

Oral Tolerance Induction in *Dermatophagoides pteronyssinus*-Sensitized Mice Induces Inhibition of IgE Response and Upregulation of TGF- β Secretion

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ABSTRACT

Oral antigen administration induces peripheral tolerance in naive animals. Studies of oral tolerance induction in sensitized mice have clinical relevance as a strategy to modulate allergy. In this study, the A/Sn mice sensitized with extract of *Dermatophagoides pteronyssinus* (Dp) and submitted to oral Dp administration showed a marked decrease in IgE anti-Dp antibody production compared with sensitized phosphate-buffered saline (PBS)-fed mice. T cells from Dp-fed mice cocultured with spleen cells from PBS-fed mice were able to inhibit IgE anti-Dp antibody production and did not interfere in IgG1 antibody levels. The analysis of cytokine profile after Dp feeding showed a significant decrease in interleukin-4 (IL-4), IL-5, and IL-13 antigen-induced secretion levels by spleen cells, without shifting to IL-2 and interferon- γ (IFN- γ) production. Both transforming growth factor- β (TGF- β) baseline and TGF- β antigen-stimulated levels were increased in Dp-fed mice. The effects of regulatory cytokines on anti-Dp IgE antibody production were investigated *in vitro*. The addition of recombinant TGF- β (rTGF- β) to spleen cell cultures stimulated by Dp inhibited IgE antibody secretion in both mouse groups. Neutralizing antibodies to IL-4, but not anti-TGF- β , induced a marked inhibition of IgE production. Therefore, a negative modulatory effect on IgE response by inhibition of the axis Th2 was observed in sensitized Dp-fed mice, possibly mediated by induction of regulatory cytokines.

INTRODUCTION

ALLERGY IS HIGHLY FREQUENT WORLDWIDE and is associated with both genetic and environmental factors. Allergens evoke Th2 responses in atopic subjects and play an important role in the induction and maintenance of the allergic inflammatory cascade.^(1,2) Research on new approaches for alternative therapies to the modulation of the IgE response has improved allergy treatment. Oral tolerance protocols that induce systemic immunologic hyporesponsiveness⁽³⁾ have been studied in a variety of autoimmune and allergic disease models.^(4–8) Most of the experimental protocols in oral tolerance are carried out with nonimmunized animals because of the difficulty of applying such protocols in primed animals, even though investigations in primed animals would improve the treatment of some diseases.^(9–14)

Clonal anergy or deletion and immunosuppressive cytokines are regulatory mechanisms described in oral tolerance related

to high-dose usage and to multiple dose administration, respectively.^(15–19) In primed animals, however, the regulatory mechanisms can be different, justifying the need to design appropriate protocols to avoid the undesirable stimulation of the onset of immune responses.

Oral tolerance could downregulate Th1-mediated autoimmunities^(5,17,19,20) both by inducing transforming growth factor- β (TGF- β)-secreting cells and making Th1 response tolerant. The allergic phenomenon in which Th2 function is predominant is an interesting system to evaluate regulation of IgE response by oral tolerance.

The present work was designed to evaluate the effects of oral administration of the dust mite (*Dermatophagoides pteronyssinus* [Dp]) to sensitized mice on the IgE antibody response and the *in vitro* effects of T cells from tolerant mice on IgE antibody production. We also investigated the cytokine profile and the effect of *in vitro* cytokine and anticytokine monoclonal antibodies (mAb) on anti-Dp IgE production of cell cultures. The

results showed that Dp administered orally to sensitized mice is capable of negatively modulating IgE antibodies and suggest the involvement of regulatory cytokines, strengthening the modulatory role of TGF- β in oral tolerance.

MATERIALS AND METHODS

Animals

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

Immunization protocol

Mice were injected subcutaneously (s.c.) with 10 μ g Dp (IFIDES-ARISTEGUI, Bilbao, Spain) (112,900 units of biological equivalents [UBE]/ml) in 6 mg Al(OH)₃. Animals were boosted intraperitoneally (i.p.) on the 14th and 21st days after immunization (d.a.i.) with 10 μ g and 50 μ g Dp, respectively, without adjuvant. Mice were bled through the retroorbital plexus on the 28th d.a.i., and sera were stored at -20°C until use.

Induction of oral tolerance

Mice were fed a total dose of 0.25 mg Dp extract in 0.5 ml phosphate-buffered saline (PBS) or PBS alone by gavage under light ether anesthesia. The feeding was performed on the 7th, 8th, and 9th d.a.i. All mice groups were boosted on the 14th and 21st d.a.i.

Passive cutaneous anaphylaxis (PCA)

IgE antibodies were estimated by the PCA reaction as described by Ovary.⁽²¹⁾ Briefly, 0.1 ml of sera dilutions from the mice were inoculated intradermally on the shaved backs of the rats. After 18 h, the rats received a tail vein injection of 0.8 mg Dp extract in 1.0 ml 0.5% Evans blue. One hour later, the rats were killed, and the reciprocal of the highest serum dilution that gave a spot >5 mm in diameter was taken as the PCA titer.

T cell purification

Spleens from Dp-fed mice on the 28th d.a.i. or from normal (nonimmunized, nontreated) mice were collected aseptically and pressed through a stainless steel wire in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT) and 40 mg/L gentamicin. The cell suspension was centrifuged on a Ficoll-Hypaque gradient (μ : 1.095). Cells were washed in RPMI medium, and $5\text{--}8 \times 10^7$ cells were loaded onto a murine anti-Ig column (R&D Systems, Inc., Minneapolis, MN). Enrichment of T cells was $>95\%$ and was shown by staining with FITC anti-CD3 ϵ mAb and phycoerythrin (PE)-labeled B220 mAb (PharMingen, San Diego, CA) by cytometry.

Cell cultures conditions

Suspensions of single splenic cell from PBS-fed mice on the 28th d.a.i. were centrifuged on a Ficoll-Hypaque gradient,

washed, and cocultured (3.5×10^6 cells) with enriched T cells (0.5×10^6) obtained from Dp-fed mice or with T cells from normal mice. Purified T cells in the absence of spleen cells were also cultured. The cells were incubated in 24-well plates (Costar, Cambridge, MA) in 0.5 ml RPMI medium supplemented with 5% FBS in the presence of 19 μ g/ml Dp extract for 12 days at 37°C in a humidified 5% CO₂ incubator. After 7 days of culture, 0.3 ml supplemented medium was added to the wells. Some cultures were performed with 4×10^6 splenic cells per well from Dp-fed or PBS-fed mice in 0.5 ml supplemented culture medium with Dp or Dp plus recombinant human TGF- β (rHuTGF- β) (0.1, 1.0, or 10 ng/ml) (R&D Systems) or with 10 μ g/ml mAb anti-TGF- β 1 (R&D Systems), anti-interleukin-4 (IL-4), or isotypic antibody control (PharMingen) for 12 days. Supernatant was collected and stored at -70°C until use.

Determination of antibody levels

An ELISA was performed to detect anti-Dp IgE and IgG1 antibodies, as well as total IgG1 and IgE levels in supernatant cell cultures. Briefly, to measure anti-Dp IgG1 antibody and total IgG1 and IgE, 96-well microplates (Costar) were coated with 10 μ g/ml Dp extract in 0.1 M carbonate-bicarbonate buffer, pH 9.5. The wells were blocked with PBS-0.5% bovine serum albumin (BSA) (PBS/BSA) before incubation of the samples. Detection was performed with biotinylated anti- γ 1 or anti- ϵ mouse chain antibodies (1:3000) (Southern Biotechnologies), Birmingham, AL) and streptavidin peroxidase (1:1500) (Sigma, St. Louis, MO), followed by tetramethylbenzidine (TMB) (Sigma). The reaction was detected at 450 nm in an ELISA microplate reader (Bio-Rad, Hercules, CA). To detect the anti-Dp IgE antibody, the wells were coated with 100 μ g/ml Dp extract, and ELISA assay was done using an ELAST amplification system (Dupont, Boston, MA).

Total IgE and IgG1 were measured by double mAb ELISA assays, using standard curves derived from purified protein (PharMingen).

Cytokine measurements by ELISA

Single spleen cell suspensions were cultured in 96-well microplates at 8×10^6 per well in 0.2 ml supplemented RPMI medium in the presence of 19 μ g/ml Dp or 5 μ g/ml concanavalin A (ConA) (Sigma) for 24 and 72 h, and cell-free supernatants were stored at -70°C until cytokine assay. IL-2, IL-4, IL-5, interferon- γ (IFN- γ) (minikits, Endogen, Woburn, MA), IL-10 (duo set, Genzyme, Cambridge, MA), IL-13 (R&D Systems), and TGF- β 1 (Promega, Madison, WI) measurements were performed following manufacturers' recommendations. Briefly, 96-well microplates (high binding, Costar) were coated with an anticytokine mAb overnight at 4°C . After the washes, samples were incubated for 2 h at ambient temperature. After new washes, biotinylated mouse anticytokine antibodies were incubated, followed by the addition of streptavidin peroxidase. The reaction was developed after TMB addition and stopped with 1 N sulfuric acid. Reading was performed at 450 nm in an ELISA assay microplate reader. Optical density (OD) units were correlated with the protein concentration using standard curves derived from provided standards. Detection limits levels of IL-2, IL-4, IL-5, IL-10, IL-13, IFN- γ , and TGF- β were

45 pg/ml, 4 pg/ml, 15.6 pg/ml, 60 pg/ml, 78 pg/ml, 0.48 ng/ml, and 15.6 pg/ml, respectively.

Statistical analysis

Differences between groups were considered statistically significant with p values <0.05 , using the Mann-Whitney U-test.

RESULTS

In vivo and in vitro modulatory effect on IgE antibody production in oral tolerance to Dp

The *in vivo* production of specific IgE antibodies in Dp-sensitized mice fed with antigen or PBS was evaluated by the PCA reaction. Oral administration of 0.25 mg Dp to sensitized mice induced a marked decrease in IgE antibody response compared with mice fed PBS (Fig. 1). This dose was chosen in view of our previous observation that oral administration of higher doses in sensitized mice decreased IgE antibodies but enhanced anti-Dp IgG1 subclass production.⁽¹⁴⁾

In parallel to the inhibition of anaphylactic IgE antibody production observed after oral Dp administration, we analyzed the *in vitro* effect of T cells from Dp-fed mice on anti-Dp IgE and IgG1 antibody production by spleen cells from PBS-fed mice. Anti-Dp IgE antibody in the supernatants was analyzed by ELISA using an amplification system. Figure 2 shows that T cells from Dp-fed mice added to the cultures of spleen cells from PBS-fed mice were able to inhibit anti-Dp IgE antibody production. In addition, T cells from normal, nonimmunized animal did not interfere with the IgE antibody production by splenic cells of PBS-fed mice. There was no evidence of IgE

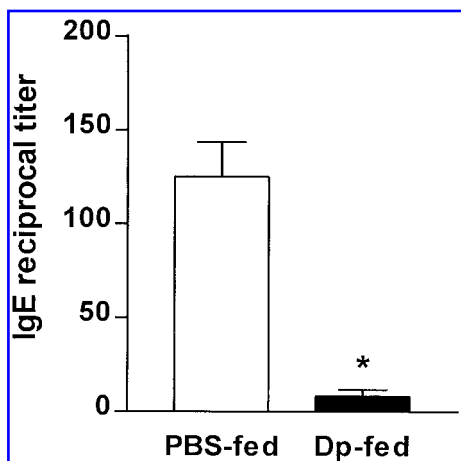


FIG. 1. *In vivo* inhibition of anti-Dp IgE antibody in sensitized mice orally administered Dp. Groups of Dp-sensitized mice were fed on the 7th, 8th, and 9th d.a.i. with a total dose of 0.25 mg Dp or PBS. After two i.p. boosts with Dp on the 14th and 21st d.a.i., the serum of each animal obtained on the 28th d.a.i. was assayed for anti-Dp IgE antibody by PCA. Results of five experiments, each using 4 mice, shown as the mean $\pm$ SEM. * $p < 0.05$ when compared with the control group fed PBS.

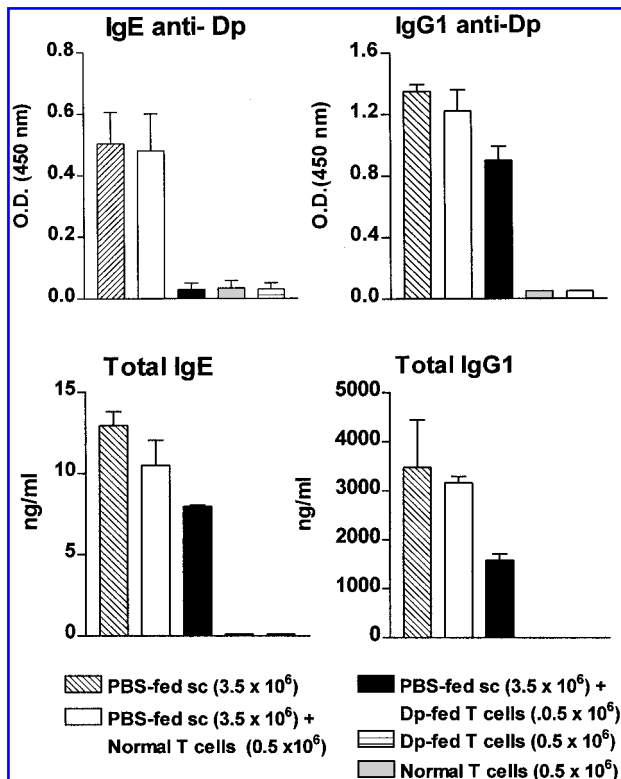


FIG. 2. *In vitro* effect of T cells from Dp-fed mice on the anti-Dp IgE and IgG1 antibodies produced by spleen cells from PBS-fed mice. Splenic cells (sc) (3×10^6) from PBS-fed mice (28th d.a.i.) were cultured for 12 days with Dp (19 μ g/ml) in the presence or absence of 0.5×10^6 T cells from Dp-fed mice (28th d.a.i.) or from normal, nonimmunized mice. Cultures with just T cells from Dp-fed mice or normal mice are shown. Anti-Dp IgE antibody in the supernatants was analyzed by ELISA using an amplification system (ELAST), and anti-Dp IgG1 and total IgE and IgG1 were determined by ELISA. Mean $\pm$ SEM of OD from three independent experiments, each using cells from 2 mice.

and IgG1 antibody production when T cells were cultured alone (Fig. 2).

The inhibitory effect mediated by T cells from Dp-fed mice on anti-Dp IgG1 antibodies and total IgE and IgG1 antibody levels by cells from PBS-fed mice was less intense than those observed with anti-Dp IgE antibodies (Fig. 2).

Cytokine production in sensitized mice fed Dp

T cells from Dp-fed mice were able to negatively modulate the *in vitro* production of anti-Dp IgE antibodies of splenic cells from PBS-fed mice. As the T cells are an important source of cytokines, the profiles of IL-2, IFN- γ , IL-4, IL-5, IL-10, IL-13, and TGF- β secretion were investigated in the supernatants of spleen cell cultures stimulated with either Dp or ConA.

The levels of IL-2 and IFN- γ , Th1-associated cytokines, were similar in both Dp-fed and PBS-fed mice (Fig. 3). However, oral Dp administration significantly decreased Th2 cytokine production, for example, IL-4, IL-5, and IL-13, when induced by antigen stimulation. The level of IL-10 secretion did

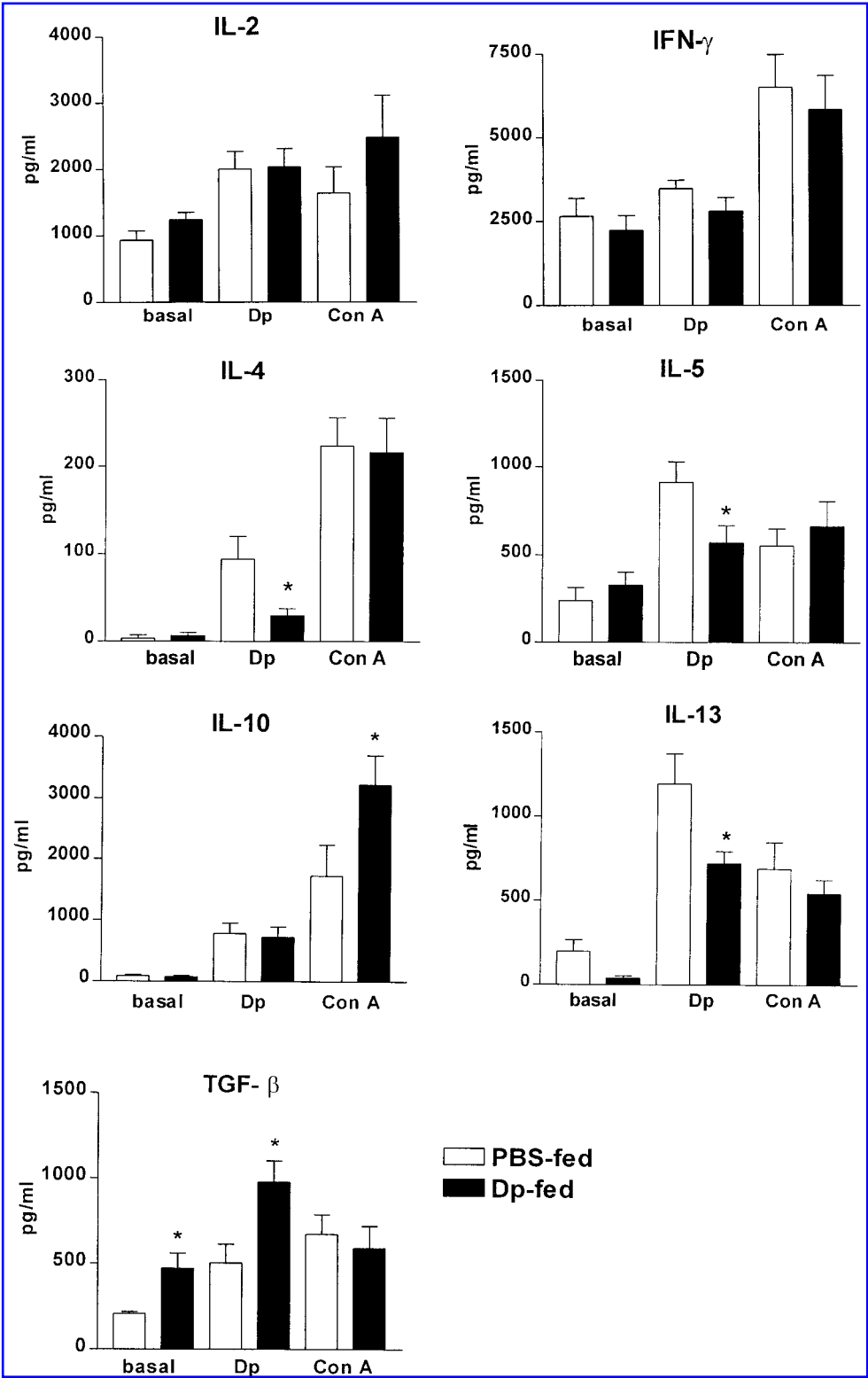


FIG. 3. Cytokine profile of sensitized mice orally administered Dp. Spleen cells from Dp-fed or PBS-fed mice (28th d.a.i.) were cultured with Dp (19 μ g/ml) or ConA (5 μ g/ml). The cell culture supernatants were harvested at 48 h for IL-4 and IL-2 and at 72 h for IFN- γ , IL-5, IL-10, IL-13, and TGF- β dosages by ELISA assay. Results are from five experiments, each using 4 mice, shown as the mean $\pm$ SEM. * $p < 0.05$ when compared with the control group fed PBS.

not change in Dp-fed mice but was significantly increased on ConA stimulus (Fig. 3). In parallel, a high baseline level of TGF- β secretion was detected in the Dp-fed mice, which increased even more with antigen stimulation (Fig. 3).

In vitro cytokine effects on anti-Dp IgE production

A downmodulation of Th2-associated cytokines in sensitized mice fed Dp was observed. Therefore, we next investigated the effect of the addition of regulatory cytokines on anti-Dp IgE antibody production by spleen cells *in vitro*. Splenic cells from Dp-fed or saline-fed mice were cultured for 12 days with Dp extract and cytokine or anticytokine mAb. The presence of IgE antibodies was analyzed in the supernatant cell cultures.

Secretion of anti-Dp IgE antibodies was markedly inhibited by the addition of anti-IL-4 neutralizing antibodies, making evident the influence of IL-4 over IgE secretion (Fig. 4). The addition of 0.1 rHuTGF- β did not interfere in IgE antibody production, and 1 ng/ml induced a slight increase in IgE by spleen cells of Dp-fed mice. However, at a concentration of 10 ng/ml, rHuTGF- β induced considerable inhibition of IgE antibody secretion by splenic cells of mice from both analyzed groups. Neutralizing anti-TGF- β antibodies as well as isotypic mAb did not modify IgE antibody secretion in both cell cultures.

DISCUSSION

Oral administration of extract of the dust mite, *Dermatophagoides pteronyssinus* (Dp), in previously sensitized mice caused negative modulation of the anti-Dp IgE antibody response. Furthermore, T cells from Dp-fed mice were able to inhibit the *in vitro* secretion of anti-Dp IgE antibodies by splenic cells from PBS-fed mice. The cytokine profile of Dp-fed mice demonstrated inhibition of Th2 cytokine secretion associated with stimulation of TGF- β production. In addition, the results

of the *in vitro* investigation with regulatory cytokines, such as TGF- β , emphasized their inhibitory role on anti-Dp IgE antibody production.

Whereas naive animals submitted to continuous antigen exposure protocols or to uniquely high antigen doses showed suppression of cellular and humoral responses,⁽³⁾ feeding after immunization could enhance humoral responsiveness.^(9,10) Our previous results demonstrate that a high dose of Dp orally administered to sensitized mice suppresses the IgE response and stimulates IgG1 antigen-specific memory cells.⁽¹⁴⁾ As IgG1 can be anaphylactic in mice and can contribute to allergic reactions,⁽²²⁾ these observations reinforce the need to carefully select an appropriate dose and protocol for administering to primed animals. In the present work, sensitized mice were administered an antigen dose that does not stimulate other antibody isotypes.

The animals in the oral administration protocol showed a significant decrease in anaphylactic IgE antibody production, evaluated by the PCA reaction. Thus, T cells from tolerant mice were analyzed for their ability to modulate *in vitro* IgE antibody production. T cells from Dp-fed mice inhibited anti-Dp IgE production from spleen cells of immunized PBS-fed animals. Furthermore, T cells from normal, nonimmunized animals did not alter the production of specific antibodies, underlying the *in vitro* modulatory capacity of T cells from Dp-tolerant animals over IgE antibody production. It is possible that *in vivo* inhibition of the IgE response from Dp-fed mice is mediated by regulatory T cells.

Oral Dp administration in sensitized mice using Dp in alum induced a marked inhibition of antigen-specific IL-4, IL-5, and IL-13 secretion, which are important cytokines in IgE isotype switching.^(23,24) These observations and the decreased *ex vivo* number of IL-4-secreting and IgE-secreting cells⁽¹⁴⁾ strongly suggest the downmodulation role of Th2 axis in Dp-fed mice. The decrease in IL-4 and IL-13 secretion is relevant because the two cytokines share common signaling pathways related to

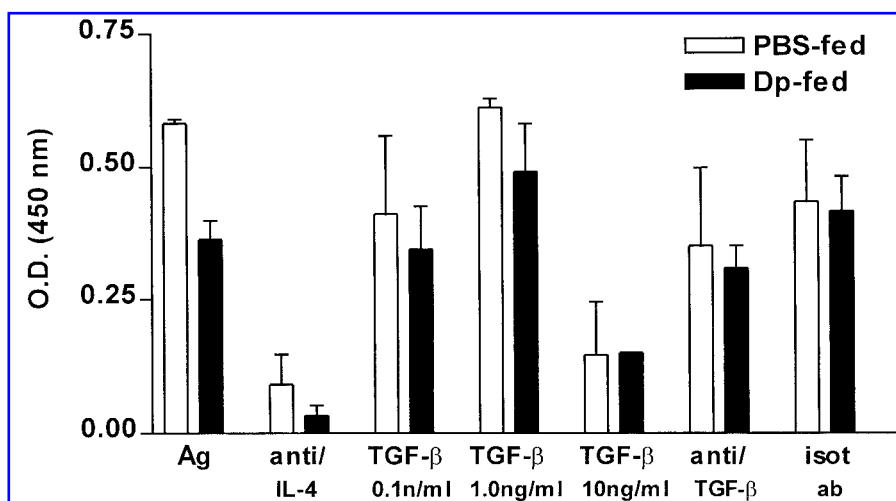


FIG. 4. *In vitro* rHuTGF- β effect on anti-Dp IgE antibody. Spleen cells (4×10^6 cells) from Dp-fed or PBS-fed mice (28th d.a.i.) were cultured in the presence of Dp (19 μ g/ml) and rHuTGF- β 1 (0.1, 1, or 10 ng/ml) or 10 μ g/ml mAb anti-IL-4 or anti-TGF- β 1 or isotypic antibody control. The supernatants were harvested at 12 days of culture and analyzed for anti-Dp IgE antibody by ELISA-ELAST. Mean $\pm$ SEM of OD from three independent experiments, each using cells from 3 mice.

IgE synthesis and inflammation.^(25–27) Thus, the compensatory mechanism to IgE synthesis is impaired in the absence of one of them. In addition, Dp-fed mice demonstrated a significant increase in IL-10 mitogen-induced secretion. IL-10 enhances *in vitro* priming of CD4 T cells for TGF- β secretion.⁽²⁸⁾ IL-10 and IL-4 and, especially, TGF- β have important regulatory roles in oral tolerance.^(5,19) In this sense, we observed an increase in baseline and antigen-specific levels of TGF- β production in Dp-fed mice. The addition of rHuTGF- β to spleen cell cultures of either Dp-fed or PBS-fed mice inhibited the production of IgE antibodies induced by Dp. TGF- β is a key regulatory cytokine in the maintenance of immunologic homeostasis because of suppressive effects on inflammation and on the immune response. One function ascribed to TGF- β is the inhibition of IgE isotype switching, and it seems that the inhibition of IgE-mediated hypersensitivity is due to its effects on mast cells.^(29,30) In addition, TGF- β induced by oral tolerance ameliorates experimental tracheal eosinophilia.⁽³¹⁾ The regulatory action of TGF- β on IgE production can be seen in TGF- β 1^{-/-} mice, which have an uncontrolled B cell response and elevated IgE antibody levels, indicating preferential Th2 cytokine environment.⁽³²⁾

Recently, our group observed that mice immunized with Dp in complete Freund's adjuvant and orally administered 1 mg antigen presented altered patterns of IFN- γ , IL-5, and TGF- β secretion using the gut-associated lymphoid tissue (GALT) system.⁽³³⁾ Oral Dp administration in mice sensitized with Dp in alum was found to induce a systemic increase in TGF- β secretion and inhibition of Th2 cytokine production. We also observed an inhibitory effect of rHuTGF- β on IgE antibody production in Dp-stimulated splenic cell cultures. Besides the differences in the immunization protocols, these results support that oral Dp administration in previously sensitized mice leads to an increase in TGF- β secretion in GALT and spleen cells. Taken together, these results suggest that one of the mechanisms involved in the control of Th2 cytokines and IgE response in Dp-fed mice is caused by TGF- β , as no shift to IL-2 and IFN- γ was observed. Furthermore, a significant decrease in anti-Dp IgG2a antibody levels was detected in Dp-fed mice,⁽¹⁴⁾ suggesting there was no influence of Th1 cytokines in oral Dp tolerance.

The involvement of multiple mechanisms described in oral tolerance studies^(13,15–19) cannot be ruled out in Dp-fed mice. Additional investigations are necessary to clarify the role of TGF- β in the variety of feeding protocols and the large bulk of evidence about regulatory cytokines in systemic tolerance.

The results demonstrate that Dp extract orally administered to primed animals leads to *in vivo* and *in vitro* downmodulation of anti-Dp IgE antibodies, inhibits the production of Th2 cytokines, and stimulates secretion of regulatory cytokines, such as TGF- β . It seems that a complex regulatory cytokine can be operating in Dp-fed mice.

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