

Roles of CD4+ and CD8+ T Cells in Adjuvant Activity of Diesel Exhaust Particles in Mice

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Key Words

Adjuvant · Diesel exhaust particles · Allergy · Th1/Th2

Abstract

Through an imbalance in Th1 and Th2 cytokine profiles, diesel exhaust particles (DEP) are thought to induce Th2-dominated IgE and IgG1 production. However, the roles of CD4+ and CD8+ T-cell subtypes in the increased immune responses to antigen in mice exposed to DEP are unclear. In the present study, we investigated whether treatment with anti-CD4 or anti-CD8 mAb abrogated the adjuvant activity of DEP. On day –1 and day 1, each group of mice was injected intraperitoneally with anti-CD4, anti-CD8, or rat IgG (vehicle). On day 0, the mice were immunized with ovalbumin (OVA) or OVA plus DEP. After 3 weeks, each mouse was boosted with 10 µg of OVA alone. On day 7 after the first injection with OVA+DEP or OVA alone, the numbers of total, IA+, CD80+/IA+ and CD86+/IA+ cells in peritoneal exudate cells (PEC) were higher in OVA+DEP-immunized mice than in OVA-immunized mice. Depletion of CD8+ cells resulted in a modulation of the production of granulocyte-macrophage colony-stimulating factor, IL-12 and PGE₂ in peritoneal exudate fluid from OVA+DEP-immunized mice. On day 28, DEP injection markedly increased

IL-4 production in the culture supernatants of spleen cells from CD4+ or CD8+-depleted mice. Depletion of CD8+ cells in OVA+DEP-immunized mice resulted in a decrease in IFN-γ production compared with that in OVA-immunized mice. Adjuvant activity of DEP was observed in anti-OVA IgE, anti-OVA IgG1, anti-OVA IgG3, and total IgE production. Depletion of CD4+ T cells abrogated the adjuvant effect of DEP on anti-OVA IgE, and anti-OVA IgG1 production in plasma. However, depletion of CD8+ T cell inhibited the upregulated anti-OVA IgG3 production. These findings suggest that DEP injection may affect not only the function of CD4+ cells but also that of CD8+ T-cell subsets to modulate the synthesis of proinflammatory cytokine in PEC and type-1 and type-2 cytokine production in spleens.

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Introduction

Some epidemiologic studies have suggested that the increase in the incidence of allergic diseases, such as asthma and allergic rhinitis, is related to exposure to environmental pollutants [1–4]. A positive relationship between the onset of allergic rhinitis and exposure to diesel exhaust particulates (DEP) has been observed [5, 6]. In animal

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experiments, intraperitoneal, intratracheal and intranasal administration of DEP with antigen significantly increased antigen-specific IgE antibody [7–9] and IL-4 production in mediastinal and cervical lymph node cells [9, 10]. Intranasal or intratracheal administration of DEP significantly increased the intranasal pressure and nasal secretion induced by histamine, and the infiltration of eosinophils and lymphocytes [11, 12]. In human studies, DEP exposure in healthy subjects enhanced nasal cytokine production and in ragweed-allergic subjects, challenge with ragweed allergen plus DEP increased expression of mRNA for Th2-type cytokines compared with challenge with ragweed alone [13, 14]. Allergen-specific IgE and IgG4 levels in nasal washes were markedly increased. In vitro experiments have shown that incubation of bronchial epithelial cells and nasal epithelial cells with DEP stimulates IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) production [15, 16]. DEP extract increased eosinophil adhesiveness to human nasal epithelial cells and induced eosinophil degranulation [17]. In vitro treatment with DEP significantly enhanced IgE-mediated IL-4 production by mouse bone marrow-derived mast cells [18]. These results suggest that DEP and DEP extract can cause adverse effects on nasal and respiratory immune defense mechanisms, resulting in induction of allergic diseases.

Since Mosmann et al. [19] reported the existence of CD4⁺ T-cell subsets and their respective functions, not only CD4⁺ T-cells subsets but also CD8⁺ T-cell subsets have been associated with cell-mediated and humoral immunity by cross-regulatory networks [20–22]. Humoral antibody production, particularly IgE production, is regulated by balance of Th1/Th2 cytokine profile. Th2 cytokines can help B cells to produce IgE. Although DEP seem to induce pathways from Th0- to Th2-like response, the mechanism by which DEP facilitate the development of the Th2-like response is unclear. The balance of Th1/Th2 cytokine profile in CD4⁺ T cells may be related to the balance of Tc1/Tc2 cytokine profile in CD8⁺ T cells. Because our previous studies and those of others suggested that DEP have a Th2 cytokine-inducing activity, exposure to DEP may modulate cytokine networks through effects on CD4⁺ and CD8⁺ T-cell subsets. However, studies addressing these effects have not been done previously.

In the present study, we sought to evaluate the role of CD4⁺ and CD8⁺ T-cell subsets in the adjuvant activity of DEP. We depleted mice of CD4⁺ or CD8⁺ T cells by using anti-CD4 and anti-CD8 mAbs, and then investigated the effects of DEP on cytokine production and the

levels of various antibody isotypes. Particularly, the production of pro-inflammatory cytokines in peritoneal exudate fluid and changes in expression of surface molecules in peritoneal exudate cells were investigated in CD4⁺- or CD8⁺-depleted mice on day 7. On day 28, the production of Th1- and Th2-type cytokines from spleen cells and IgE and IgG antibody titers in plasma were determined as systemic events.

Materials and Methods

Animals

Male BALB/c mice (7 weeks old), purchased from Charles River Japan, Co. Ltd. (Kanagawa, Japan), were used in this study. Male Sprague-Dawley rats (8 weeks old), from Japan Clea (Tokyo, Japan), were used for IgE assay. Food and water were given ad libitum. This study was performed with approval of the ethics committee for experimental animals at the National Institute for Environmental Studies.

DEP

DEP were collected on a glass fiber filter at the end of a dilution tunnel connected to a four-cylinder diesel engine (4JB1 type, Isuzu Automobile Company, Tokyo, Japan). As measured by Andersen Air Sampler, the mean diameter of the particles was 0.4 μ m [23]. The carbon content of DEP was 26% of the total DEP concentrations. DEP (40 mg/ml) were dissolved in saline containing 0.1% Tween 80 by sonicating the mixture for 10 min.

Antigen and Antibodies

Ovalbumin (OVA) was obtained from Sigma (St. Louis, Mo.). The anti-CD4 (clone GK1.5) and anti-CD8 (clone 3.155) mAbs were purchased from Leinco Technologies, Inc. (St. Louis, Mo.). Isotype-matched negative antibody control was obtained from Chemicon International Inc. (Temecula, Calif.).

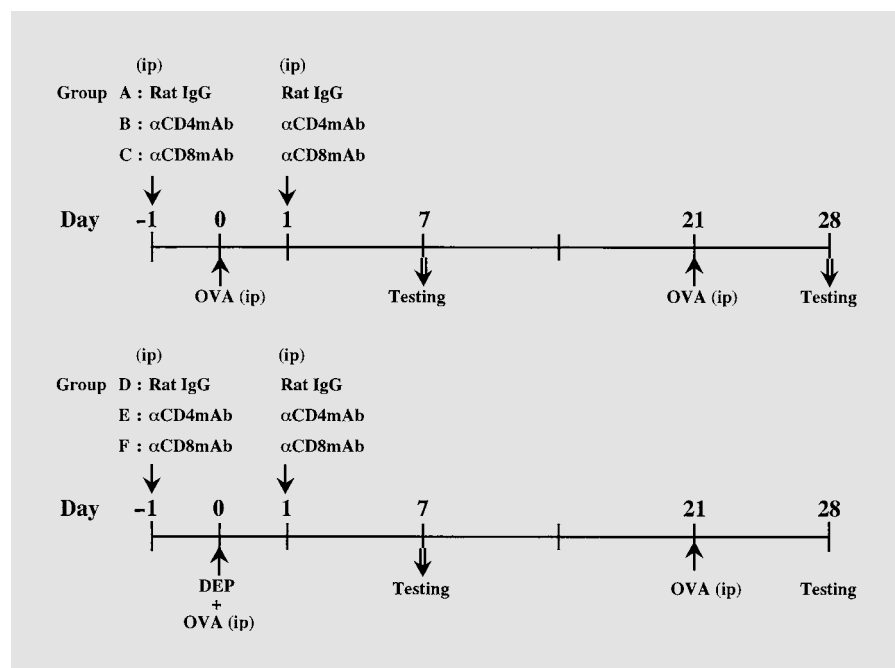
Immunization with OVA plus DEP and Depletion of CD4⁺ and CD8⁺ Cells

On day –1 and day 1, each group of 7–8 mice was injected intraperitoneally with 100 μ g anti-CD4, 100 μ g anti-CD8, or 100 μ g rat IgG (vehicle) [24]. On day 0, the mice were immunized with 10 μ g OVA or 10 μ g OVA plus 2 mg DEP. After 3 weeks, each mouse was boosted with 10 μ g of OVA alone. The experimental protocol is shown in figure 1.

Cell Preparation, Assay for Cell Growth and Cytokine Production

On day 7, peritoneal exudate fluid was collected from mice by injecting 3 ml sterilized saline; peritoneal exudate cells (PEC) were harvested by centrifugation. Spleens were collected. On day 28, spleens were collected, and the cell growth was measured by using a colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Chemicon International). Spleen cells from each mouse at a final density of 2.5×10^5 cells/well were cultured with OVA using 96-well flat-bottomed microplates (Nunc Ltd., Roskilde, Denmark) in 200 μ l RPMI-1640 medium (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine (Gibco RBL, Life Technologies,

Fig. 1. Experimental protocol. The horizontal axis indicates times in days. Arrows above the line show mAb injection by the intraperitoneal route, whereas arrows below the line show antigen and/or alum injection by the intraperitoneal route. On day 7, each group of mice was euthanized, and peritoneal exudate fluid and spleens were collected. On day 28, each group of mice was euthanized, and blood and spleens were collected.



Grand Island, N.Y.), 0.1 mM nonessential amino acids (Gibco RBL) and 5 mM HEPES (NacalaiTesque, Kyoto, Japan), penicillin (100 units/ml) and streptomycin (100 µg/ml; ICN Biomedicals, Inc., Aurora, Ohio), pH 7.2–7.3. After 72 h, the optical density at 570 and 630 nm was measured with microplate reader (Bio-Rad Lab., Hercules, Calif.). Cytospin preparations of cells were stained with Diff-Quik (International Reagents Co., Kobe, Japan) to obtain differential cell counts.

In another experiment, spleen cells from each mouse were plated at a final density of 1×10^6 cell/well in 96-well flat-bottomed microplates and were cultured with 10 µg OVA in 200 µl RPMI-1640 medium supplemented as described previously for 48 h. The supernatants were collected and stored at -70°C .

Cytokine levels (GM-CSF, TNF- α , IL-1 α , IL-10, IL-12p70) in peritoneal exudate fluid and the levels of IL-2, IL-4, IL-10 and IFN- γ in the culture supernatants were analyzed with commercially available mouse cytokine ELISA kit (Endogen, Inc., Woburn, Mass.; Amersham International plc, Little Chalfont, UK). The absorbance at 450 and 550 nm was measured by using a microplate reader. The average absorbance of the standards was plotted against the standard concentration, and the standard curve was drawn through these points. The cytokine concentration in each sample was determined by interpolating from the standard curve. PGE₂ in the samples were measured by using a competitive enzyme immunoassay system (Amersham).

Flow Cytometric Analysis

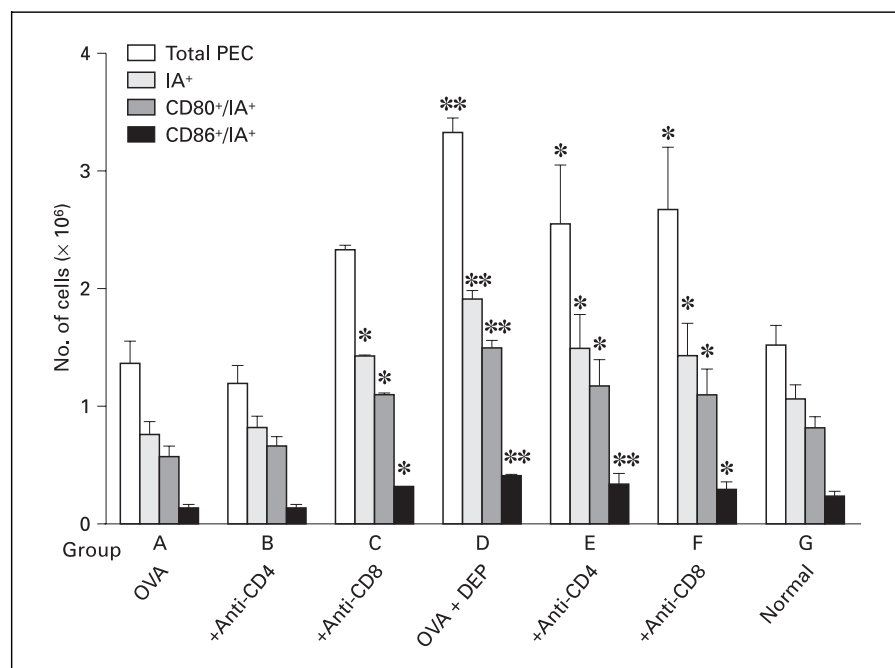
The immunophenotype of the PEC and splenic cells from each group of mice on day 7 and of splenic cells on day 28 was analyzed by using flow cytometry. The mAbs used, purchased from Pharmingen (San Diego, Calif.), were as follows: anti-mouse I-A^d (clone AMS-32.1); phycoerythrin (PE)-labeled rat IgG anti-CD4 (clone GK1.5);

FITC-labeled rat IgG anti-CD8a (clone 53-6.7); hamster IgG anti-CD3 PE (clone 145-2C11); hamster IgG anti-CD80 FITC (clone 16-10A1); rat IgG2a anti-CD86 FITC (clone GL-1); rat IgG anti-CD45R/B220 FITC (clone RA3-6B2), and the corresponding isotype-matched controls. Goat anti-mouse IgG2b PE was purchased from Caltag Laboratories (Burlingame, Calif.). Stained cells were resuspended in buffered salt solution containing 0.1% sodium azide. Cell samples were analyzed on a FACSCalibur flow cytometry (Becton Dickinson Immunocytometry Systems, Mansfield, Mass.). For each sample, 10,000 events were collected and data analysis was performed with Lysis II software.

Assay for Antibody Titers in Plasma

One week after the last immunization, plasma samples were collected from the mice under ether anesthesia. To determine the antigen-specific IgE antibody titers in plasma, the passive cutaneous anaphylaxis (PCA) reaction assay was performed with rat dorsal skin and 24-hour sensitization. Antigen-specific IgE titers in the plasma were expressed as PCA titers. Antigen-specific IgG1, IgG2a, IgG3 and total IgE antibody titers in plasma were measured by using ELISA. Briefly, for titration of total IgE, 96-well microimmunoplates (Nunc) were treated overnight with anti-mouse IgE monoclonal antibody, blocked by Block Ace (Dainippon Pharmaceutical Co., Ltd.) and incubated with 100-µl aliquots of the diluted samples or standards for 1 h at 37°C . After 6 washings, 100 µl biotinylated anti-mouse IgE (Pharmingen) diluted 1/1,000 was added to each well for 30 min at 37°C . After washing, each well was incubated with 100 µl streptavidin-horseradish peroxidase conjugate (Gibco) for 1 h at room temperature. For titration of OVA-specific IgG1, IgG2a or IgG3, microplates were treated overnight with OVA, blocked with Block Ace and incubated with plasma samples diluted 1:100 or standards for 1 h at 37°C . After 6 washings, HRP-labeled anti-mouse IgG1, IgG2a or

Fig. 2. Effects of DEP on the proportion of CD80+ and CD86+ cells in PEC. On day 7, PEC were collected from normal, OVA- or OVA+DEP-injected mice depleted of CD4+ or CD8+ cells. Aliquots of these populations were evaluated by trypan blue dye exclusion test and flow cytometry. Data represent the mean $\pm$ SE (n = 3–4), and are expressed as the total number of PEC, IA+ and cells of CD80+/IA+ or CD86+/IA+ that were recovered per mouse from each treatment group. Group A = OVA+rat IgG-treated group; group B = OVA+anti-CD4-treated group; group C = OVA+anti-CD8-treated group; group D = OVA+DEP+rat IgG-treated group; group E = OVA+DEP+anti-CD4-treated group; group F = OVA+DEP+anti-CD8-treated group, and group G = normal (untreated) group. * p < 0.05 vs. group A; ** p < 0.01 vs. group A.



IgG3 antibodies (Southern Biotechnology, Birmingham, Ala.) were added and plates incubated for 1 h. After an additional wash, the substrate solution containing O-phenylenediamine and H₂O₂ in substrate buffer (7.74 g of citric acid and 17.93 g of Na₂HPO₄ per liter of distilled water) was added and developed. The OD at 490 and 595 nm was determined with a microplate reader (Model 550; Bio-Rad).

Statistical Analysis

All data are presented as the mean $\pm$ SE, which is indicated by bars in the figures. Statistical analysis was performed with StatMate II Statistical analysis system for Microsoft Excel version 5.0 (Nankodo Inc., Tokyo, Japan). All data were compared with parametric methods: Student's t test for comparison of 2 groups, and one-way analysis of variance for multiple comparisons. A p value of < 0.05 was considered significant.

Results

Early Changes in Peritoneal Exudate Cells

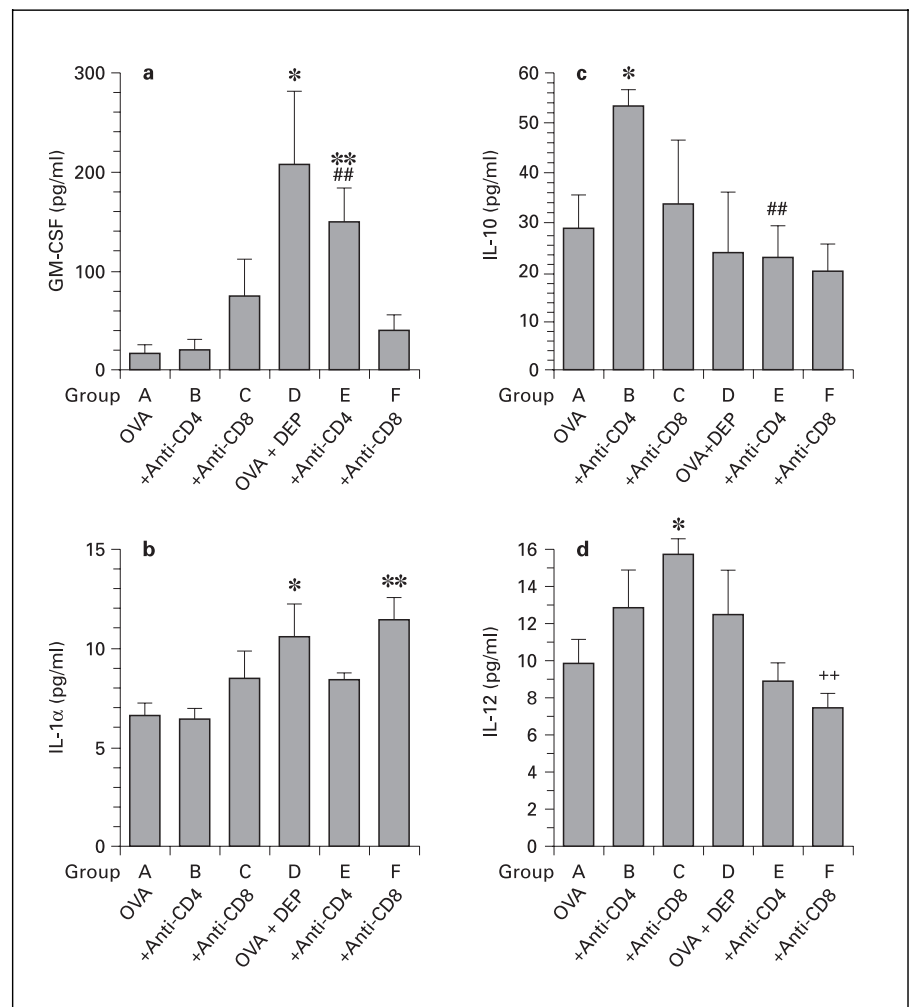
To investigate the changes in early responses of macrophages at the site of immunization, the number of PEC was counted using a hemocytometer. On day 7, the number of exudate cells had increased more significantly in DEP+OVA-immunized mice than in OVA-immunized mice (fig. 2). Compared with cell counts in OVA-immunized mice, OVA+DEP-immunized mice had significantly more macrophages, but the numbers of eosinophils, neutrophils and lymphocytes did not differ significantly (data

not shown). Expression of costimulatory molecules on PEC was evaluated by FACS analysis. As shown in figure 2, the number of IA+, CD80+/IA+ and CD86+/IA+ cells in PEC from OVA+DEP-immunized mice was increased significantly compared with that of OVA-immunized mice (p < 0.01). Treatment with anti-CD4 and anti-CD8 mAbs in PEC from DEP-injected mice retained the increased expression of costimulatory molecules on PEC.

Cytokine Production in Peritoneal Exudate Fluids

To evaluate the effect of DEP on the production of proinflammatory cytokines and the modulation of cytokine production by depletion of CD4+ and CD8+ cells, cytokine levels were assessed on day 7. Compared with levels in OVA-immunized mice, GM-CSF (fig. 3a) and IL-1 α secretion (fig. 3b) in the peritoneal exudate fluid was significantly enhanced in DEP+OVA-immunized mice (p < 0.05). Although treatment with anti-CD4 mAb found the adjuvant activity of DEP on GM-CSF secretion, depletion of CD8+ cells returned to the control level (rat IgG). In contrast, depletion of CD8+ cells in OVA+DEP-immunized mice resulted in increase IL-1 α secretion compared with control population treated with vehicle (rat IgG) in OVA-immunized mice. Treatment with anti-CD4 mAb had no influence on this pattern of IL-1 α secretion.

Fig. 3. GM-CSF (a), IL-1 α (b), IL-10 (c) and IL-12 (d) production in peritoneal exudate fluids. On day 7, peritoneal exudate fluids were collected from OVA- or OVA+DEP-injected mice depleted of CD4+ or CD8+ cells and levels of cytokine production were determined by ELISA. Each value is mean $\pm$ SE (n = 4). * p < 0.05 vs. group A; ** p < 0.01 vs. group A; # p < 0.05 vs. group B; ## p < 0.01 vs. group B; + p < 0.05 vs. group C; ++ p < 0.01 vs. group C.



Comparison of OVA-immunized mice with OVA+DEP-immunized mice showed that both IL-10 (fig. 3c) and IL-12 production (fig. 3d) in peritoneal exudate fluids of DEP-injected mice did not differ from those of controls. Treatment with anti-CD4 mAb significantly decreased IL-10 production in DEP-injected mice ($p < 0.01$), whereas treatment with anti-CD8 mAb significantly reduced IL-12 production in these mice ($p < 0.01$). In OVA-immunized mice, depletion of CD4+ cells and depletion of CD8+ cells markedly enhanced IL-10 and IL-12 production, respectively ($p < 0.05$).

PGE₂ enhances Th2-dominated antibody production via suppression of IFN- γ production [25, 26]. The PGE₂ levels were examined in peritoneal exudate fluids. There was no detectable production of PGE₂ in control fluids of both OVA+DEP- and OVA-immunized mice. Treatment with anti-CD8 mAb induced higher PGE₂ production in

peritoneal exudate fluid from OVA+DEP-immunized mice than that in OVA-immunized mice (fig. 4).

Effect of DEP on Cell Population in Spleen

The effect of anti-CD4 and anti-CD8 mAbs on spleens of mice exposed to DEP was investigated. On days 7 and 28, the number of spleen cells in mice immunized with OVA+DEP after in vivo administration of anti-CD4 and anti-CD8 mAbs was similar to that in mice immunized with OVA (data not shown). Adjuvant activity of 2 mg DEP on the number of spleen cells was not observed.

To examine the persistence of T-cell depletion, the number of CD4+ and CD8+ T cells in the spleen was analyzed using FACS on day 7 and day 28. As shown in table 1, cell depletion in spleens of anti-CD4-treated mice immunized with OVA and OVA+DEP was 96% (vs. group 1) and 97% (vs. group 4) on day 7, respectively. Cell

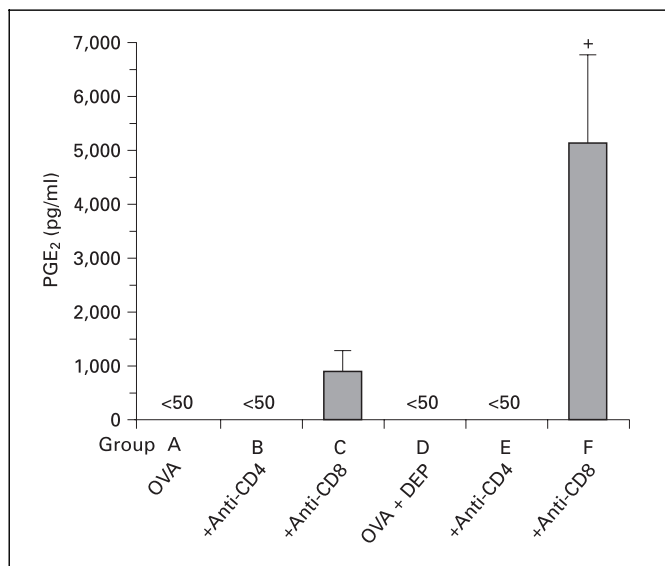


Fig. 4. PGE₂ production in peritoneal exudate fluids. On day 7, peritoneal exudate fluids were collected from OVA- or OVA+DEP-injected mice depleted of CD4+ or CD8+ cells and levels of cytokine production were determined by ELISA. Each value is mean $\pm$ SE (n = 4). + p < 0.05 vs. group C.

depletion in anti-CD8-treated mice immunized with OVA and OVA+DEP was 91% (vs. group 1) and 95% (vs. group 4), respectively. On day 28, cell depletion in anti-CD4-treated mice immunized with OVA and OVA+DEP was 60% (vs. group 1) and 59% (vs. group 4), respectively. Cell depletion in anti-CD8-treated mice immunized with

OVA and OVA+DEP was 55% (vs. group 1) and 45% (vs. group 4), respectively. The CD4/CD8 ratio in mice immunized with OVA+DEP was lower than that in mice immunized with OVA (group 1 vs. group 4) on day 7; however, on day 28, this ratio was higher in mice immunized with OVA+DEP than in the OVA-immunized mice. These results indicate that DEP may stimulate the differentiation of CD4+ helper T cells rather than of CD8+ cytotoxic T cells.

Proliferation of Spleen Cells from DEP-Treated Mice

The effects of DEP on the proliferative response of spleen cells from each group of mice were determined by culturing the spleen cells with OVA in vitro. After 72 h, the proliferative response to OVA did not differ between OVA- and OVA+DEP-immunized mice that received the treatment with vehicle (rat IgG). The proliferative response to OVA in the spleen cells from anti-CD4-treated mice immunized with OVA and from anti-CD8-treated mice immunized with OVA+DEP was decreased significantly (p < 0.05) relative to each control (data not shown).

Effects of DEP on Antigen-Stimulated Cytokine Production by Spleen Cells

Because DEP is a potent Th2 cytokine-inducing adjuvant, we investigated whether the effects of DEP on cytokine production were mediated by CD4+ and CD8+ T cells. Mice were treated with anti-CD4 or anti-CD8 mAb, immunized with OVA+DEP or OVA alone the next day, and retreated with each mAb the following day. At 21 days after receiving OVA or OVA+DEP, all mice

Table 1. In vivo depletion of T cells with anti-CD4 or anti-CD8 mAb

In vivo treatment	T-cell subset, %					
	day 7			day 28		
	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+
1 OVA+rat IgG	27.7 $\pm$ 0.9	10.3 $\pm$ 0.7	2.70	18.3 $\pm$ 0.9	7.8 $\pm$ 0.6	2.35
2 OVA+anti-CD4	1.2 $\pm$ 0.1	12.6 $\pm$ 0.4	0.30	7.4 $\pm$ 0.2	12.3 $\pm$ 0.6	0.60
3 OVA+anti-CD8	29.9 $\pm$ 1.4	0.9 $\pm$ 0.2	33.22	23.8 $\pm$ 0.4	3.5 $\pm$ 0.3	6.80
4 OVA+DEP+rat IgG	24.0 $\pm$ 0.4	10.3 $\pm$ 0.4	2.33	20.4 $\pm$ 1.9	7.5 $\pm$ 0.2	2.72
5 OVA+DEP+anti-CD4	0.7 $\pm$ 0.1	9.8 $\pm$ 1.6	0.07	8.4 $\pm$ 0.3	10.5 $\pm$ 0.5	0.80
6 OVA+DEP+anti-CD8	26.2 $\pm$ 0.8	0.5 $\pm$ 0.2	52.40	24.5 $\pm$ 1.3	4.1 $\pm$ 0.2	5.98

Mice were immunized intraperitoneally with OVA (10 μ g) or OVA (10 μ g) + DEP (2 mg). One day before and after immunization, mice were injected intraperitoneally with anti-CD4, anti-CD8 or rat IgG (control) mAb.

On days 7 and 28, the proportion of CD4+ and CD8+ cells in spleens was analyzed. Each value is the mean $\pm$ SEM of 3–4 mice per group. One representative experiment out of 3 is shown.

Fig. 5. IL-4 and IFN- γ production in culture supernatants during in vitro OVA stimulation of spleen cells. On day -1 and day 1, each group of 4 mice was injected intraperitoneally with 100 μ g of anti-CD4, 100 μ g of anti-CD8 or 100 μ g of rat IgG (control). On day 0, mice were immunized with 10 μ g of OVA, or 10 μ g of OVA plus 2 mg of DEP. After 3 weeks, each mouse was boosted with 10 mg of OVA alone. On day 28, spleens were collected from each treatment group and spleen cells were cultured with OVA. Supernatants were collected after 48 h of culture. IL-4 (**a**) and IFN- γ (**b**) production was determined by ELISA. Each value is mean $\pm$ SE. * $p < 0.05$ vs. group A; ** $p < 0.01$ vs. group A; # $p < 0.05$ vs. group B; ## $p < 0.01$ vs. group B; + $p < 0.05$ vs. group C; ++ $p < 0.01$ vs. group C.

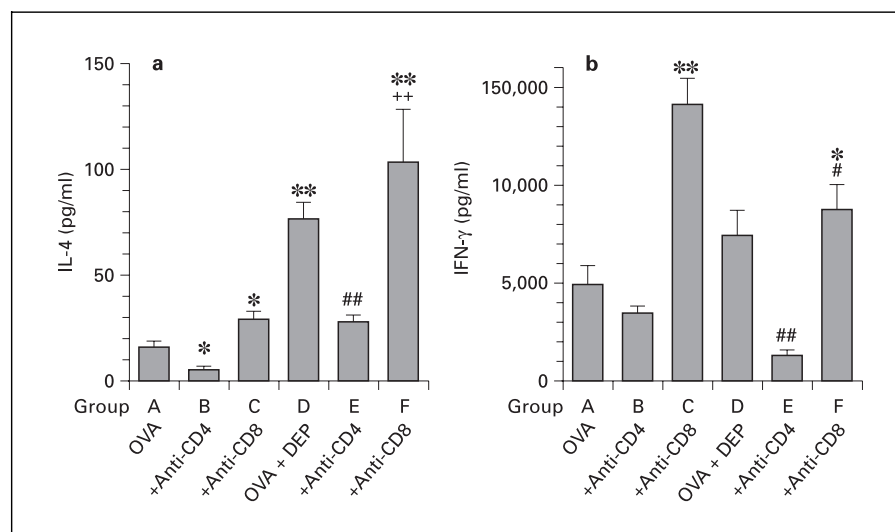
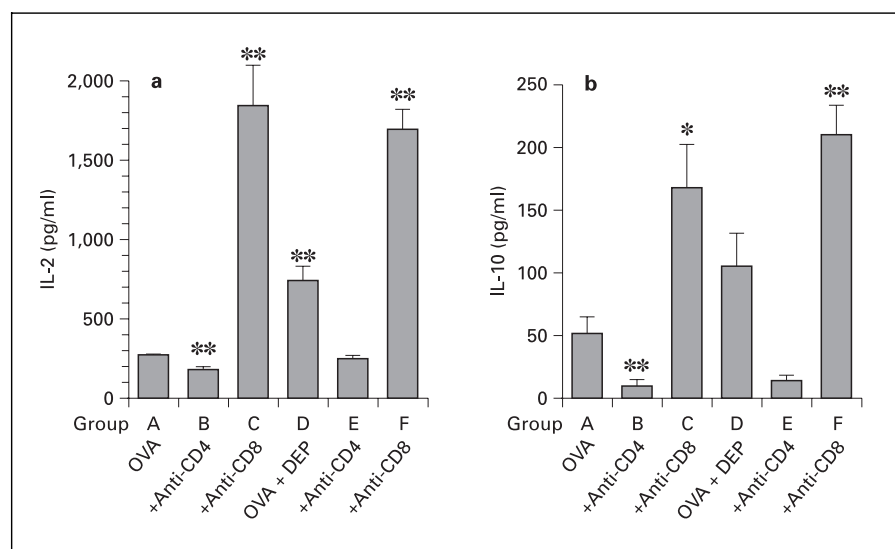


Fig. 6. IL-2 (**a**) and IL-10 (**b**) production in culture supernatants during in vitro OVA stimulation of spleen cells. On day -1 and day 1, each group of 4 mice was injected intraperitoneally with 100 μ g of anti-CD4, 100 μ g of anti-CD8 or 100 μ g of rat IgG (control). On day 0, mice were immunized with 10 μ g of OVA, or 10 μ g of OVA plus 2 mg of DEP. After 3 weeks, each mouse was boosted with 10 mg of OVA alone. On day 28, spleens were collected from each treatment group and spleen cells were cultured with OVA. Supernatants were collected after 48 h of culture. Cytokine production was determined by ELISA. Each value is mean $\pm$ SE. * $p < 0.05$ vs. group A; ** $p < 0.01$ vs. group A; + $p < 0.05$ vs. group C.



received a booster immunization of OVA. On day 28, spleen cells from each group of mice were placed in culture with OVA. IL-4 production in culture supernatants had significantly increased in the DEP-injected mice treated with vehicle (rat IgG; $p < 0.01$) and anti-CD8 ($p < 0.01$) mAb (fig. 5a).

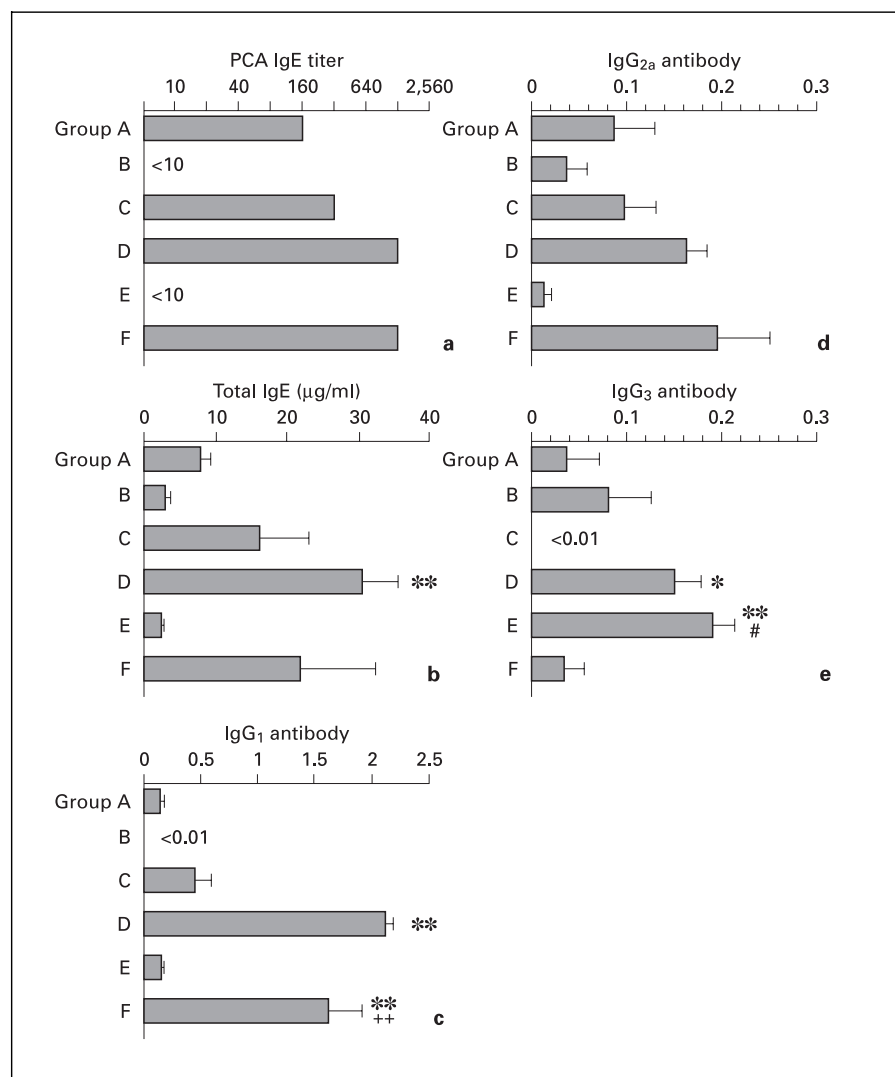
At 48 h, IFN- γ production in OVA+DEP-injected mice showed no significant increase compared with OVA-injected mice. Treatment with anti-CD8 mAb markedly increased IFN- γ production in the DEP+OVA- and OVA-injected mice (fig. 5b). Between the groups, reduced IFN- γ production was observed in DEP-injected mice. Depletion of CD4 $^{+}$ cells in DEP-injected mice resulted in marked decrease of IFN- γ production ($p < 0.01$).

At 48 h, DEP injection significantly increased the IL-2 production ($p < 0.01$), a Th1 cytokine, as compared with vehicle level in OVA-immunized mice (fig. 6a). Although treatment with anti-CD4 mAb in OVA-injected mice significantly reduced IL-2 production, treatment with anti-CD4 mAb in DEP-injected mice had no influence in this cytokine. In IL-10 production, DEP injection to mice failed to enhance the production, but treatment with anti-CD8 mAb resulted in a significant increase more in DEP-injected mice than in OVA-injected mice (fig. 6b).

Effect of DEP on IgE and IgG Subclass Synthesis

To investigate the systemic effect of DEP after treatment with anti-CD4 and anti-CD8 mAbs, we asked

Fig. 7. Effects of DEP on IgE and IgG antibody responses. On day -1 and day 1, each group of 4 mice was injected intraperitoneally with 100 µg of anti-CD4, 100 µg of anti-CD8 or 100 µg of rat IgG (control). On day 0, mice were immunized with 10 µg of OVA, or 10 µg or OVA plus 2 mg of DEP. After 3 weeks, each mouse was boosted with 10 mg of OVA alone. On day 28, plasma was collected from each treatment group and levels of anti-OVA IgE (**a**), total IgE (**b**), anti-OVA IgG1 (**c**), anti-OVA IgG2a (**d**) and anti-OVA IgG3 (**e**) were measured by PCA and ELISA. Each value is mean $\pm$ SE. Group A = OVA+rat IgG-treated group; group B = OVA+anti-CD4-treated group; group C = OVA+anti-CD8-treated group; group D = OVA+DEP+rat IgG-treated group; group E = OVA+DEP+anti-CD4-treated group; group F = OVA+DEP+anti-CD8-treated group. * $p < 0.05$ vs. group A; ** $p < 0.01$ vs. group A; # $p < 0.05$ vs. group B; ++ $p < 0.01$ vs. group C.



whether the isotype and IgG subclass of anti-OVA antibody changed after injection with DEP. In the OVA+DEP-immunized, vehicle-treated mice, anti-OVA IgE and total IgE levels in plasma were markedly increased (fig. 7a, b) compared with OVA-immunized, vehicle-treated mice. Treatment with anti-CD4 mAb in OVA+DEP- and OVA-injected mice markedly reduced OVA-specific and total IgE levels. In addition, OVA+DEP-injected, vehicle-treated mice showed a significant increase ($p < 0.01$) in anti-OVA IgG1 levels compared with levels in OVA-injected controls (fig. 7c). Treatment with anti-CD8 mAb in OVA+DEP-injected mice significantly increased ($p < 0.01$) the IgG1 levels, but treatment with anti-CD4 mAb resulted in complete loss of the IgG1 production.

Levels of anti-OVA IgG2a in OVA+DEP- and OVA-injected mice were similar regardless of the treatment with anti-CD4 and anti-CD8 mAbs (fig. 7d). In the OVA+DEP-immunized, vehicle-treated mice, anti-OVA IgG3 levels in plasma were significantly ($p < 0.05$) increased compared with OVA-immunized, vehicle-treated mice (fig. 7e). Levels of anti-OVA IgG3 in DEP-injected anti-CD4-treated mice also significantly increased ($p < 0.01$). However, treatment with anti-CD8 mAb markedly reduced anti-OVA IgG3 levels in either OVA- or OVA+DEP-injected mice. These findings suggest that DEP differently affect humoral immune response and that increased IgE and IgG1 antibody production may be due to the effect on CD4+ cells.

Discussion

Administration of DEP has enhanced antigen-specific IgE production and increased Th2-type cytokines in animal and human studies [7–9, 14]. However, it is uncertain whether the adjuvant activity of DEP is due to modulation of the function of CD4+ and CD8+ T-cell subtypes which regulated type-1 and type-2 cytokine profiles. In the present study, we investigated the effects of DEP on cytokine networks involved in antigen-presenting cell (APC) and systemic immune response in spleen and plasma from mice depleted of CD4+ or CD8+ cells.

To examine the relation between CD4+ and CD8+ T-cell depletion and proinflammatory cytokine secretion in the peritoneal cavity of DEP-injected mice as an early host defense mechanism, we analyzed the expression of CD80 and CD86 costimulatory molecules on APC and cytokine production from PEC on day 7. Although PEC from DEP-injected mice had increased numbers of IA+, CD80+/IA+ and CD86+/IA+ cells, DEP injection and CD4+ and CD8+ T-cell depletion did not synergize to induce the synthesis of the costimulatory molecules. The real role of CD80 and CD86 molecules in the immune response remains controversial. CD86 provides a signal to naive T cells to differentiate to IL-4 producers, whereas CD80 induces more neutral differentiation [27]. *Listeria* infection causes upregulation of both CD80 and CD86 on macrophages [28]. Schweitzer and Sharpe [29] demonstrated that induction of IL-4 production and Th2 differentiation by naive T cells is highly dependent on B7 molecules, but does not appear to selectively regulate Th1/Th2 balance. DEP and DEP extract induced the release of oxygen radicals from activated APC and increased CD80 expression in human nasal lavage cells [30]. Together with our work, these studies suggest that DEP injection may activate expression of the costimulatory molecules CD80 and CD86, which may promote a differentiation of T cells and lead to upregulation of immune response. Expression of CD80 and CD86 on APC interacted with CD28 and CTLA-4 on T cells plays an important role in the regulation of immune response to allergen. Whether the expression of CD28 and CTLA-4 molecules on T cells is involved in the adjuvant activity of DEP is presently unknown.

APC-derived proinflammatory cytokines, including IL-1, IL-6, IL-8 GM-CSF and TNF- α , are important modulators in the immune and inflammatory reactions. Among these cytokines, IL-1 is released at an early stage of allergic response, and induces macrophage and neutrophil emigration, initiation of cytokine cascades, and stimulates release of GM-CSF from endothelial cells [31, 32].

GM-CSF is a hematopoietic growth factor that stimulates eosinophil proliferation and maturation and enhances the survival of both neutrophils and eosinophils [33, 34]. In fact, DEP enhances IL-1 production by rat alveolar macrophages in vitro [35]. In vitro culture with DEP stimulates GM-CSF release from human bronchial epithelial cells [15, 16]. Our present study indicated that GM-CSF and IL-1 α secretion in the peritoneal cavity from DEP-injected mice was significantly increased. However, treatment with anti-CD8 and anti-CD4 mAbs disturbed the enhancement of GM-CSF and IL-1 α production induced by DEP injection, respectively. These findings suggest that enhanced GM-CSF and IL-1 α production may be partly related to the modulation of CD4+ and CD8+ cell function in DEP-injected mice. Holmes et al. [36, 37] suggested that some CD8+ T cells may help initiate an IgE response. CD8+ T cells likely modulate the CD4+ T-helper cell function of inducing and maintaining the IgE response. We found that depletion of CD8+ cells abrogated the adjuvant activity of DEP in GM-CSF production, and this result may point to an early helper function of CD8+ cells. The interaction of CD8+ cells with APC needs to be addressed in light of the role of APC for studying adjuvant activity of DEP.

Regarding IL-10 and IL-12 as inhibitory cytokines in allergic inflammation, DEP injection caused no change in peritoneal cavity (fig. 3c, d). IL-10 serves as a feedback inhibitory mechanism to damp down the inflammatory response [38]. Treatment with anti-CD4 mAb significantly increased the production of IL-10 in the peritoneal cavity of OVA-injected mice. However, the same treatment in DEP+OVA-injected mice had no influence on IL-10 secretion. IL-12 is one of the macrophage-derived cytokines that can promote Th1-type T-cell differentiation and induces IFN- γ production [39]. Depletion of CD8+ cells led to enhancement of IL-12 levels in the peritoneal cavity from OVA-injected mice, but DEP injection returned to the control levels.

When Th0-like responses are shifted toward a Th subset development, PGE₂ may play a role in suppression of IL-12 production and in facilitating the production of Th2-like cytokines [25, 26, 40]. On day 7, there is no increase in production of PGE₂ in peritoneal exudate fluid from both OVA- and DEP+OVA-injected mice. However, treatment with anti-CD8 mAb resulted in an increase in PGE₂ in OVA-injected mice and DEP injection enhanced its production markedly, suggesting that DEP have an ability to retain the production of PGE₂ in the presence of CD4+ cells. Previous results showed that intraperitoneal, intratracheal and intranasal instillation

Table 2. Summary of cytokine production from in vitro spleen cell cultures

	OVA			OVA+DEP		
	RIgG	+anti-CD4	+anti-CD8	RIgG	+anti-CD4	+anti-CD8
IL-2		↓↓*	↑↑	↑↑		↑↑
IFN-γ			↑↑			↑
IL-4		↓	↑	↑↑		↑↑
IL-10		↓↓	↑			↑↑

↑ = increase (p < 0.05); ↑↑ = increase (p < 0.01); ↓ = decrease (p < 0.05); ↓↓ = decrease (p < 0.01).
* Versus OVA+RIgG.

with OVA plus DEP enhanced anti-OVA IgE production, suggesting that DEP have an adjuvant activity regardless of the route used [7–9]. Therefore, the present protocol for immunization and treatment with anti-CD4 or anti-CD8 mAb was adopted from Yang et al. [24]. One day before and after intraperitoneal immunization with or without DEP in the presence of OVA, mice were treated with anti-CD4 or anti-CD8 mAb. Efficient depletion of CD4+ and CD8+ cells from spleens was confirmed by FACS analysis on day 7 (table 1). Further studies will be required to determine time dependency in the production of PGE₂ induced by DEP injection.

The mechanisms by which DEP exert their adjuvant activity to induce a Th2-dominated cytokine production are as yet unclear. Our present results in the production of cytokines from spleen cells are summarized in table 2, and clearly demonstrate for the first time that DEP inhibit the suppressive potential of CD8+ T cells, but enhance the activated potential of CD4+ T cells. Moreover, compared with Th1-type cytokines (IFN-γ, IL-2), DEP increase Th2-type cytokine production from CD4+ T cells. The production of IFN-γ in culture supernatants from vehicle-treated spleen cells of DEP+OVA-injected mice showed no significant difference from that of vehicle-treated spleen cells of OVA-injected mice (fig. 5b). The reason why DEP injection did not reduce the production of IFN-γ in culture supernatants might be due to the different route of antigen administration. Depletion of CD8+ T cells in a mouse model for IgE immune responsiveness and a rat model of asthma led to a significant reduction in the mRNA expression of Th1 cytokines, whereas Th2 cytokine mRNA expression was unchanged [22, 41]. In humans, DEP+ragweed antigen challenge into ragweed-sensitive subjects resulted in increased expression of IL-4, IL-5, IL-6, IL-10 and IL-13 mRNAs in nasal lavage cells, but had an inhibitory effect on IFN-γ mRNA

[14]. These observations with our results suggest that the adjuvant activity of DEP in the regulation of OVA-induced cytokine production modulates the function of both CD4+ and CD8+ T-cell subtypes.

In mice, production of both Th1-dominated antibody, such as IgG2a and IgG3, as well as Th2-dominated antibody, such as IgE and IgG1, is associated with a balance in the Th1/Th2-type cytokine production [42–44]. Adjuvant activity of DEP was observed in anti-OVA IgE, anti-OVA IgG1, anti-OVA IgG3, and total IgE production. Depletion of CD4+ T cells abrogated the adjuvant effect of DEP on anti-OVA IgE and anti-OVA IgG1 production in plasma. However, depletion of CD8+ T cells inhibited the upregulated anti-OVA IgG3 production. Because IgG3 is a Th1-dominated antibody, the effect of DEP on the CD8+ subset known as Tc1 cells may be related to the above reduction. However, we were unable to document an anti-CD8-associated decrease in cytokine production. Another possibility is supported by the fact that IgG3 might be regulated by a T-independent mechanism [45, 46], suggesting that B cells appear to play an important part in enhanced IgG3 production induced by DEP injection. Taken together, CD4+ T-cell subtypes appear to contribute to the increased levels of IgE and IgG1 by DEP, and increased levels of IgG3 may be due partly to the contribution of the CD8+ T-cell subtype.

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