

Oral administration of specific antigens to allergy-prone infant dogs induces IL-10 and TGF- β expression and prevents allergy in adult life

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Background: Oral administration of allergens can induce immune tolerance to specific allergens in rodents and hence might be a possibility to prevent and treat allergic diseases in human subjects. However, the gastrointestinal tract of mice is different from that of human subjects. The absorption of specific antigens and subsequent antigen presentation to intestinal T cells is different in both species, making it difficult to extrapolate results.

Objective: We investigated primary oral tolerance to ovalbumin (OVA) in an IgE high-responder dog model, which is more predictive for human allergic diseases than corresponding rodent models.

Methods: Oral tolerance was induced by means of a 28-day treatment with OVA dissolved in cow's milk.

Results: We observed reduced OVA-specific IgE and IgG production in response to ensuing subcutaneous challenges. Allergic conjunctivitis induced by means of ocular and airway provocation was significantly reduced in tolerized animals compared with that seen in nontolerized control animals. In addition, eosinophilia and neutrophilia in bronchoalveolar lavage fluid and bronchoconstriction after airway allergen challenge were significantly suppressed in tolerized animals. Cytokine analysis by means of real-time PCR on bronchoalveolar fluid cells after allergen challenge revealed a high-level expression of IL-10 and transforming growth factor β , predominantly in the CD14⁺ population.

Conclusion: Feeding infant beagles with OVA for 4 weeks is sufficient to prevent hallmark manifestations of asthma and allergy in adult life. The mechanism of oral tolerance involved an increased expression of IL-10 and transforming growth factor β cytokines. (*J Allergy Clin Immunol* 2003;111:1069-75.)

Key words: Oral tolerance, allergy, regulatory cells, IL-10, TGF- β

Abbreviations used

BAL: Bronchoalveolar lavage
HPRT: Hypoxanthinephosphoribosyltransferase
OVA: Ovalbumin
TGF: Transforming growth factor

Allergic diseases, such as seasonal rhinoconjunctivitis and allergic asthma, are chronic inflammatory conditions usually occurring in genetically predisposed individuals after exposure to ubiquitous antigens. These diseases are associated with increased levels of antigen-specific IgE antibodies. In atopic patients IL-4 induces allergen-specific precursor T_H cells to differentiate into T_H2 cells. Activated T_H2 cells release IL-4 and IL-13, which, together with surface-bound CD40 ligand, activate B cells and induce their differentiation into IgE-producing plasma cells. IgE antibodies bind to high-affinity Fc ϵ R present on mast cells, which, after subsequent encounter with allergens, are activated and release mediators of allergic inflammation.

The higher susceptibility to allergies observed in atopic individuals is not fully understood, but the environment and genetic background of these individuals play an important role.¹ Therefore children of atopic parents have a higher risk of having an allergic disorder. The great number of factors influencing the development of allergy makes it very difficult to decide whether to focus research efforts on the therapeutic or preventive aspects of the disease. Therapeutic approaches to treat allergic diseases include treatment with corticosteroids, immunosuppressants, bronchodilators, antihistaminic agents, general anti-inflammatory agents, and, more recently, cytokine modulatory agents.² In contrast, allergy prevention is mostly achieved by means of allergen avoidance early in life, but efforts to find oral tolerogenic treatments capable of producing allergen tolerance have been attempted.³⁻⁶ This approach was first described in 1911, when Wells and Osborne⁷ found that feeding cow's milk, dried ox blood, and dried egg white for 3 to 8 weeks to guinea pigs rendered them insensitive to subsequent challenge with these proteins. Since then, antigen feed-

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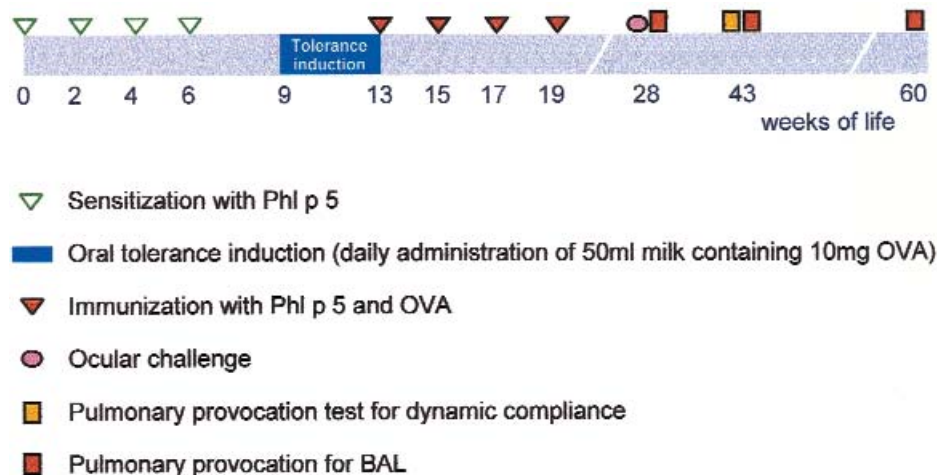


FIG 1. Experimental set-up. Newborn beagles were sensitized to Phl p 5 (weeks 0, 2, 4, and 6). Starting at the age of 9 weeks, one group (tolerized group) received 10 mg of OVA dissolved in 50 mL of milk orally once a day for 28 consecutive days. At the age of 13 weeks and subsequently thereafter (weeks 15, 17, and 19), all dogs (tolerized and control animals) were immunized with OVA and Phl p 5.

ing over a prolonged period of time has been used to induce peripheral tolerance in a variety of allergy⁸⁻¹⁰ and autoimmune models¹¹ in rodent systems, including mouse, rat, and guinea pig systems. The encouraging results obtained with these rodent models prompted a number of human trials in adult patients with established allergies,¹²⁻¹⁴ but there is no guarantee that reverting established conditions will be effective for preventive purposes. On the other hand, oral tolerance protocols used in rodents cannot be extrapolated to human subjects because the digestive systems of human subjects and rodents are very different. Thus a large animal species with a human-like digestion system and capable of allergy development, such as the dog, would be more appropriate to assess the efficacy of preventive treatments against allergies in human subjects.

Here we report that oral allergen immunotherapy in atopic beagles results not only in the suppression of the IgE responses but also in attenuation of other clinical parameters of allergy, such as allergic bronchial hyperresponsiveness and conjunctivitis. Furthermore, we demonstrate a link between the suppression of allergic symptoms and the induction of regulatory IL-10 and transforming growth factor (TGF) β cytokines.

METHODS

Animals

A beagle colony was established at the Novartis Research Institute as a model for human atopy.¹⁵ By means of selective breeding, these dogs could be differentiated into high and low IgE responders, which, after allergen immunization, produced 50 ± 18 and 3.5 ± 2.5 relative units, respectively, compared with values produced by a standard pool of specific IgE. For the purpose of this study, 12 newborn beagles from 4 different litters and the offspring of parents with known high IgE backgrounds were selected. These dogs received a strict ovalbumin (OVA)-free diet, and the allergen was introduced deliberately during the tolerization and immunization procedures, as described in Fig 1. These offspring were split into 2

groups (tolerized and control animals) of 6 dogs each. During all experiments, the guidelines of the Austrian law for the care and use of experimental animals were strictly followed.

Allergen sensitization and tolerance induction

Recombinant timothy grass pollen (Phl p 5, *Phleum pratense*) and OVA were obtained from Sigma Aldrich and Biomay, respectively. Induction of allergen-specific IgE was achieved according to an established immunization protocol.¹⁶ Immunization with Phl p 5 was performed as a specificity control (ie, OVA-tolerized dogs should be able to respond to challenge with Phl p 5). Briefly, both control and test groups were sensitized immediately after birth (week 0) with Phl p 5 at a dose of 1 μ g per dog. The allergen was dissolved in aluminum hydroxide (1.3% in water, Alu Gel S; Serva Feinbiochemica GmbH), mixed with saline solution (0.9%, 1:1), and administered subcutaneously in a total volume of 2.0 mL per dog. This procedure was repeated 3 times (weeks 2, 4, and 6), as indicated in Fig 1. OVA was selected as the test antigen because of its potential to induce allergic responses, its low cost, and its ability to control allergen exposure. Tolerance was achieved by using a 4-week treatment (weeks 9-13) consisting of daily feeding with 10 mg of OVA dissolved in 50 mL of fresh pasteurized cow's milk with a 3.6% fat content. Cow's milk protected acid-induced denaturation of the allergen in the gastrointestinal tract. One day after the last feeding, all dogs were immunized subcutaneously with Phl p 5 (1 μ g per dog) and OVA (10 μ g per dog) in aluminum hydroxide (1.3%) and subsequently at weeks 15, 17, and 19 (Fig 1).

Antibody determinations

Blood samples were collected once before the feeding period (week 9) and weekly after oral tolerance induction. The serum samples were stored at -20°C , and allergen-specific IgE and IgG levels in serum were determined by means of a standard sandwich ELISA. Briefly, microtiter plates were coated with specific allergen at 5 μ g/mL. Allergen-specific serum IgE or IgG levels were detected by using biotinylated monoclonal anti-dog IgE antibody (CMG-Heska) or polyclonal sheep anti-dog IgG antibody (Bethyl Laboratories), respectively, followed by horseradish peroxidase (Sigma Aldrich) and *o*-phenylenediamine dihydrochloride (SIGMA FAST OPD

tablets, Sigma Aldrich). IgE and IgG results from dog sera were compared with results from a standard serum pool and expressed as relative units.

In vivo challenges

Ocular challenge, pulmonary provocation tests, and bronchoalveolar lavage (BAL) tests were carried out, as presented below, after achievement of general anesthesia.

Ocular challenge. The conjunctival provocation test was performed 15 weeks after tolerance induction by adding 3 drops of OVA (1.0%) dissolved in a buffered 0.9% saline solution into one eye and an equal volume of diluent into the other (control) eye. Clinical symptoms (hyperemia, edema, and tearing) were evaluated 20 minutes after challenge. Each symptom was scored from 0 to 4 according to the following scheme: 0, absent; 1, mild; 2, moderate; 3, severe; and 4, very severe.

Pulmonary provocation tests. Changes in lung function were measured at week 43, approximately 7 months after OVA feeding. Severe bronchoconstriction and a decrease in dynamic compliance (Δ volume/ Δ pressure) was used as an indicator for lung tissue elasticity. Aerosols were generated by the use of a nebulizer (Mark 7 Respirator, Bird Products Corp) directly connected to the endotracheal tube of the dogs. Baseline was set after 15 breaths of aerosolized PBS to compare the parameters before and after antigen challenge. Changes in compliance caused by inhalation of 0.05% OVA in PBS were recorded by using the software BioSystem XA (Version 1.5.7 f1, Buxco Electronics Inc).

BAL after OVA challenge. Beagles were aerosol challenged with OVA 24 hours before BAL. Then a flexible fiberoptic bronchoscope (Olympus) was advanced to the right cardiac lobe and wedged into a segmental bronchus, which was lavaged by means of instillation and immediate aspiration of 2 aliquots (maximum of 4 mL/kg body weight with 0.9% NaCl). After total cell counting, cytospin preparations of BAL fluid were stained with May-Grünwald-Giemsa, and differential cell counts were performed on the basis of morphology and staining characteristics. BAL experiments were performed at weeks 28, 43, and 60.

Quantitation of cytokine gene expression in BAL fluid cells after OVA challenge

Cytokine gene expression analysis of the BAL fluid cells was performed 24 hours after OVA challenge. Total RNA was isolated from BAL fluid cell pellets by using Trizol (Gibco BRL, Life Technologies), according to the manufacturers' instructions. The following primers (Gibco BRL) were designed from published sequences by using PrimerExpress Software (Applied Biosystems): human conserved hypoxanthinephosphoribosyltransferase (HPRT; GB:M31642), sense 5'-AGA CTT TGC TTT CCT TGG TCA GG-3' and antisense 5'-TCA AGG GCA TAT CCT ACA ACA AAC-3'; canine IL-10 (GB:U33843), sense 5'-GAC AAG CTG GAC AAC ATA CTG CTG-3' and antisense 5'-GTC CTA GAG TCG AGA AGA GTT GCC-3' and canine TGF- β (GB:L34956), sense 5'-AGT TAA AAG AGG AGC AGC ATG T-3' and antisense 5'-CCA TGA GGA GCA GGA AGG G-3'. TAMRA/FAM-labeled TaqMan probes were from Eurogentech (Seraing): HPRT, 5'-CAA GCT TGC TGG TGA AAA GGA CCC C-3'; IL-10, 5'-TGG AGG AGG TGA TGC CCC GG-3' and TGF- β , 5'-TGC TGG CGC CCA GCG ACA-3'. Quantitative real-time PCR was performed on an ABI7700 thermocycler (Applied Biosystems) by using the Superscript One-Step RT-PCR kit (Gibco BRL) with a standard protocol of 2 minutes at 50°C plus 30 minutes at 60°C for reverse transcription, followed by 5 minutes at 95°C and 40 to 45 cycles of a 2-step PCR with 20 seconds at 94°C and 1 minute at 62°C. All primers and probes were titrated before use with the HPRT primers and probe

and showed RNA concentration-dependent signals. Cytokine signals were expressed as a percentage of HPRT on the basis of threshold cycle differences.

Magnetic-activated cell sorting of BAL fluid cells

BAL fluid cells from OVA-challenged dogs (60 weeks old) were washed with PBS containing 5.0% FCS and 2 mmol/L EDTA and stained with anti-human CD14-PE (clone TÜK4, cross-reactive to canine CD14, DAKO). The CD14⁺ macrophages were separated from the CD14⁻ cells (mainly T cells) by using a MACS cell-separator (Auto MACS, Miltenyi Biotec).

Statistical analysis

Serum IgE and IgG levels were compared by calculating the area under the curve. If the data sets contained zero values, comparisons were made by using the Wilcoxon Mann-Whitney test. Otherwise, area-under-the-curve values were logarithmically transformed to achieve normal distribution and homogeneous variances and compared by using the Student *t* test. All other parameters were evaluated by using the Wilcoxon Mann-Whitney test with exact *P* values (StatXact, Cytel Software Corp).

RESULTS

Oral treatment with low doses of OVA inhibits OVA-specific IgE and IgG responses

The presence of high levels of IgE in serum is a key parameter in the diagnosis of immediate-type allergies in human subjects. Thus effective tolerogenic treatments should diminish the serum levels of specific IgE, even in the presence of subsequent allergen challenges. Beagles sensitized at birth with Phl p 5 were rendered tolerant by means of daily feeding with OVA dissolved in milk and were subsequently challenged by means of 4 consecutive subcutaneous injections of OVA or control Phl p 5 allergens to test this hypothesis, as described in Fig 1.

Nontolerized control dogs had a significant OVA-specific IgE titer after the third injection of OVA, which increased during the following weeks (Fig 2, A). Similarly, OVA-specific IgG levels were also increased in these animals, although the response was slightly delayed compared with IgE levels (Fig 2, B). In contrast to the control group, the dogs that were tolerized by feeding with OVA did not have any significant OVA-specific IgE or IgG responses within the experimental period (Fig 2, A and B). The lack of response to OVA was specific and resulted from the tolerization protocol because both the IgE and IgG control responses of these dogs to Phl p 5 antigen were indistinguishable from those elicited in control animals (data not shown).

Oral treatment with OVA prevents conjunctivitis in OVA-challenged animals

Conjunctivitis is a common manifestation occurring in atopic individuals on encounter with specific allergens, and therefore another important parameter by which the efficacy of our oral tolerance protocol could be assessed. In the conjunctival provocation test dogs fed with OVA early in life showed a significantly weaker reaction than those of the control group. Control animals consistently

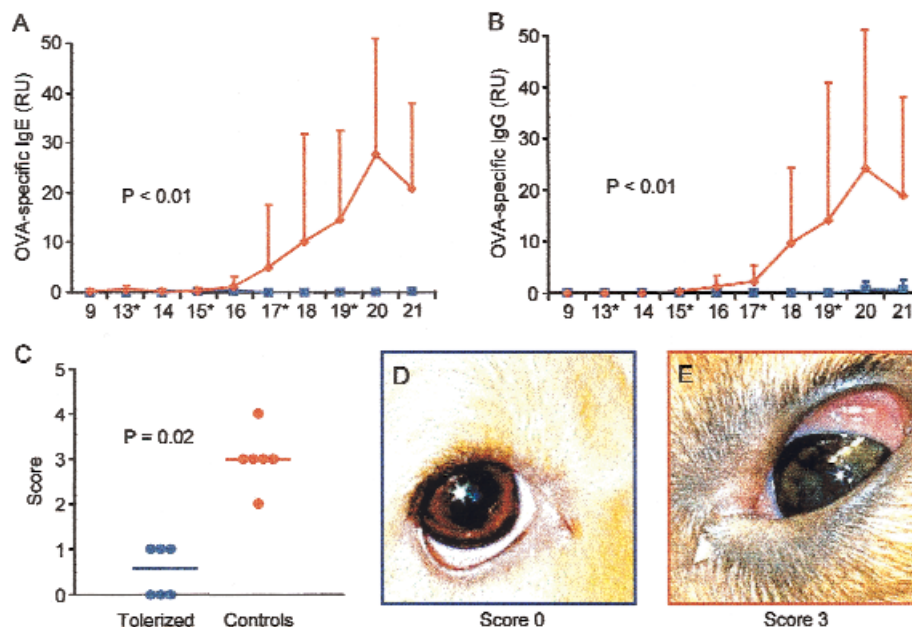


FIG 2. **A** and **B**, OVA-specific serum IgE (Fig 2, **A**) and IgG (Fig 2, **B**) levels. Data show mean $\pm$ SD values of 6 tolerized (blue squares) and 6 nontolerized control (red diamonds) animals. All values are given as relative units with respect to a serum pool. Stars indicate time points of immunization. **C-E**, Response to conjunctival provocation tests. Clinical score from tolerized (n = 6) and nontolerized control (n = 6) dogs (Fig 2, **C**). The horizontal line represents the average value for each group. The P value indicates the significance of the statistical test. A representative picture from a nonaffected eye (Fig 2, **D**) and from an eye with edema and hyperemia (Fig 2, **E**) is shown for comparison.

had hyperemia and edema of the lid, symptoms that were frequently accompanied by tearing; these signs were absent or very mild in the tolerized group (Fig 2, **C-E**).

Oral administration of allergen suppresses asthma-like responses

Because allergic asthma is a clinical manifestation commonly observed in atopic patients, our experimental dogs were also tested for the predisposition to airway hyperresponsiveness after aerosol challenge with OVA (week 43). Changes in lung function were evaluated in terms of dynamic compliance as the deviation of baseline levels measured in the absence of antigen. Whereas tolerized dogs had limited responses that were within 20% of normal deviation, 5 of 6 control dogs had significant constriction of the respiratory tract (negative values in Fig 3, **A**). Furthermore, 3 of these animals displayed a severe bronchoconstriction that resulted in respiratory arrest and required administration of oxygen for recovery (Fig 3, **A**).

Twenty-four hours after lung provocation with nebulized OVA, BAL fluid was also obtained from all animals. There were no significant differences in the volumes recovered or in the total cell counts obtained from control and OVA-tolerized dogs. However, 2 control dogs exhibited pronounced eosinophilia, and 2 additional control animals had a moderate increase in eosinophil counts (Fig 3, **B**). In contrast, there was only one dog from the OVA-tolerized group with eosinophilia. Simi-

larly, neutrophil counts were increased in most of the control dogs, whereas they were remarkably low in the tolerized animals (Fig 3, **C**). Macrophage counts were slightly increased in the tolerized group but only 24 hours after challenge (data not shown). Furthermore, lung reactivity in control and tolerized dogs correlated with the serum levels of OVA-specific IgE (Fig 3, **D**), which were markedly increased in the control group but insignificant in the majority of OVA-tolerized animals (Fig 3, **D**). The outlier points in OVA-specific IgE production and eosinophil counts were observed in different dogs and are due to the normal variations within these experiments.

Oral tolerance is associated with increased expression of TGF- β and IL-10

To provide some insights into the mechanism of action of the tolerogenic treatment, we compared BAL fluid cell mRNA expression of the cytokine IL-2, IL-4, IL-5, IL-10, IFN- γ and TGF- β expression from aerosol-challenged control dogs with that of tolerized animals. IL-10 and TGF- β expression were consistently higher in the samples obtained from tolerized animals than in those obtained from the control group (Fig 4). The expression of IL-2, IL-4, IL-5, and IFN- γ was less than the detection limits in both groups. These results, together with the observation that the macrophage counts were slightly increased in the tolerized animals, suggested that the

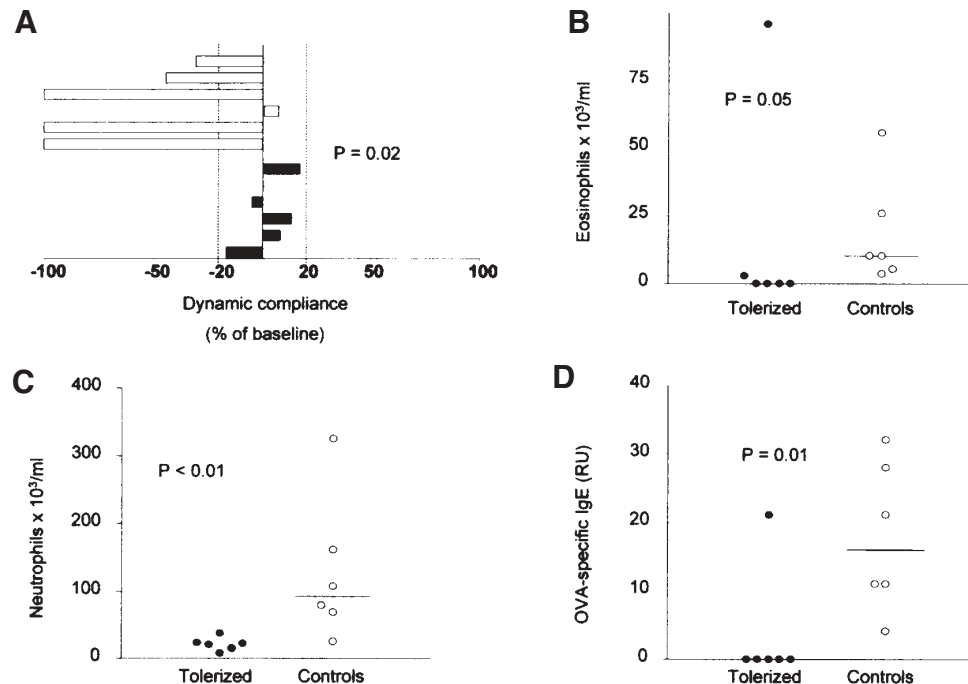


FIG 3. A, Pulmonary provocation test. Black and white bars indicate changes in lung function of individual tolerized and nontolerized control dogs, respectively, after provocation with aerosolized OVA. Each bar represents the mean percentage deviation in dynamic lung compliance compared with baseline values (dotted lines). Broken bars indicate severe bronchoconstriction in combination with respiratory arrest; in this case the assigned compliance value was -100% . **B** and **C,** Total numbers of eosinophils and neutrophils in the BAL fluids of tolerized and control dogs 24 hours after OVA challenge. **D,** OVA-specific serum IgE levels (in relative units) 1 week after OVA respiratory challenge. The horizontal line represents the median value for each group.

source of immunoregulatory cytokines could be the monocyte-macrophage population rather than the few lymphocytes present in the BAL fluid. Indeed, real-time PCR analysis of the CD14⁺ fraction of tolerized dogs showed that these cells are high producers of IL-10 and TGF- β (Fig 4). In contrast, the CD14⁻ population (not shown) did not generate significant IL-10 or TGF- β mRNA expression levels.

DISCUSSION

The primary goal of the present study was to determine whether oral administration of an allergenic substance during infancy is sufficient to induce tolerance to this antigen and prevent the development of allergic reactions to the same molecule in adult life. Our results demonstrate that feeding beagles with OVA allergen during the first weeks of life is sufficient to block hallmark manifestations of asthma and allergy, such as IgE production, lung eosinophil and neutrophil inflammation, bronchoconstriction, and allergic conjunctivitis, after specific allergen challenge. It is worth noting that repeated administration of OVA in adjuvant through a good immunogenic route (subcutaneous route at weeks 13, 15, 17, and 19) after the feeding period with the same antigen did not break the tolerance status achieved during the early weeks of life.

Our results are in partial agreement with those of the report by Russo et al.¹⁷ The authors demonstrated a suppression of asthma-like symptoms after oral OVA administration in mice. However, these authors could not find an association between oral tolerance and increased IL-10 and TGF- β production. On the other hand, Zhang et al¹⁸ have correlated the induction of T-regulatory cells producing IL-10 and TGF- β with oral tolerance to the same antigen. These differences could be related to different experimental designs but clearly indicate that there is controversy about the mechanism of induction of oral tolerance. Our experiments were performed with a dog model, which, despite the complexity associated with the large size of the animals, has several advantages over small rodent models. Large-animal models offer the possibility to investigate several different clinical parameters in the same individual. In addition, the clinical features of allergy observed in allergy-prone sensitized dogs are very similar to those manifested by atopic patients. Furthermore, the anatomy of the gastrointestinal system, the pH and composition of the gastric fluids, and the diet of human subjects are very different from those of rodents. In contrast, the canine and human gastrointestinal systems are very similar, which offers the possibility to study galenic formulations of allergens. On the other hand, the elucidation of the exact mechanism responsible for the tolerogenic effects observed in the dog is ham-

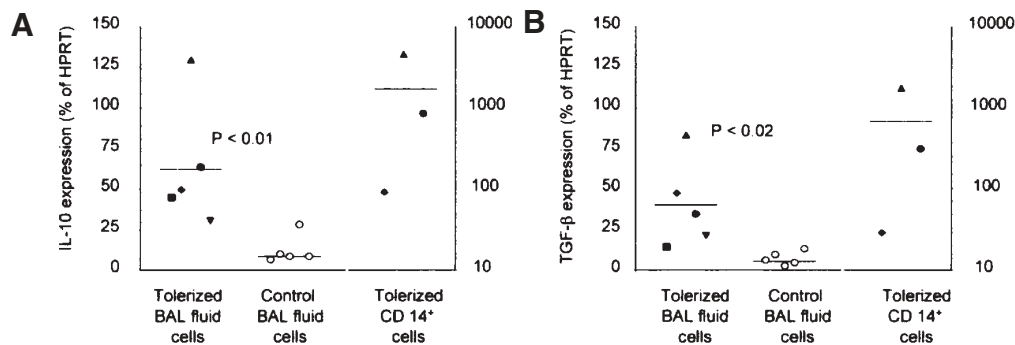


FIG 4. IL-10 (A) and TGF- β (B) expression in BAL fluid cells of tolerized and control dogs after OVA challenge. IL-10 and TGF- β transcripts were determined 24 hours after allergen challenge and expressed as a percentage of HPRT mRNA. The horizontal line represents the average value for each group. The *P* values indicate the significance of the statistical tests comparing mRNA levels of unsorted BAL fluid cell populations. Cytokine mRNA expression on isolated CD14⁺ populations was performed on selected dogs at later time points. Matching symbols from total BAL fluid and CD14⁺ cells indicate that the samples were obtained from the same dog.

pered by the lack of reagents specific for cytokines and cellular receptors of this species. This problem was partially overcome by the use of RT-PCR techniques. Our experimental results clearly implicated IL-10 and TGF- β as important mediators of suppression.

Both IL-10 and TGF- β act by dampening general immune responses and can promote the differentiation of T-regulatory cells capable of suppressing antigen-specific responses in vitro and in vivo.^{19,20} Multiple subsets of CD4⁺ T regulatory cells have been recently described in mice and human subjects according to their cytokine production profiles and surface CD25 and CTLA-4 expression.²¹ At present, it is still unclear whether these different T-regulatory cells represent one or multiple T-cell subsets, and there are conflicting data on whether they exert their regulatory-suppressive functions through direct cell-cell interactions or by the release of soluble cytokine mediators. Nevertheless, both IL-10- and TGF- β -secreting regulatory T cells have been isolated after oral administration of antigens in mice.^{5,18,22} Accordingly, we found that unsorted BAL fluid cells from tolerized dogs had a significantly high expression of IL-10 and TGF- β . However, sorting these cells according to CD14 expression demonstrated that the main producers of these regulatory cytokines were among the CD14⁺ subset, which was predominantly comprised of alveolar macrophages. These findings do not exclude the presence of CD4⁺ T regulatory cells, which could be induced by these cytokines and further contribute to the homeostasis of the challenged tissues; they rather suggest that macrophage-derived IL-10 and TGF- β are crucial players in this process. These data are also supported by a recent study by Akbari et al,²³ demonstrating that IL-10 production is critical for the induction of tolerance in a mouse model. The authors showed that IL-10 produced by mature pulmonary dendritic cells was the responsible mediator of tolerance after intranasal application of OVA. Similarly, Thepen et al²⁴⁻²⁷ have also demon-

ed that depletion of alveolar macrophages leads to a significant increase in the pulmonary immune response and serum IgE levels after administration of antigen in the respiratory tracts.

In summary, this report demonstrates that strong primary oral tolerance to allergens can be induced in dogs, as evidenced by the inhibition of antigen-specific antibody reduction and marked reduction of clinical symptoms for allergic responses. Contrary to the current paradigm of allergen avoidance, our results suggest that early feeding of allergens could be an effective prophylactic approach to prevent the development of allergies later in life.

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