

# Modulation of IgE Response and Cytokine Production in Peyer's Patches and Draining Lymph Nodes in Sensitized Mice Made Tolerant by Oral Dust Mite Administration

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## ABSTRACT

Such allergic diseases as rhinitis and asthma are IgE-mediated type I reactions and are controlled primarily by Th2 cells. One of the major dust mites, *Dermatophagoides pteronyssinus* (Dp), is considered to cause allergic reactions. Oral tolerance, largely used to modulate immune response, opens the possibility of modulating Th2 allergic responses. We observed downmodulation of total and specific IgE antibody levels as well as the number of specific IgE-secreting cells with Dp feeding in previously sensitized mice. Analysis of the cytokine profile in mucosal lymphoid tissues in the protocol revealed altered patterns of interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-5 (IL-5), and transforming growth factor- $\beta$  (TGF- $\beta$ ) secretion in Dp-fed animals. The results suggest that both the Th and B cell populations are modulated in mice made tolerant by oral Dp feeding. Understanding the mechanisms at the mucosal level that underlie oral tolerance can improve its use in allergy immunotherapy.

## INTRODUCTION

TYPE I HYPERSENSITIVITY MEDIATED BY IgE is prevalent in the world population. One of the major indoor allergens is the dust mite *Dermatophagoides pteronyssinus* (Dp), which can cause such diseases as rhinitis and asthma. Subcutaneous (s.c.) immunotherapy using Dp is prolonged and not innocuous, thus supporting the search for other routes. Mucosal antigen administration can induce humoral modulation and decreased lymphoid proliferation after parenteral antigen reexposure.<sup>(1)</sup> This procedure, known as oral tolerance, has been studied in experimental murine models of autoimmunity and allergy.<sup>(2,3)</sup> The vast majority of experimental protocols of oral tolerance use naive animals, leading to results that possibly can differ from those in primed animals.

Different mechanisms have been proposed to mediate oral tolerance, such as active suppression when low antigen doses are administered and anergy and clonal deletion by apoptosis when high doses are used.<sup>(4–6)</sup> These mechanisms are not mutually exclusive and can be cooperative. In addition, other regulatory mechanisms, such as the production of autoantibodies against IgE, have been suggested in mice sensitized with Dp and submitted to an oral tolerance protocol.<sup>(7)</sup>

The mucosal immune tissues are the principal sites at which the orally administered antigen first encounters the immune system and initiates an immune response, leading to oral tolerance.

In the mucosal lymphoid tissue of orally tolerant mice, an initial increase in interleukin-2 (IL-2) production in Peyer's patches has been observed, followed by higher IL-4, IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ ) production.<sup>(8)</sup> This cytokine response could be related to the modulation of other cytokine secretions, expansion of T cell clones, and downmodulation of specific responses. An understanding of events following antigen exposure on mucosal surfaces is of fundamental importance to better understand the regulatory mechanisms of oral tolerance.

We studied the effect of feeding Dp on humoral response in a murine model of immediate hypersensitivity to Dp and studied cytokine production in mucosal tissues, such as Peyer's patches (PP), mesenteric lymph nodes (MLN), and draining lymph nodes (DLN). We observed a decreased number of specific IgE-secreting cells and downregulation of the anti-Dp IgE serum level, with concomitant stimulation of regulatory cytokines. Modulation of a predominant allergic Th2 response by oral tolerance could be a potential strategy of immunotherapy using oral administration of allergens in clinical situations.

## MATERIALS AND METHODS

### Animals

Inbred female A/Sn mice (8–10 weeks old) were provided by the animal facility of São Paulo University School of Med-

icine. Wistar Furth rats (RT<sup>u,u</sup>) of both sexes, 3–4 months old and bred in our own laboratory's animal facility, were employed for passive cutaneous anaphylactic reactions.

### *Immunization and oral administration of Dp*

Mice were sensitized s.c. at the tail base and intramuscularly (i.m.) in the two hind footpads with a total of 10  $\mu$ g Dp (112,900 units of biological equivalents [UBE]/ml) (Ifidesa-Aristegui, Bilbao, Spain) emulsified v/v with complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO). They received two intraperitoneal (i.p.) boosters on days 21 (10  $\mu$ g) and 28 (50  $\mu$ g) after priming. Under light ether anesthesia, mice were fed by the intragastric (i.g.) route using a urethral tube long enough to reach the stomach on days 6, 7, and 8 after priming with a total of 1.0 mg Dp dissolved in 0.5 ml saline. The control group was fed with saline solution. Each mouse was bled on days 28 and 35 after priming from the retroorbital plexus, and individually collected sera were frozen at  $-20^{\circ}\text{C}$  until use.

### *Passive cutaneous anaphylaxis (PCA)*

IgE antibodies were estimated by passive cutaneous anaphylaxis (PCA) reaction as described by Ovary<sup>(9)</sup> and modified by Mota and Wong.<sup>(10)</sup> Briefly, 0.1 ml of mouse serum dilutions was inoculated intradermally (i.d.) into the shaved backs of rats. After 18 h, the rats received 50  $\mu$ g Dp in 1.0 ml 0.5% Evans blue intravenously (i.v.). The rats were killed 1 h later, and the reciprocal of the highest serum dilution that showed a reaction  $>5$  mm was taken as the PCA titer.

### *Cell collection*

Splenic mesenteric, and draining lymph nodes (periaortic and inguinal) as well as Peyer's patches were removed aseptically from mice on day 12, 28, or 35 after priming, put into RPMI 1640 medium (GIBCO, Grand Island, NY), and pressed through a stainless steel wire mesh to produce a single cell suspension. Mononuclear spleen cells were obtained after Ficoll-Hypaque gradient ( $\mu$ :1096) centrifugation. The cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT), 40 mg/L gentamicin, and 200 U/L Penicillin. The final concentration was determined according to the assay.

### *Measurement of total IgE and anti-Dp subclasses of IgG antibodies*

To measure specific IgG antibodies by ELISA, 96-well microplates (Costar, Cambridge, MA) were coated with 5  $\mu$ g/ml Dp extract in 0.1 M carbonate-bicarbonate buffer, pH 9.5. The wells were blocked with phosphate-buffered saline (PBS)/0.5% gelatin before incubation of the serum sample dilutions. Detection was performed with biotinylated anti- $\gamma$ 1, anti- $\gamma$ 2a, anti- $\gamma$ 2b, and anti- $\gamma$ 3 mouse chain antibodies (1:3000) (Southern Biotechnologies, Birmingham, AL) and streptavidin  $\beta$ -galactosidase (1:1500) (Sigma), followed by 0.8 mg/ml ortho-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) (Sigma) in 0.1 M phosphate buffer, pH 7.0, containing 0.0056% (v/v) 2- $\beta$ -mercaptoethanol. The reaction was detected at 415 nm in an ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA). Total IgE measurement was performed by double monoclonal antibody (mAb)

ELISA, using 3  $\mu$ g/ml antimouse IgE (PharMingen, San Diego, CA) to coat the wells. The individual sera were serially diluted and incubated as described. After six washes, a biotinylated anti-IgE antibody (1:1000) (PharMingen) was added, and plates were incubated for 1 h at  $37^{\circ}\text{C}$ , followed by incubation with streptavidin  $\beta$ -galactosidase and ONPG as described. The OD units were correlated to the protein concentration with standard curves derived from purified IgE (PharMingen).

### *ELISPOT assay for estimation of IgE-secreting cells*

An improved ELISPOT method to estimate the number of total and anti-Dp IgE antibody-secreting cells was developed as previously described.<sup>(11)</sup> Briefly, circular nitrocellulose membranes (45  $\mu$ m pore size) (Schleicher & Schuell, Keene, NH) previously placed in 24-well culture plates (Costar) were coated with 10  $\mu$ g/ml anti-IgE antibodies (PharMingen) for detection of total IgE-secreting cells or with 25  $\mu$ g/ml Dp for anti-Dp IgE-secreting cells and incubated overnight. After washing with PBS, the membranes were saturated with PBS/1% bovine serum albumin (BSA)/1% FBS for 2 h. Cell suspensions ( $0.25 \times 10^6$  cells for total IgE-secreting cells and  $4 \times 10^6$  for anti-Dp-secreting cells) in RPMI 1640 medium supplemented with 10% FBS were added in volumes of 400  $\mu$ l per well. In some experiments, 10  $\mu$ g/ml cycloheximide (Sigma) was added during incubation. The plates were incubated 18 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . After four washes with PBS/0.01% Tween-20, biotinylated anti-IgE antibody (1:500) (PharMingen) was added, and plates were incubated overnight at  $4^{\circ}\text{C}$ . After a similar washing procedure alkaline streptavidin phosphatase (1:1000) (PharMingen) was added, and the plates were incubated for 2-h at ambient temperature. The reaction was developed with addition of 400  $\mu$ l per well 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP-NBT) (Sigma), after incubation for 20 min at ambient temperature. Washes with distilled water were used to stop the reaction. The spot readings were done with an inverted microscope, and estimates are expressed per  $10^6$  cells for total IgE-secreting cells or per  $10^7$  for anti-Dp IgE-secreting cells.

### *ELISPOT assay for estimation of cytokine-secreting cells*

Cytokine-secreting cells were analyzed by ELISPOT assay as described by Sacks and Klinman.<sup>(12)</sup> Briefly, each flat-bottomed 96-well microplate was coated with 8  $\mu$ g/ml anti-interferon- $\gamma$  (IFN- $\gamma$ ) (clone RA-6A2) or 4  $\mu$ g/ml anti-IL-4 (clone 11B11), anti-IL-2 (clone JES6-1A12), or anti-IL-5 (clone TRFK5) monoclonal antimouse antibody (PharMingen) and incubated overnight at  $4^{\circ}\text{C}$ . The plates were blocked with PBS/4% BSA for IFN- $\gamma$  assays and with 2% BSA for IL-2, IL-4, and IL-5 assays and incubated for 2 h at ambient temperature. After adding  $1.0 \times 10^6$  cells per well in RPMI 1640 medium supplemented with 10% FBS, the plates were incubated overnight at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator. Three washes were done by adding PBS/0.01% Tween to the wells for 10 min. Subsequently, 1.25  $\mu$ g/ml biotinylated anti-IFN- $\gamma$  (clone XMG1.2) or 2  $\mu$ g/ml biotinylated anti-IL-2 (clone JES6-5H4), anti-IL-4 (clone BVD6-024G2), or anti-IL-5 (clone TRFK4) mAb (PharMingen) was added, and the plates were incubated overnight at  $4^{\circ}\text{C}$ . After similar washings, alkaline streptavidin

phosphatase (1:1000) was added, and plates were incubated for 2 h at ambient temperature. Finally, 0.1% BCIP in 2-amino-2-methyl-1-propanol, pH 10.25, buffer solution (Sigma) was added for 2 h at 37°C. The colorimetric reaction was halted with distilled water. Spots were enumerated under 40× magnification, and results are expressed per 10<sup>6</sup> cells.

### ELISA for TGF- $\beta$ measurement in supernatants

Single cell suspensions from PP, or MLN, or DLN ( $10 \times 10^6$  cells/ml) were cultured in the presence of Dp ( $50 \mu\text{g/ml}$ ), concavalin A (ConA) ( $5 \mu\text{g/ml}$ ), or medium alone for 72 h for TGF- $\beta$  detection, whereupon cell-free supernatants were collected and assayed using the cytokine-specific ELISA assay. TGF- $\beta$  (Promega, Madison, WI) ELISA assays were performed following the manufacturer's recommendations. Briefly, 96-well microplates (Costar) were coated with an appropriate concentration of anticytokine mAb overnight at  $4^\circ\text{C}$ . After decanting, the microplates were blocked with TGF- $\beta$  block solution for 35 min at  $37^\circ\text{C}$ . The block solution was decanted, and the samples were incubated for 2 h with constant shaking at ambient temperature. The plates were washed, the specific secondary anti-TGF- $\beta$  antibody was added, and the plates were incubated for 2 h with constant shaking at ambient temperature. After additional washings,  $50 \mu\text{l}$  anti-TGF- $\beta$  antibody conjugated with peroxidase (1:1000) (Sigma) was added, followed by incubation for 2 h with constant shaking at ambient temperature. The reaction was started by adding tetramethylbenzidine (TMB) (Promega) and stopped with  $25 \mu\text{l}$  phosphoric acid (1.0 M). The reading was performed at 450 nm in an ELISA assay microplate reader (Bio-Rad). The OD units were correlated to the protein concentration using standard curves. The detection limit level of TGF- $\beta$  was  $15.6 \text{ pg/ml}$ .

## RESULTS

### *Humoral response after oral Dp administration in sensitized mice*

To investigate the effect of oral administration in sensitized mice, we selected a murine strain able to produce high amounts of anti-Dp IgE antibodies in response to Dp immunization.

Control A/Sn mice were sensitized with Dp emulsified in CFA and received two boosters on days 21 and 28 after priming. Anti-Dp IgE antibodies were detectable only on day 35 (Fig. 1). Mice sensitized using the same procedure were administered oral Dp on days 6, 7, and 8 after priming (a total of 1 mg). The mice fed Dp showed a significant decrease in production of anti-Dp IgE antibodies (Fig. 1) and total IgE antibodies when compared with control saline-fed mice on day 35 after priming (Fig. 2). Total IgE antibody levels were not measurable on day 21 after priming but were first detected on day 28, when both groups showed similar levels (Fig. 2).

When the IgG response was analyzed on the 28th day, the levels of anti-Dp antibodies, belonging to subclasses IgG1, IgG2a, and IgG2b, were significantly decreased in Dp-fed mice when compared with controls (Fig. 3). The level of specific IgG3 antibodies, which was lower than those of other subclasses by immunization, did not alter with the oral Dp administration

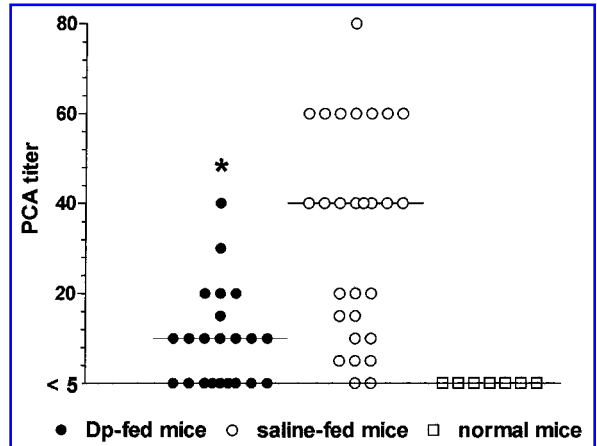


**FIG. 1.** Anti-Dp IgE antibody levels of previously sensitized Dp-fed mice. A/Sn mice were immunized with Dp/CFA and fed with Dp or saline on the 6th, 7th, and 8th days after priming (dap). After two boosters, the animals were bled on the 35th dap, and the individual sera were assayed for specific IgE antibodies by PCA. \* $p < 0.001$  when compared with control saline-fed mice. Data are means of 4–7 mice in each group of four independent experiments.

schedule. On day 35 after priming, Dp-fed mice and controls showed similar levels of all IgG subclasses (Fig. 3).

To evaluate the effect of oral Dp administration on antibody-secreting cells, the number of total and anti-Dp IgE-secreting cells from spleen were estimated by the ELISPOT assay (Table 1). This assay had to be adapted to detect IgE-secreting cells,<sup>(11)</sup> as this isotype-secreting cell is usually found at a lower frequency than other isotype-producing cells. The number of anti-Dp IgE-secreting cells of Dp-fed mice was decreased in the spleen, in agreement with the low IgE serum levels (Table 1).

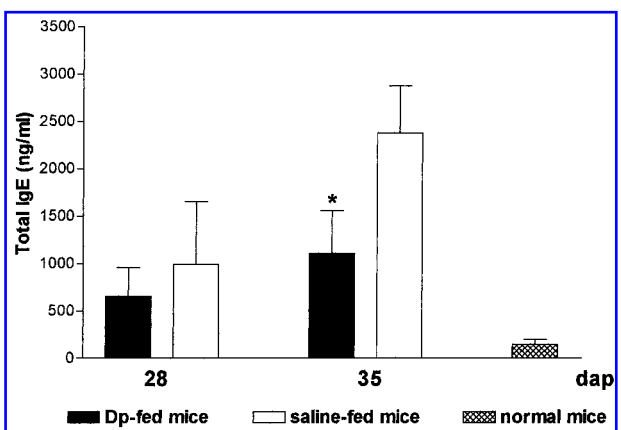


**FIG. 2.** Total IgE antibody of previously sensitized Dp-fed mice. A/Sn mice were immunized with Dp/CFA and fed with Dp or saline on the 6th, 7th, and 8th days after priming (dap). The animals were boosted on the 21st and 28th dap. They were bled on the 28th and 35th dap, and the individual sera were assayed for total IgE antibody concentrations by ELISA. \* $p < 0.05$  when compared with control saline-fed mice. Data are means of 4–7 mice in each group of four independent experiments.

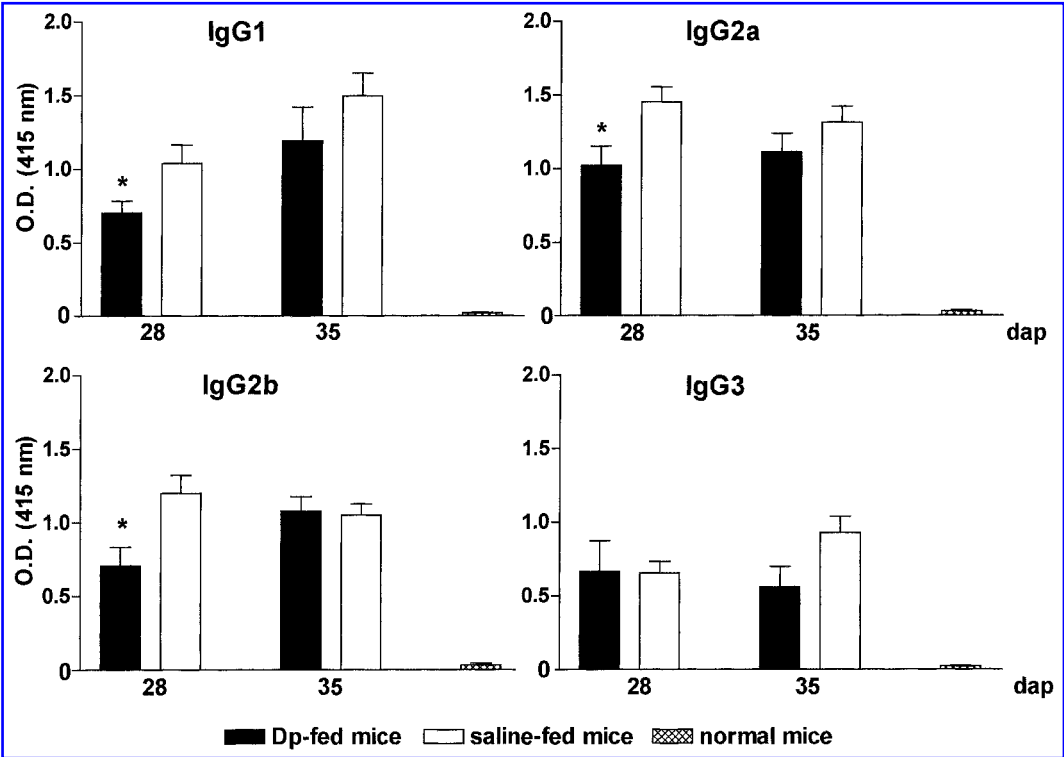


**FIG. 3.** IgG subclasses of anti-Dp antibodies in previously sensitized Dp-fed mice. The sera of Dp-fed and saline-fed mice obtained on the 28th and 35th days after priming (dap) were assayed for IgG1 (1:160,000 and 1:320,000, respectively), IgG2a (1:3000 and 1:3000, respectively), IgG2b (1:6400 and 1:2000, respectively), and IgG3 (1:100 and 1:400, respectively) antibodies by ELISA. \**p* < 0.05 when compared with control saline-fed mice. Data are means of 4–7 mice in each group of four independent experiments.

However, similar numbers of total IgE-secreting cells were observed in the spleen of both groups. To check the *in vitro* synthesis of this isotype, some assays were performed with the addition of cycloheximide, which blocks *de novo* synthesis. These assays showed a marked reduction in the spot number (>95%), indicating that no previously antibody-coated cells were present in the samples (data not shown).

*Effects of oral tolerance on cytokine-secreting cells in sensitized mice*

The inhibition found in the humoral response of sensitized mice led us to analyze the number of cytokine-secreting cells, as cytokines can actively modulate antibody production. Fresh isolated cells from PP, MLN, and DLN were analyzed for IL-2, IFN- $\gamma$ , IL-4, and IL-5 production by the ELISPOT assay on day 12 (4 days after the last gavage) and day 28 after priming (7 days after the first booster).

The DLN showed the highest number of IL-2-secreting cells when compared with the other sites and an increase on day 28 after priming, mainly in Dp-fed mice (Fig. 4). A similar profile of IL-4-secreting cells was detected in all lymphoid compartments analyzed in control as well as Dp-fed mice, showing an increased cell frequency on day 28 (Fig. 4).

Decreased numbers of IL-5-secreting cells were found in MLN of Dp-fed mice compared with saline-fed mice on day 12 after priming. On day 28, both groups showed similar numbers of IL-5-secreting cells in MLN as well as PP and DLN (Fig.

4). A significant decrease in IFN- $\gamma$ -secreting cell numbers was observed in PP soon after gavage in Dp-fed mice compared with the control saline-fed mice (Fig. 4), and a similar number of IFN- $\gamma$ -secreting cells was observed in MLN and DLN in both mouse groups (Fig. 4).

*Cytokine profile in vitro*

In parallel to the *ex vivo* cytokine analysis, TGF- $\beta$  secretion by PP, MLN, and DLN cells induced by Dp and ConA on day 12 after priming was analyzed *in vitro*. The TGF- $\beta$  secretion level by nonstimulated MLN cells was significantly higher in

TABLE 1. NUMBER OF TOTAL IgE-SECRETING CELLS IN Dp-SENSITIZED MICE ADMINISTERED Dp OR SALINE ORALLY

| Group           | Anti-Dp IgE-secreting cells <sup>a</sup><br>(/10 <sup>7</sup> ) | Total IgE-secreting cells <sup>a</sup><br>(/10 <sup>6</sup> ) |
|-----------------|-----------------------------------------------------------------|---------------------------------------------------------------|
| Dp-fed mice     | 25.7 $\pm$ 5.3 <sup>b,*</sup>                                   | 485 $\pm$ 89.6                                                |
| Saline-fed mice | 55.9 $\pm$ 10.4                                                 | 550 $\pm$ 69.6                                                |

<sup>a</sup>The number of IgE-secreting cells was quantified in the spleen on day 35 after priming by ELISPOT assay.

<sup>b</sup>Median  $\pm$  SEM for 3 mice in each group of three independent experiments.

\**p* < 0.05 when compared with saline-fed mice (Mann-Whitney test).

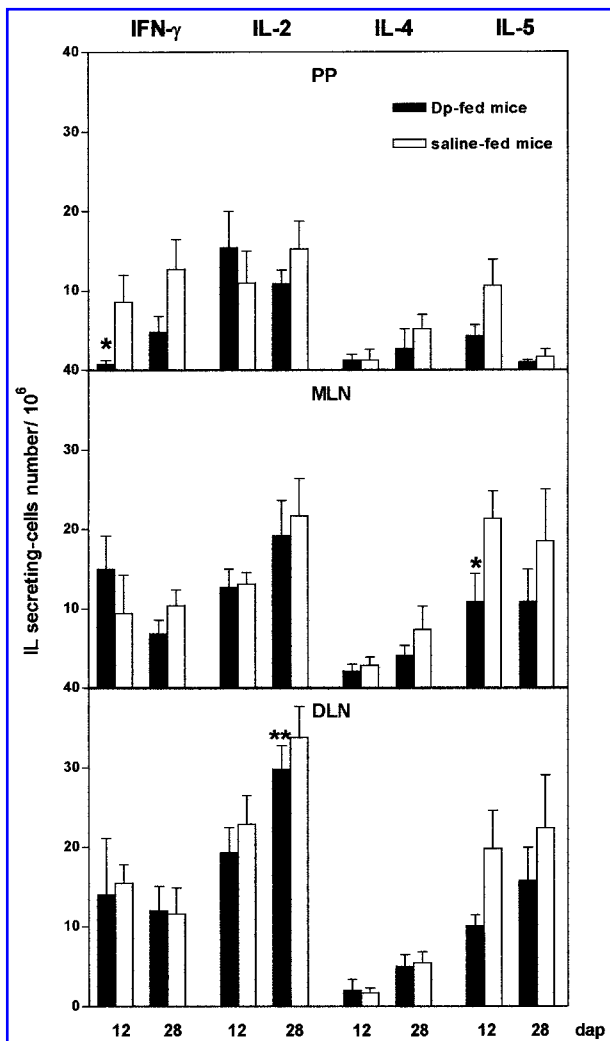


**FIG. 4.** Cytokine-secreting cells in PP, MLN, and DLN of previously sensitized Dp-fed mice. A fresh single cell suspension isolated from PP, MLN, or DLN was tested by ELISPOT assay to enumerate IFN- $\gamma$ , IL-2, IL-4, and IL-5-secreting cells on the 12th and the 28th day after priming (dap). \* $p < 0.05$  when compared with control mice. \*\* $p < 0.05$  when compared with Dp-fed mice on the 12th day. Data are means  $\pm$  SEM for 3 mice in each group of three independent experiments.

cell cultures of Dp-fed mice than those of control mice (Fig. 5). Both antigen-specific and mitogenic stimulation induced similar levels of TGF- $\beta$ .

## DISCUSSION

The effects of Dp feeding on humoral response and on cytokine production in sensitized mice were studied at the mucosal site of antigen entrance, that is, PP, MLN, and DLN.

The oral Dp administration schedule, consisting of three consecutive doses given after sensitization with Dp/CFA, led to a significant decrease in the production of anti-Dp IgE, IgG1, IgG2a, and IgG2b antibody levels. The low number of splenic

anti-Dp IgE-secreting cells found in Dp-fed mice reflected the downmodulation of IgE antibody serum levels. Despite a large number of works focusing on the induction of oral tolerance in unsensitized animals,<sup>(1,4-6)</sup> we considered the possibility of modulating the IgE antibody response of sensitized mice by Dp oral administration, an approach more consistent with the clinical situation. It is known that the time between immunogenic and tolerogenic stimuli is critical, and oral tolerance induction seems to be difficult to establish after immunization.<sup>(13)</sup> The data concerning antigen feeding in experimental protocols of autoimmune diseases in primed animals are conflicting, suggesting either amelioration<sup>(14-16)</sup> or induction/exacerbation of the disease.<sup>(17,18)</sup> Furthermore, the dosage and condition of oral exposure to antigen strongly influence the outcome of the ex-

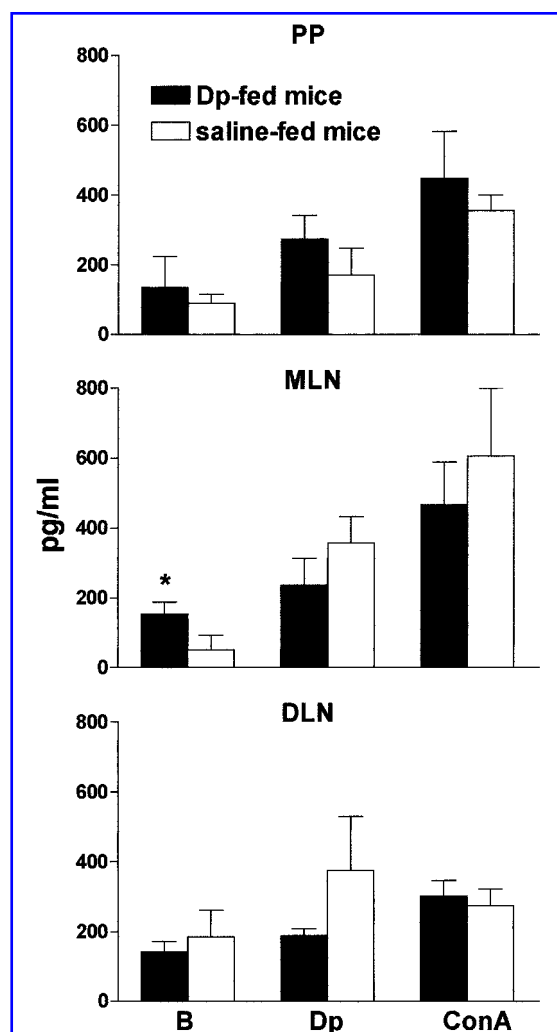


**FIG. 5.** Profile of TGF- $\beta$  secretion *in vitro* by PP, MLN, or DLN cells of previously sensitized Dp-fed mice. Mice were fed with either Dp or saline on the 6th, 7th, and 8th days after priming (dap). The PP, MLN, and DLN cells were harvested on the 12th dap and cultured in the presence of Dp (50  $\mu$ g/ml) or ConA (5  $\mu$ g/ml) or medium alone. Supernatants obtained after 72 h were assayed for TGF- $\beta$  production. \* $p < 0.05$  when compared with controls. B, baseline level. Data are means  $\pm$  SEM of 3 mice in each group of three individual experiments.

periments. Previous results showed that a high oral Dp dosage could enhance IgG antibody production in Dp/alum-sensitized mice, drawing attention to a possible undesirable effect of such protocols in clinical trials.<sup>(7)</sup>

It is well known that the antibody isotype profile is considered a relative indicator of murine Th subset activity.<sup>(19)</sup> In Dp-fed mice analyzed on day 28 after priming, both Th2 and Th1 subsets seem to have been downmodulated, affecting IgG1 and IgG2a antibody production. The IgG3 antibody level did not change, probably as a consequence of the proteic nature of Dp antigen that calls for T cell and B cell cooperation and because this isotype usually is raised by T-independent antigens.<sup>(20)</sup> After the second booster, we observed on day 35 after priming that Dp-administered mice produced IgG subclasses, mainly IgG1, in amounts similar to those found in control mice. This finding suggests an independent regulation of IgE and IgG1 antibody production, as the IgE response in Dp-fed mice was decreased at this time. Differences in the regulation of IgE-specific and IgG1-specific responses to T cell-dependent antigen have been described using an intranasal tolerance-inducing protocol.<sup>(21)</sup> The mechanisms involved in Dp-tolerant mice remain to be defined, but it is possible that the decreased number of specific IgE-secreting cells could be related to active suppression by cytokines or even clonal deletion, such as apoptosis.

An initial burst of cytokines, such as IL-2 and IFN- $\gamma$ , in the DLN seems to precede specific T cell tolerance.<sup>(22)</sup> In our experiments, the Dp-fed animals showed a significant increase in IL-2-secreting cells in the DLN and a decrease of IFN- $\gamma$ -secreting cells in the PP. The reduction in IFN- $\gamma$ -secreting cells in Dp-fed mice seems to agree with data showing that mucosal tolerance can be reversed with minute amounts of IFN- $\gamma$  by the nasal route.<sup>(23)</sup> Furthermore, the neutralization of IFN- $\gamma$  with mAb at the time of feeding has no effect on oral tolerance induction, and tolerance normally can be induced in both IFN- $\gamma$  receptor knockout (IFN- $\gamma$ R<sup>-/-</sup>) mice and IL-12 p40 knockout (IL-12<sup>-/-</sup>) mice.<sup>(24)</sup> These data indicate that the decrease in IFN- $\gamma$  does not seem to be a primary event in oral tolerance induction. Although a lower IgE response could be ascribed to Th2 modulation, these findings do not suggest a shift to the Th1 axis and underline the concept of a regulatory cytokine network operating in the orally Dp-administered animals.

It has been demonstrated that IL-4 is not essential to make tolerant sensitized mice fed with ovalbumin,<sup>(13)</sup> although altered secretion of IL-4 in murine models of oral tolerance has been demonstrated.<sup>(8,11)</sup> We did not observe any difference in the animal groups regarding IL-4-secreting cells in spite of their increased numbers after booster. It is possible that the addition of splenic adherent cells could permit detection of IL-4 secretion in culture supernatants, as was observed for IL-3, IFN- $\gamma$ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion in PP cells.<sup>(25,26)</sup>

IL-5 can provide, together with IL-4, a signal for isotype switching to IgE.<sup>(27)</sup> In this sense, the decrease in IL-5-secreting cells observed in MLN of Dp-fed mice after feeding could be partially ascribed to IgE downmodulation. Moreover, it has been demonstrated that oral tolerance can diminish IL-5 secretion<sup>(13)</sup> and prevent eosinophil recruitment into pulmonary tissue,<sup>(28)</sup> a desirable effect for allergic immunotherapy. In addition, an increase in IgA-secreting cells was observed in the

MLN of Dp-fed mice (data not shown), and considering that IL-5 is an IgA switching factor, that increase might be related to other cytokines, such as TGF- $\beta$ . Four days after antigen administration, MLN cells showed a significantly higher baseline level of TGF- $\beta$  secretion, suggesting that this cytokine could be one of the factors involved in the induction of oral tolerance to Dp in sensitized mice. TGF- $\beta$  has been described largely as participating in oral tolerance models<sup>(2)</sup> and seems to down-regulate the eosinophilic inflammatory infiltrate.<sup>(29)</sup> The immunosuppressive role of TGF- $\beta$  (and IL-4) in mucosal inflammatory responses has been described as counteracting IFN- $\gamma$  proinflammatory properties.<sup>(30,31)</sup> The IFN- $\gamma$  decrease in Dp-fed mice could favor TGF- $\beta$  secretion by gut-associated lymphoid tissue (GALT) cells after oral antigen administration.

In summary, our data demonstrate that sensitized mice can be made tolerant orally, as judged by decreased IgE antibody serum levels and secreting cells, suggesting that T and B cells populations are modulated. This modulation might be in part due to TGF- $\beta$  stimulation, and it was associated with a concomitant decrease in IFN- $\gamma$ -secreting and IL-5-secreting cells in mucosal-associated lymphoid tissues.

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