4⁺ T cells. Moreover, our results point out differences in the regulation of T cell-dependent Ag-specific IgE and IgG1 responses. *The Journal of Immunology*, 1997, 159: 3009–3015.

Infancy and early childhood are generally acknowledged to be a high risk period for primary allergic sensitization to environmental allergens (1). Several studies on postnatal appearance of serum IL-4 and allergen-specific IgE Ab levels to dietary allergens have shown that both levels peak by an age of 6 to 9 mo, which is a period of strong antigenic stimulation (2, 3). Moreover, it has been demonstrated that T cells derived from neonates show a decreased capacity of IFN- γ production, compared with adult levels (4), and furthermore, this maturational defect is most marked in children with a genetic predisposition to atopy (5). In fact, lack of sufficient IFN- γ production may contribute to impaired neonatal antiviral immune responses and facilitate allergic sensitization as a result of abnormal T cell function. Studies on neonatal B and T cell functioning have indeed demonstrated defective Th cell activity until at least 6 mo of age, characterized by low expression of the IL-2R (6).

Evidence has been presented that priming of T cells to environmental allergens might occur in utero or during very early infancy (1). Characteristically, responses to food allergens are found mainly during neonatal life in both atopic and normal children. Although atopic children usually display longer lasting and more

profound allergic responses, IgE serum levels to food allergens usually wane within the first 3 yr of life (3). These findings suggest that normal environmental exposure results in the induction of a very efficient down-regulating mechanism in both atopic and normal individuals, eventually switching off IL-4-driven IgE responses (1). In fact, food-allergic responses hardly ever survive into adulthood, which suggests that tolerance to food allergens is established very efficiently. However, failure or a delay in this natural tolerance induction mechanism is thought to result in persistent allergen-specific IgE responses.

It has been suggested that increased concentrations of dietary Ag, e.g., at time of weaning, efficiently induce complete T cell anergy as well as T cell-mediated immune suppression, characterized by increased IFN- γ production and elevated plasma IL-2R concentration (1, 5, 6). Several studies in experimental animals have demonstrated that mucosal administration of allergen before systemic sensitization with the same Ag favors long lasting immune suppression instead of priming (7–9). Characteristically, these tolerizing protocols result in decreased T cell proliferative responses and decreased IL-2 production, a situation that inhibits isotype switching to IgE. Tolerance induction via the nasal or oral route was found to be mediated by CD4⁺ T cells (8, 9), whereas in case of aerosol-induced tolerance, CD8⁺ T cells expressing the $\gamma\delta$ TCR are held responsible (7).

In the present study, we focus on the application of mucosal tolerance induction, i.e., intranasal and oral tolerance induction, to abrogate IgE, IgG1, and mast cell responses to subsequently ingested allergen. Previous reports have shown that administration of high doses of Ag via the oral mucosa favors clonal anergy (10, 11), while low dose tolerance induction via the respiratory tract is thought to be based on immune deviation or active suppression (7). Based on these distinct mechanisms, results on mucosal tolerance induction performed either before or after primary Th2 sensitization will be presented.

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Materials and Methods

Mice

Specific pathogen-free female BALB/c mice were obtained from Harlan CPB (Zeist, The Netherlands) or from Culas (Sydney, Australia) and were maintained under standard animal housing conditions. The mice were bred and kept on an OVA-free diet and were used at 8 to 12 wk of age. All experiments were approved by The Animal Experiments Committee of Vrije Universiteit and Animal Experimentation Ethics Committee of the Institute for Child Health Research.

Tolerance induction and subsequent Ag sensitization

To induce oral tolerance, mice were given a single feed of 20 mg of OVA type VII (Sigma Chemical Co., St. Louis, MO) in 0.2 ml of PBS. Ag sensitization was started 7 days later. To induce intranasal tolerance, mice received 10 μ l of PBS containing 100 μ g of OVA type VII intranasally on 3 consecutive days. One day later, Ag sensitization was started. Ag sensitization consisted of two i.p. OVA injections of 1 μ g of OVA adsorbed on 1 mg of alum (F2200 Reheis Chemical Company, Berkeley Heights, NJ) with a 2-wk interval. Four days after the second injection, mice were challenged intragastrically (i.g.)³ with 1 mg of OVA dissolved in 0.2 ml of PBS (12). Serum and spleens were collected at 4 days after i.g. challenge for determination of OVA-specific IgE and IgG1 levels and cytokine production *in vitro*. Small bowel tissue was collected for histamine analysis.

Measurement of OVA-specific serum responses by ELISA

Falcon 3912 Microtest III plates were coated overnight at 4°C with 2 μ g/ml of rat anti-mouse IgE (EM95) (12) in PBS. After washing with PBS/0.1% BSA/0.05% Tween-20, the plates were incubated for 1 h at room temperature with PBS containing 1% BSA to block nonspecific protein-binding sites. After blocking, the plates were washed, and samples, diluted in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) containing 1% BSA, were added in a volume of 100 μ l and incubated for 3 h at room temperature. As a positive standard, pooled serum obtained from multiple OVA-boosted mice was used with an arbitrary concentration of 1000 U/ml. The plates were washed three times, and 1 μ g/ml of OVA-digoxigenin in PBS/1% BSA/0.05% Tween-20, coupled as described previously (12), was added. After incubation for 1 h at room temperature, the plates were washed three times, and 150 mU/ml of peroxidase-coupled sheep anti-digoxigenin Fab fragments (Boehringer Mannheim Corp., Mannheim, Germany) diluted in PBS/1% BSA/0.05% Tween was added. After a final incubation for 1 h at room temperature, the plates were washed three times before 100- μ l substrate of 2 mg/ml *O*-phenylenediamine-dihydrochloride (Sigma Chemical Co.) in 0.1 M phosphate-citrate buffer containing 0.015% hydrogen peroxide was added. The plates were read at 490 nm, and IgE concentrations were determined with reference to a standard curve constructed using serial dilutions of the positive standard serum. The detection limit of the ELISA is 0.5 U/ml.

To determine OVA-specific IgG1 serum levels, plates were coated with 10 μ g/ml of OVA VII in PBS. For detection, biotinylated rabbit anti-mouse IgG1 (Zymed, San Francisco, CA) and peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) were used at a concentration of 0.3 and 1.6 μ g/ml, respectively. The same positive standard serum as described above was used as a standard, at an arbitrary IgG1 concentration of 1000 U/ml. The detection limit of the ELISA is 0.005 U/ml.

Induction and measurement of IFN- γ production *in vitro*

To measure IFN- γ production, spleens were collected and erythrocyte-depleted single cell suspensions were obtained by suspending the cells in RPMI 1640 medium, containing 5% FCS, 1 mM glutamine, 2500 IU/ml penicillin, 2500 IU/ml streptomycin, and 5×10^{-5} M 2-ME. Spleen cells were cultured in 24-well tissue culture plates (Greiner, Alphen a/d Rijn, The Netherlands) at 2×10^6 cells/ml in 1-ml aliquots stimulated with either 5 μ g/ml Con A (ICN, Zoetermeer, The Netherlands) or medium alone. Supernatants were harvested after 48 h of culture and stored at -70°C until assayed.

IFN- γ was quantified using a sandwich ELISA. Plates were coated overnight at 4°C with 1 μ g/ml of rat anti-mouse IFN- γ Ab XMGI.2 (kindly provided by Dr. R. L. Coffman, DNAX, Palo Alto, CA) in PBS. After washing the plates with PBS/Tween-20, nonspecific protein binding was blocked by incubating the plates with PBS/1% BSA for 1 h at room temperature. Samples were incubated for 3 h at room temperature, diluted in

RPMI 1640 medium containing 0.1% BSA. After washing the plates three times, biotinylated rat anti-mouse IFN- γ Ab R46.A2 (provided by Dr. R. L. Coffman) was added at a concentration of 0.75 μ g/ml and the plates were incubated for an additional hour. Finally, streptavidin-peroxidase and *O*-phenylenediamine dihydrochloride were used for color development, as described above. Cytokine concentrations were determined with reference to a standard curve constructed using serial dilutions of murine rIFN- γ (Genzyme Corp., Sanbio, Uden, The Netherlands). The detection limit of the ELISA is 55 pg/ml.

Histamine analysis in whole gut tissue homogenates

To study histamine content in the small intestines to determine mast cell activation, the first 5 cm of duodenum, distal from the ligament of Treitz, was collected 4 days after i.g. challenge. In a previous study, we have reported detectable histamine depletion in the small intestines at this time point (12). Intestinal samples were flushed with 5 ml of cold 0.9% NaCl solution, before homogenizing with 1 ml of saline. After thoroughly mixing, 200 μ l of 12% perchloric acid in saline was added and the samples were centrifuged for 10 min at $10,000 \times g$. Subsequently, 1 ml of supernatant was collected and again precipitated with 200 μ l of 12% perchloric acid. Finally, supernatant was collected after centrifugation and stored at -20°C until further analysis. Histamine concentration was measured by fluorometric analysis, as described previously (13).

Transfer experiments

Donor mice were tolerized by three daily intranasal OVA administrations. One day after the last administration, mice were primed with 1 μ g of OVA on alum. After 2 wk, the mice were boosted, followed by i.g. challenge 4 days later. Spleens of tolerized and control, naive mice, were collected and pooled 4 days postchallenge. Erythrocyte-depleted single cell suspensions were prepared as described above.

For total splenocyte transfer, cells were washed in sterile PBS and 60×10^6 cells were transferred via i.p. injection to primed and boosted recipients that received an i.g. challenge immediately upon transfer. For partial purification, cells were incubated for 15 min on ice with B cell-specific rat mAb (RA3-6B2, anti-B220 and 187-1, anti- κ). After washing the cells in RPMI 1640 medium containing 1% BSA, sheep anti-rat IgG Dynabeads (Dyna, Oslo, Norway) were added in a 2:1 bead to cell ratio. Spleen cells were incubated for 15 min on a rotator at 4°C. Positively stained cells were depleted by using the magnet. The remaining cell population contained 60% CD4⁺ T cells, as tested by FACS analysis (FACStar; Becton Dickinson, Mountain View, CA), using PE-conjugated anti-CD4 mAb (GK1.5, kindly provided by Dr. R. E. Mebius, Vrije Universiteit). Partially purified CD4⁺ T cells were washed in sterile PBS, and 1×10^6 cells were transferred by i.v. tail injection to primed and boosted recipients.

For further purification, cells were washed and incubated with PE-conjugated anti-CD4 for an additional 15 min on ice after initial B cell depletion. After washing, the cells were positively sorted by FACS, resulting in 95% purity for CD4⁺ T cells, as tested by reanalysis. Highly purified CD4⁺ T cells were washed in sterile PBS, and 1×10^6 cells were transferred by i.v. tail injection to primed and boosted recipients. In case of CD8⁺ T cell transfer experiments, cells were purified and transferred, as described, using PE-conjugated anti-CD8 mAb (53-6.7, provided by Dr. R. E. Mebius) and positive selection by FACS, resulting in 95% purity for CD8⁺ T cells, without contaminating CD4⁺ T cells, as tested by immunohistochemical staining of cytopins.

Ab treatment *in vivo*

Primed and boosted recipient mice were injected i.p. with rat IgG1 mAb specific for murine IFN- γ (XMGI.2, 1 mg/mouse) (14) before i.g. challenge and transfer of spleen cells, derived from either tolerized or naive donors. Control mice received an isotype-matched rat IgG1 Ab, directed to *Escherichia coli* β -galactosidase (GL113, 1 mg/mouse) (15). Both mAbs were cultured and purified as described elsewhere (16).

Statistic analysis

Results are represented as mean $\pm$ SEM, where indicated, and were analyzed by using Student's *t* test. Values of *p* < 0.02 were considered significant.

Results

Effects of tolerance induction on OVA-specific IgE and IgG1 production

Previously, we have shown in mice that low dose OVA priming, followed by an i.g. OVA challenge, results in a rapid increase of

³ Abbreviations used in this paper: i.g., intragastric; CD40L, CD40 ligand; PE, phycoerythrin.

Table 1. Effects of intranasal or oral tolerance induction on serum antibody production and splenic IFN- γ production^a

Group	OVA-Specific IgE	OVA-Specific IgG1	Con A-Induced IFN- γ
Nontolerant	1039 $\pm$ 148	594 $\pm$ 80	352 $\pm$ 50.9
Oral tolerant	130 $\pm$ 14.3*	0*	654 $\pm$ 100*
Intranasal tolerant	267 $\pm$ 9.9*	535 $\pm$ 103	641 $\pm$ 120.3*

^a Serum levels of OVA-specific IgE (U/ml) and IgG1 (U/ml) Abs measured by ELISA at 4 days after previous systemic priming, boosting, and subsequent intragastric challenge. Prior to systemic sensitization, mice were tolerized by either a single 20-mg OVA VII feed or three daily intranasal OVA VII applications of 100 μ g. Corresponding splenic IFN- γ production (ng/ml) after 48 h culture with 5 μ g/ml Con A is shown. Results presented are mean $\pm$ SEM of five mice per group. *, $p < 0.01$ vs nontolerant, but OVA primed, boosted, and challenged mice. Similar results were obtained in two replicate experiments.

OVA-specific IgE, peaking 4 days after provocation (12). These data are compatible with a selective induction of a Th2-type response. Moreover, i.g. provocation results in increased OVA-specific IgG1 serum levels as well, which remain high over a longer period when compared with IgE levels. We wished to study the effects of tolerance induction via the mucosa on the generation of both isotypes when using our immunization protocol. We therefore compared both oral and intranasal tolerance induction.

Mice that were fed with a high dose OVA before priming and subsequent i.g. challenge showed significantly suppressed OVA-specific IgE and IgG1 serum levels compared with nontolerized, but OVA-sensitized and -challenged mice (Table 1). In addition, when mice were tolerized intranasally, significantly suppressed Ag-specific IgE responses were found. In contrast to the oral tolerance induction protocol, no suppressive effect on OVA-specific IgG1 levels was detected upon intranasal tolerance induction. These results suggest different regulatory mechanisms for Ag-specific Ab production upon Th2 sensitization or different effector mechanisms after tolerization via these different routes.

Based on these results, we next studied the capacity of intranasal tolerance induction to suppress Ag-specific IgE and IgG1 production when applied at different time points during systemic sensitization. Mice that received OVA intranasally before either systemic priming or boosting showed significantly reduced IgE levels upon subsequent i.g. challenge, compared with nontolerized mice (Fig. 1, *top*). However, tolerance induction after the systemic boost, shortly before i.g. challenge, was not able to block IgE production after subsequent i.g. provocation. Irrespective of the time of administration, intranasal tolerance induction never led to a significant decrease of serum OVA-specific IgG1 levels (Fig. 1, *bottom*). In fact, additional intranasal administration of OVA before boost or challenge resulted in even enhanced IgG1 production, compared with nontolerized mice.

Effects of tolerance induction on IFN- γ production in vitro

As intranasal tolerance induction was found to result in decreased IgE serum levels, the role of IFN- γ , the key regulatory cytokine that inhibits IL-4-induced IgE isotype switching (17), was studied. Total splenocyte cultures were set up at 4 days after i.g. challenge, when IgE levels peak, and cells were stimulated with Con A or medium alone for 48 h. Consistent with their suppressed IgE production, tolerant mice showed significantly increased IFN- γ levels when stimulated in vitro (Table 1). Moreover, intranasal tolerance induction after systemic priming, but before boosting, resulted in significantly enhanced IFN- γ production as well when compared with control, nontolerized but OVA-sensitized and -challenged mice (Fig. 2). However, systemic OVA priming and boosting, followed by intranasal OVA administration and subsequent i.g. chal-

lenge, which did not lead to tolerance induction (Fig. 1, *top*), resulted only in a moderate, not significant, increase in IFN- γ production and corresponding enhanced serum IgE levels. These results suggest an important role for IFN- γ as effector cytokine in the induction and maintenance of tolerance. However, increased IFN- γ production does not affect OVA-specific IgG1 production.

Tolerance induction inhibits local mast cell activation and subsequent histamine release

As tolerance induction was found to be most effective early in the sensitization phase, we were interested to study whether mast cell degranulation could still occur in tolerant mice upon i.g. challenge. Previously, we observed enhanced mast cell degranulation in the small intestines of mice that had been challenged i.g. after systemic OVA sensitization (12). As shown in Figure 3A, both intranasal and oral tolerance induction, before systemic priming and i.g. challenging, inhibited histamine release in small bowel tissue. Similar results were obtained when either oral or intranasal tolerance induction was applied after systemic priming, but before boosting (Fig. 3B).

Transfer of intranasal tolerance blocks the effect of i.g. challenge in sensitized recipients

To test the hypothesis that intranasal tolerance induction is based on active T cell-mediated suppression, transfer experiments were performed, using spleen cells from intranasally tolerized mice. As shown in Figure 4, transfer of 60×10^6 total splenocytes from tolerant mice significantly blocks the increase in OVA-specific IgE induced by subsequent i.g. challenge in recipient mice. Transfer of cells from tolerant mice initially inhibited IgG1 production, but after a delay, IgG1 levels rise rapidly and significantly, when compared with OVA-specific IgG1 levels in animals receiving cells from naive donors (Fig. 4, *bottom*). These results suggest that the transferred suppression specifically acts on the IgE production, but has no inhibitory effect on IgG1 production, suggestive for different regulatory mechanisms for Ag-specific IgE and IgG1 production.

Further purification of tolerant splenocytes showed that intranasally induced tolerance can be transferred into sensitized recipients with CD4⁺ T cells (Fig. 5). Transfer of 1×10^6 partially or highly purified CD4⁺ T cells significantly blocks OVA-specific IgE production in i.g. challenged recipients. Again, purified cells from tolerant donors were not able to significantly suppress IgG1 levels in recipient mice (Fig. 5).

To exclude the possibility that the results obtained from these transfer experiments with highly purified CD4⁺ T cells are due to contaminating CD8⁺ T cells, transfers were performed with purified CD8⁺ T cells. Transfer of 2.5×10^5 purified CD8⁺ splenic T cells, a number of which is at least a fivefold excess of the possibly contaminating CD8⁺ T cells in the CD4⁺ T cell transfer studies, did not inhibit IgE responses in recipient mice, nor did they affect IgG1 responses (Fig. 6).

IFN- γ Ab treatment in vivo blocks transfer of tolerance to sensitized recipients

In addition to the results shown in Figure 2, we observed that total spleen cells from intranasally tolerized mice produced IFN- γ upon restimulation with OVA in vitro (86 ± 4.2 ng/ml) at day of adoptive transfer, compared with control, naive, transferred cells that did not secrete any detectable levels of IFN- γ . Based on these results, we wished to demonstrate that IFN- γ is indeed the crucial cytokine in the induction and maintenance of intranasal tolerance

FIGURE 1. Effects of intranasal Ag administration at different time points during systemic sensitization. Mice received three daily administrations of 100 μ g of OVA dissolved in 10 μ l of PBS intranasally (I.N.), before either priming (prime), boosting (boost), or i.g. challenge (chall). Control nontolerized animals received PBS intranasally (-). All groups were systemically primed and boosted with 1 μ g of OVA and received an i.g. 1-mg OVA challenge on day 18. OVA-specific IgE (top) and IgG1 (bottom) serum levels at 4 days after challenge are shown as mean levels (U/ml) $\pm$ SEM for four mice per group, *, $p < 0.02$ vs control nontolerant mice.

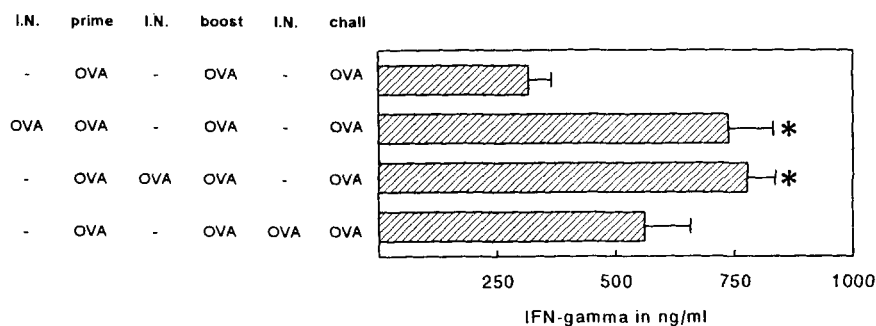
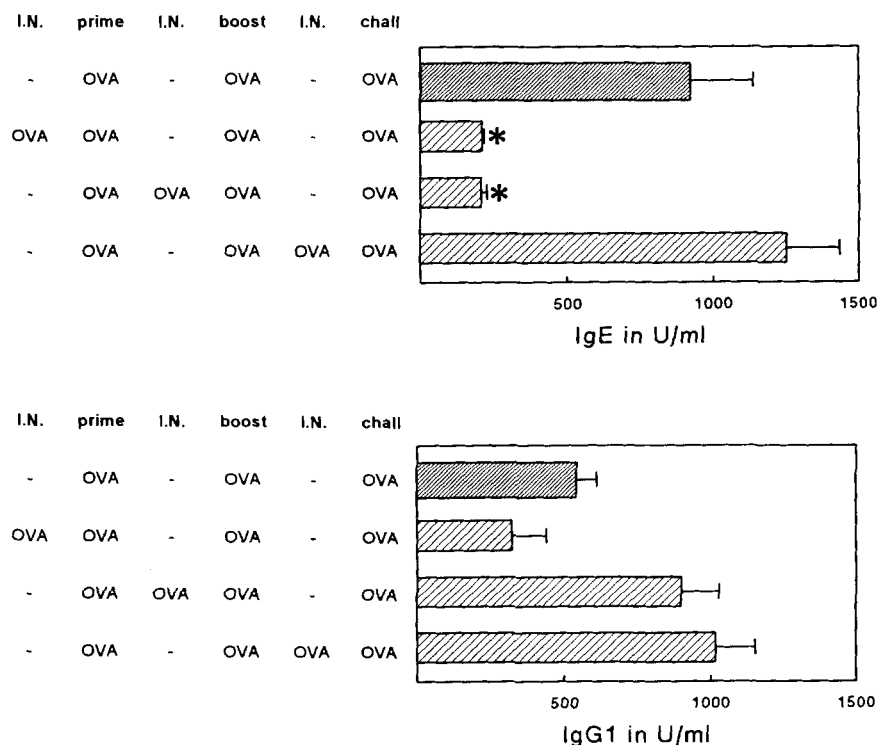


FIGURE 2. Effects of tolerance induction on Con A-induced IFN- γ production in vitro. Mice were systemically primed and boosted with 1 μ g of OVA, followed by an i.g. challenge on day 18. Mice received three intranasal OVA administrations (I.N.) before either priming (prime), boosting (boost), or i.g. challenge (chall). Control, nontolerant mice were primed, boosted, and challenged, but received PBS intranasally (-). Four days after the challenge, erythrocyte-depleted spleen cells were cultured for 48 h in the presence of 5 μ g/ml Con A, and IFN- γ production was measured by ELISA. Results of five mice per group are shown as mean levels of IFN- γ (ng/ml) $\pm$ SEM. *, $p < 0.01$ vs control nontolerant mice.

induction. For this purpose, recipient mice were injected with neutralizing IFN- γ or control Abs before transfer of spleen cells derived from intranasally tolerized donor mice. Control mice received the same Ab treatment, combined with spleen cells derived from untreated, naive donor mice. As shown in Figure 7, IFN- γ -treated recipient mice demonstrated a complete abrogation of suppression of IgE production, when compared with recipients that received cells from the same tolerant donors, but were treated with control Abs. In addition, recipients that received naive cells in combination with IFN- γ Abs showed unaffected, increased IgE serum levels, indicating that the IFN- γ Abs specifically acted on the Ag-specific T cells that transfer tolerance. Again, no suppression of IgG1 production was observed. In fact, IFN- γ Ab-treated recipients were found to have significant increased OVA-specific IgG1 serum levels upon transfer of spleen cells from intranasally tolerized donors.

Suppression of IgE responses by tolerant spleen cells depends on cell numbers transferred

Based on previous reports on dose-response experiments on transfer of tolerant cells (7), we were interested to study whether tol-

erance could still be transferred with 2-fold or 10-fold less cells, as used in our previous experiments. As shown in Table II, a minimum of 50×10^6 total spleen cells from intranasally tolerized donors is required to inhibit IgE responses in sensitized recipient mice. On the contrary, a minimum of 1×10^6 total spleen cells from orally tolerized mice was found to be capable of suppressing IgE production upon transfer.

Discussion

Although previous reports have shown that low dose oral tolerance, followed by high dose parenteral priming in CFA, results in the development of regulatory Th2 clones, producing high levels of IL-4 and IL-10, but minimal IFN- γ (18), we demonstrate in this study that three consecutive intranasal Ag applications, followed by parenteral Th2 priming, induce regulatory CD4 $^{+}$, but not CD8 $^{+}$, T cells. These results are in line with a recent report, demonstrating that transient activation of CD4 $^{+}$ T cells precedes the development of intranasal tolerance in vivo (9). Moreover, intranasal tolerance induction is not deficient in CD8 $^{-/-}$ mice, suggesting that CD8 $^{+}$ T cells are not the major effector T cell subset in this tolerizing protocol (9).

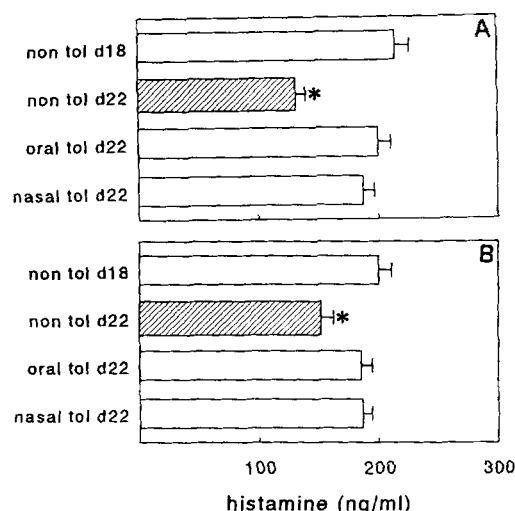


FIGURE 3. Effects of tolerance induction before priming (A) or before boosting (B) on histamine depletion in the small bowel, measured at 4 days after i.g. provocation (d22). Mice received either 20 mg of OVA orally (oral tol) or $3 \times 100 \mu\text{g}$ of OVA intranasally (nasal tol). Control nontolerized mice (non tol) were primed, boosted, and challenged, but received PBS intranasally. Results are shown as mean histamine levels (ng/ml) $\pm$ SEM in small bowel specimens of four mice per group. *, $p < 0.02$ vs control levels of nontolerant mice determined at day of challenge (d18).

Our results show that these regulatory CD4^+ T cells are able to suppress both primary and ongoing IgE responses in Th2 skewed recipients and subsequently inhibit mast cell responses in the small intestines. Furthermore, inhibition of IgE production in tolerant mice was associated with an increased production of $\text{IFN-}\gamma$, as measured in vitro. Although Con A-induced $\text{IFN-}\gamma$ production, as shown in Table I and Figure 2, might not fully represent OVA-induced cytokine production, adoptive transfer was abrogated completely by using $\text{IFN-}\gamma$ -neutralizing Abs. Together, these results point out a key role for $\text{IFN-}\gamma$ in the induction and maintenance of intranasal tolerance.

The general concept of high dose oral tolerance is complete T cell unresponsiveness, e.g., T cell anergy (19). Although it is generally believed that this state of nonresponsiveness cannot be transferred into naive recipients (20), we observed partial suppression of OVA-specific IgE responses, induced by systemic Th2 priming followed by i.g. challenge, after transfer of a minimum of 1×10^6 total splenocytes from donors tolerized orally with a high dose of OVA. Thus, upon high dose oral tolerance induction, some allergen-specific CD4^+ T cells probably evade the anergizing process (21), and can transfer suppression into either naive or Th2-primed recipients.

Taken together, mucosal tolerance induction involves the activation of cross-regulatory T cell subsets, depending on subsequent Ag exposure. Therefore, we believe that mucosal tolerance induction per se is not a Th2-driven process, demonstrated by the fact that Th2 cells themselves can be subjected to either high dose oral or low dose intranasal tolerance, as demonstrated in this study. This hypothesis is supported by the fact that high dose oral tolerance can still be induced successfully in $\text{IL-4}^{-/-}$ mice (8).

A possible mechanism, contributing to the observed decreased serum IgE and IgG1 levels induced by high dose oral tolerance induction before systemic Th2 priming, might be impaired costimulatory molecule expression, like CD40L, on anergic T cells. Activated T cells express high levels of CD40L, and cross-linking

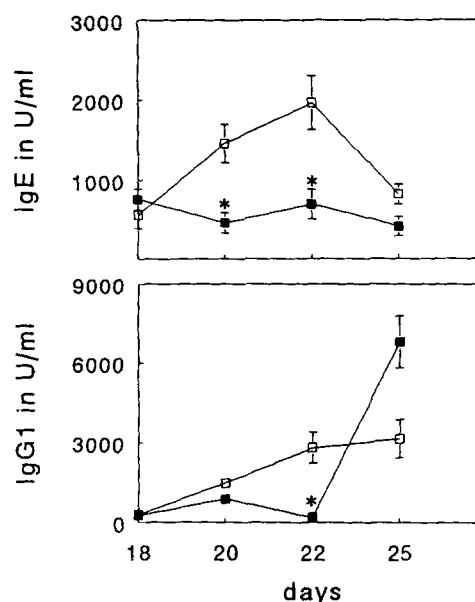


FIGURE 4. Transfer of total splenocytes from intranasally tolerized mice blocks subsequent IgE, but not IgG1, responses in Th2-primed recipients. Donor mice were tolerized intranasally before systemic priming, followed by i.g. challenge. Spleen cells were collected 4 days after i.g. challenge, and 60×10^6 cells were transferred into primed and boosted recipients (closed squares) that were challenged i.g. immediately upon transfer. Serum was collected at the day of transfer (day 18) and the days thereafter, as indicated. Results of serum IgE (top) and IgG1 (bottom) levels are shown as mean levels (U/ml) $\pm$ SEM of four mice per group. *, $p < 0.02$ vs control mice that were primed, boosted, and challenged, but received spleen cells from naive donors (open squares).

of this molecule, in conjunction with ligation of the TCR/CD3 complex and CD28, up-regulates IL-4 synthesis (22). Moreover, blockade of CD28-mediated costimulation is known to result in T cell anergy in vitro (23), indicating the importance of costimulatory molecules in T cell priming. Both IL-4 and costimulation via CD40-CD40L interaction induce steady state levels of germline $\gamma 1$ and ϵ transcripts in B cells, necessary for subsequent further isotype switching (24). Impaired induction of CD40L expression is thought to result in fewer responder B cells and suppressed serum Ab levels (25), and could account for the decreased serum IgE and IgG1 levels, as described in this work.

Strikingly, intranasal tolerance induction had no influence on OVA-specific IgG1 levels, indicative of different regulatory mechanisms for IgE and IgG1 production. In this respect, our results confirm previous data on decreased IgE, but not IgG levels, upon transfer of splenocytes from aerosol-tolerized donors into naive recipients and subsequent parenteral challenge (7). Although IL-4 is crucial for the induction of both isotypes, as demonstrated in $\text{IL-4}^{-/-}$ mice (26), it has been shown that less IL-4 is required for obtaining peak IgG1 responses compared with peak IgE responses (27). This may explain why in our model increased levels of $\text{IFN-}\gamma$ clearly suppressed OVA-specific IgE, but did not affect IgG1 levels. In fact, while IgE production in sensitized recipients could not be suppressed upon transfer of tolerant T cells in the presence of neutralizing $\text{IFN-}\gamma$ Abs, IgG1 production was increased significantly in these mice. It could be speculated that blocking $\text{IFN-}\gamma$ in these recipients results in optimum IL-4 production that facilitates both IgE and IgG1 isotype switching (27) by OVA-committed B cells that were cotransferred, based on the fact that increased IgG1

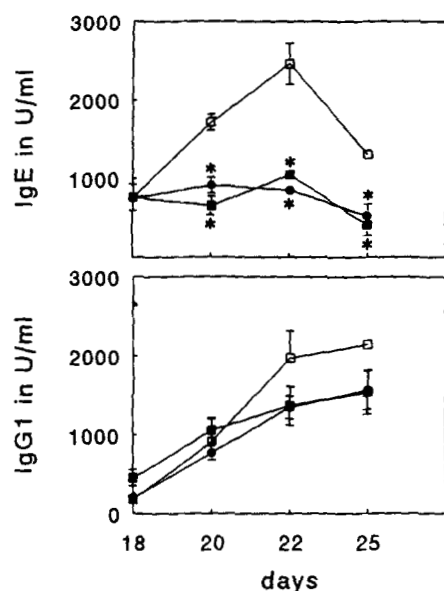


FIGURE 5. Intranasal tolerance induces regulatory CD4⁺ T cells that transfer tolerance into primed recipients. Donor mice were tolerized intranasally before systemic priming and boosting. At 4 days after i.g. challenge, spleen cells were collected and purified by using B cell depletion and a FACS sorter. After purification, 1×10^6 partially purified (60%, closed squares) or highly purified (95%, closed circles) CD4⁺ T cells were transferred to primed and boosted recipients that were challenged i.g. immediately upon transfer. Results of serum IgE (top) and IgG1 (bottom) levels in recipients are shown as mean levels (U/ml) $\pm$ SEM of four mice per group, * $p < 0.02$ vs control mice that were primed, boosted, and challenged, but received purified spleen cells from naive donors (95%, open squares).

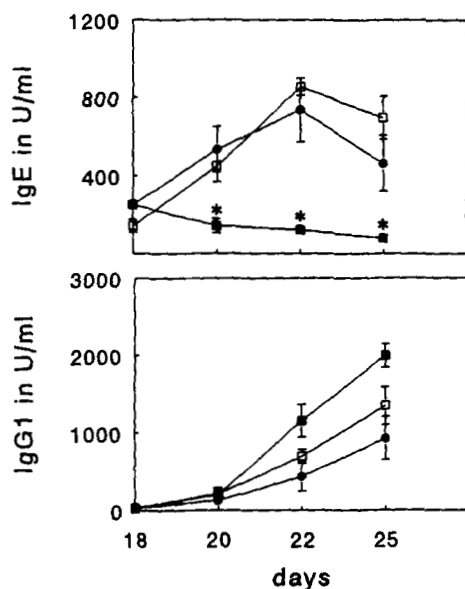


FIGURE 6. CD8⁺ T cells do not transfer tolerance. Donor mice were tolerized intranasally before systemic priming and boosting. At 4 days after i.g. challenge, spleen cells were collected and purified by using B cell depletion and a FACS sorter. After purification, 2.5×10^5 highly purified (95%, closed circles) CD8⁺ T cells were transferred to primed and boosted recipients that were challenged i.g. immediately upon transfer. Additionally, 50×10^6 total spleen cells, derived from intranasally tolerized donors, were transferred to a second group of recipient mice (closed squares). Results of serum IgE (top) and IgG1 (bottom) levels in recipient mice are shown as mean levels (U/ml) $\pm$ SEM of four mice per group, * $p < 0.02$ vs control mice that were primed, boosted, and challenged, but received 50×10^6 total spleen cells from naive donors (open squares).

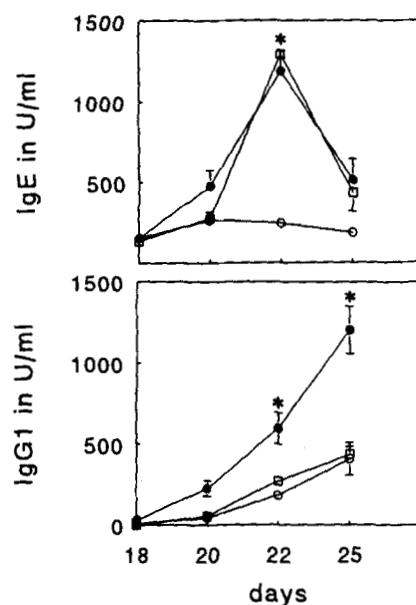


FIGURE 7. IFN- γ Ab treatment abrogates transfer of tolerance. Donor mice were tolerized intranasally before systemic priming and boosting. At 4 days after i.g. challenge, total spleen cells were collected, and 55×10^6 were transferred to primed and boosted recipients that were challenged i.g. immediately upon transfer. Before transfer, recipient mice were injected i.p. with 1 mg of either neutralizing IFN- γ (closed circles) or control Ab (open circles). Control recipient mice were primed, boosted, and challenged, but received 55×10^6 total spleen cells from naive donor mice together with IFN- γ Abs (open squares). Results of serum IgE (top) and IgG1 (bottom) levels (U/ml) $\pm$ SEM of four mice per group are shown for IFN- γ Ab-treated recipient mice that received spleen cells derived from tolerant donors, * $p < 0.01$ vs recipient mice that received the same spleen cells, but control Abs.

Table II. Percentage of inhibition of IgE responses in recipients upon transfer of different cell numbers of total splenocytes from tolerized donors^a

Source of Transferred Cells	1×10^6 Cells	5×10^6 Cells	10×10^6 Cells
Naive donor mice	0%	0%	0%
Oral tol donor mice	34.5%	ND	49.6%
Nasal tol donor mice	0%	67.3%	79.3%

^a Levels of OVA-specific IgE antibodies measured by ELISA in pooled sera (5 mice per group) collected at 4 days after previous systemic priming, boosting, and subsequent intragastric challenge. Prior to intragastric challenge, mice received numbers of spleen cells as indicated, derived from either orally (oral tol) or intranasally (nasal tol) tolerized donors. Results are presented as percentage of inhibition of IgE levels compared to IgE levels in control recipients that received equal numbers of cells derived from untreated, naive donors.

serum levels were not observed in anti-IFN- γ -treated mice that received naive spleen cells. Moreover, a similar dissociation of IL-4-dependent regulation has been reported for human IgE and IgG4 (21, 28). While IgE responses to dietary and most inhaled allergens are usually switched off during infancy and childhood, IgG4 responses are preserved and slowly increase in titer and affinity during life. It was suggested that IgG4 responses, as a memory B cell product, may be resistant to tolerance induction or alternatively, less dependent on T cell help (28).

Although low dose oral feeding or high dose intranasal application was not tested in this study, the potential mechanisms associated with these altered doses remain to be defined. Nevertheless, low dose intranasal tolerance induction, when applied early during primary allergic sensitization, might have long lasting effects with respect to IgE,

but not to IgG production, with potential application for immunotherapy in newborn, atopic children.

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