Unique dose-dependent effects of the human pregnancy hormone estriol on the ratio of blood IgM to IgG in female mice

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Abstract. The present study aimed to investigate the dose-dependent modulating effect of estriol (E_3), an estrogen predominantly produced during human pregnancy, on antigen-induced production of specific antibodies in female BALB/c mice. The animals were immunized either with bovine serum albumin (BSA) or the pneumococcal polysaccharide serotype-14 (PPS-14), and the levels of specific serum antibodies were determined using ELISA kits. E_3 was found to have very different effects on antigen-induced production of specific antibodies in animals immunized with these two antigens. While E_3 stimulated the production of PPS-14-specific antibodies, it suppressed the production of BSA-specific antibodies. The results also demonstrated that the modulating effect of E_3 on the production of antigen-specific antibodies depends on the dose of E_3 used. For BSA-induced antibody production, E_3 had a dose-dependent inhibitory effect, whereas for PPS-14-specific antibody (Ab) production, E_3 exerted the strongest stimulation at a lower dose, and produced less stimulation at higher doses. E_3 caused thymus atrophy in animals immunized with either PPS-14 or BSA, but only induced spleen atrophy in BSA-injected mice. These observations suggest that E_3 increases the ability of a pregnant female to avoid bacterial infections while decreasing the incidence of autoimmune responses against circulating components from either the fetus or pregnant female.

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Introduction

One of the most significant changes during pregnancy is the favorable adjustment of the maternal immune system toward the semi-allogeneic fetus so that it will not be rejected. It has been suggested that there is a T helper type 2 (Th-2)-biased shift in the maternal immune system during pregnancy (1,2). This shift has been demonstrated in pregnant mice by showing a stronger antibody (Ab) response but a decreased delayed-type hypersensitivity against paternal major histocompatibility complex and other foreign antigens (1,3,4). In addition, it has been observed that the conditions of certain autoimmune diseases, including systemic lupus erythematosus, that are predominantly mediated by Th-2 cells often worsen during pregnancy whereas the conditions of other autoimmune diseases, such as rheumatoid arthritis that are predominantly mediated by T helper type 1 (Th-1) cells often regress (5). Similarly, preferential production of Th-2 cytokines over Th-1 cytokines in the placenta has been observed during pregnancy with non-immune cells contributing to the Th-2 predominance (2,6-8). This differential induction of cytokines appears to be consistent with their known functions during pregnancy, whereas Th-1 cytokines, including IFN-γ, IL-2 and TNF, promote fetal loss (9-12), Th-2 cytokines (IL-10) are protective against fetal mortality in a murine model of spontaneous reabsorption (11,13).

The exact causes for these marked local and systemic immunological alterations occurring during pregnancy remain to be elucidated. It has been suggested that alterations in endogenous hormones during pregnancy may contribute to some of the changes. A number of previous studies have investigated the potential roles of progesterone, 17β-estradiol (E_2), chorionic gonadotropin and chorionic somatomammotropin in mediating immunosuppression (14-18). Progesterone, an essential hormone for maintaining pregnancy, was found to have diverse effects on different populations of immune cells, including an immunosuppressive effect in favor of fetal survival (14). Notably, E_2 has pro- and anti-inflammatory effects depending on the dose used (18). During pregnancy, the quantitatively-predominant estrogen produced in the body is estriol (E_3). However, relatively little is known about its modulating effect on the immune system during pregnancy. Our previous study reported that treatment of female BALB/c mice with a subcutaneous pellet containing 2.5 mg E_3 alters the serum levels of specific Abs against bovine

serum albumin (BSA), a circulating protein, and pneumococcal polysaccharide serotype-14 (PPS-14), a bacterial wall component, in a divergent manner, whereas E_2 (at 0.36 mg E_2 per pellet) did not have the same effect (19). It is suggested that these alterations elicited by E_3 may increase the ability of a pregnant female to ward off bacterial infections while decreasing the incidence of autoimmune responses against circulating components from either the fetus or pregnant females. The present study aimed to determine the detailed dose-response relationship of E_3 (at selected doses of 0, 0.5, 2.5, 5, 10 or 15 mg E_3/pellet) in modulating the changes of these two specific Abs.

Materials and methods

Chemicals and reagents. Cholesterol, o-phenylenediamine, Tween-20 and BSA were obtained from Sigma-Aldrich (St. Louis, MO, USA). E_3 was purchased from Steraloids (Newport, RI, USA). Hydrogen peroxide and gelatin were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Purified *Streptococcus pneumoniae* capsular PPS-14 was obtained from the American Type Culture Collection (Manassas, VA, USA). Polyclonal goat anti-mouse immunoglobulin (Ig)G was obtained from Chemicon (Temecula, CA, USA), and monoclonal rat anti-mouse IgM and rat anti-mouse IgA were obtained from eBioscience (San Diego, CA, USA). The horseradish peroxidase (HRP)-conjugated polyclonal goat Abs against mouse IgM, IgA, IgG, IgG1, IgG2a or IgG2b were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

The pellets containing either E_3 or vehicle alone were hand-pressed using the Pellet Presser (PARR Instrument Co., Moline, IL, USA). Each pellet (weighing 25 mg) contained 5 mg of sodium chloride and a combined 20 mg of E_3 plus cholesterol. Five different doses of E_3 were used in the present study. In each pellet, the quantity of E_3 was 0.5, 2.5, 5, 10 or 15 mg. Dry crystals of sodium chloride, cholesterol and estrogen were mixed thoroughly by grinding the mixtures to fine powders. The pellets were produced by the same individual by applying approximately the same quantity of pressing force.

Animal experiments. All animal experimental procedures described in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kansas Medical Center at Kansas City (Kansas, USA). Female 7-8-week-old BALB/c mice (6 animals per treatment group), purchased from Harlan Laboratories (Indianapolis, IN, USA), were housed in a room with controlled conditions of temperature (22±2˚C) and light (12-h-light and 12-h-dark cycle), and had free access to food and water. Animals were allowed to acclimate to the new environment for 1 week prior to the beginning of the experiment.

Each animal was subcutaneously implanted with a 25-mg pellet containing different doses of E_3 (0, 0.5, 2.5, 5, 10 or 15 mg/pellet) under the dorsal skin. Our previous study demonstrated that the average plasma concentration of free E_3 at 5 days after implantation of a 10-mg E_3 pellet was 12.5 nM (20). Notably, the basal circulating levels of E_3 in non-pregnant females were around 50 nM (21). Thus, the doses of E_3 used in the present study were expected to provide circulating concentrations of E_3 in physiologically-relevant ranges. The animals (5 per group) were injected with BSA or PPS-14 7 days after implantation of the estrogen-containing pellet. For BSA immunization, three intraperitoneal (i.p.) injections of 400 g BSA in 50 μl phosphate-buffered saline (PBS) were administered to the animals once every 14 days. For PPS-14 immunization, two i.p. injections of 10 μg PPS-14 in 50 μl PBS were administered once every 14 days. All the solutions injected into animals were sterilized.

The animals were sacrificed by CO_2 inhalation and the blood samples were collected 1 week after the last antigen injection. The sera were prepared by centrifugation at 1,500 xg for 10 min. Aliquots of the serum samples were stored at -80˚C until further assessment. The thymus, spleen and heart were collected from each animal and the wet weight of the organs was measured for evaluation of the effect of estrogen.

ELISA. The levels of BSA-specific Abs in mouse sera were determined by using the standard ELISA method. Briefly, 96-well plates (Nunc™ Maxisorp™; Nalge Nunc International, Rochester, NY, USA) were coated with 50 μl BSA (50 μg/ml in 0.1 M $NaHCO_3$, pH 9.6) overnight at 4˚C. After 1 h blocking of the wells with 150 μl of 0.25% (w/v) gelatin in PBS, a series of diluted sera were added into each well and incubated for 1 h at room temperature. The HRP-conjugated goat anti-mouse Ig Abs with specificity for different Ig classes were used as the secondary Ab at a dilution of 1:1,000 for anti-mouse IgG1 and 1:2,000 for anti-mouse IgG2a, IgG2b and IgM. Following 1 h incubation with the secondary Ab, freshly prepared substrate (1 mg/ml o-phenylenediamine in 0.1 M citrate buffer + 1.5 μl/ml hydrogen peroxide) was added and the optical density (OD) value in each well was measured at 450 nm using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). Every well was washed three times with 200 μl of PBS containing 0.05% (v/v) Tween-20 following incubation with the primary and secondary Abs.

Levels of the PPS-14-specific Abs in the mouse sera were determined as described above. A total of 50 μl of PPS-14 (5 μg/ml) in PBS was added to each well for coating for 2 h at 37˚C. A BSA solution (10 mg/ml in PBS) was used as the blocking buffer. Serially diluted serum, secondary Abs and the substrate solution were added similarly as mentioned above. The OD value at 450 nm was then measured.

The total Ab levels present in the sera were determined by using the capture ELISA method. A pre-determined optimal concentration of the capture Ab was used instead of a specific antigen. Following coating with the capture Abs, the plates were developed in the same way as mentioned above. The goat anti-mouse IgG polyclonal Ab was used as the capturing Ab for IgG1, IgG2a and IgG2b at 1 μg/ml, and the rat anti-mouse IgM monoclonal Ab was used at 2 μg/ml to capture IgM.

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical significance was analyzed using Student's t-test with Microsoft Excel (Microsoft, Seattle, WA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

E_3 inhibits the production of BSA-induced specific Abs. Dose-response experiments were conducted to determine

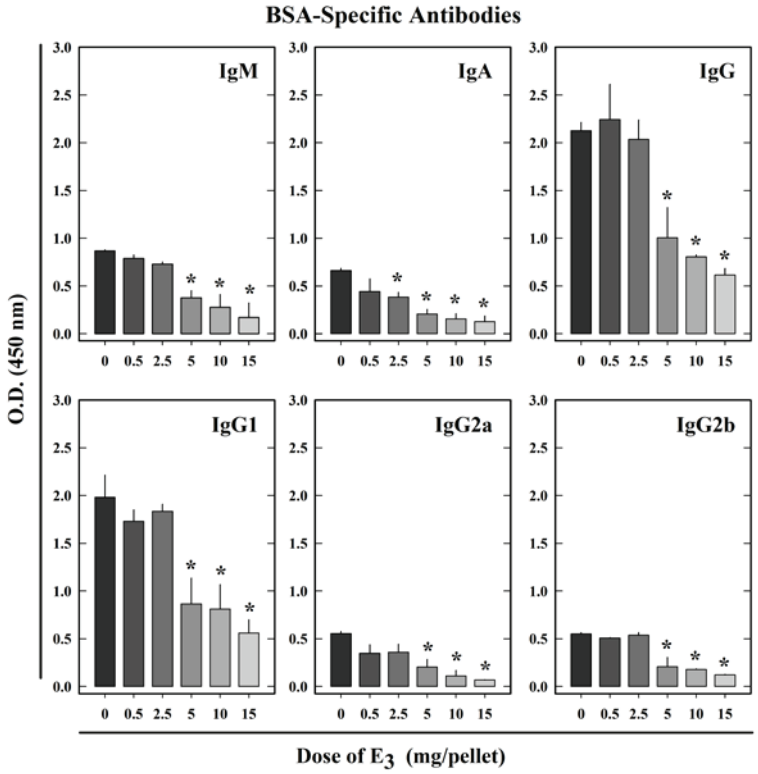

Figure 1. Dose-dependent inhibition of BSA-induced specific Ab production by E_3. Female 7-8-week-old BALB/c mice (n=6 per group) were implanted with a vehicle pellet (control) or a pellet containing different doses of E_3 plus immunization with intraperitoneal injections of BSA as described in the Materials and methods. The levels of BSA-specific Ig classes in the E_3-treated animals were expressed as a percentage of the corresponding levels in vehicle-treated control animals. Each value shown in the figure is the mean ± standard deviation of six animals. Note that the serum sample of each animal was determined at several dilutions and then the average value was used to represent that individual animal. *P<0.05, compared with the corresponding control. E_3, estriol; BSA, bovine serum albumin. Ig, immunoglobulin; OD, optical density.

the modulating effect of E_3 on the production of specific Abs against BSA, a circulating protein antigen, using female BALB/c mice as a model. Animals were initially implanted with a pellet containing the vehicle alone (no estrogen) or five different doses of E_3 (0.5, 2.5, 5, 10 or 15 mg/pellet), and then immunized with BSA. As shown in Fig. 1, treatment of animals with E_3 + BSA resulted in dose-dependent inhibition of the production of BSA-specific IgM, IgA, IgG, IgG1, IgG2a and IgG2b compared with the control animals treated with the vehicle + BSA. At relatively lower doses (0.5 and 2.5 mg), E_3 had little or no effect on the production of BSA-specific IgM, IgG, IgG1 and Ig2b, however, the production of IgA and IgG2a was more sensitive to inhibition. At higher doses (5, 10 and 15 mg), E_3 decreased serum levels of all classes and subclasses of specific Igs in BALB/c mice in a dose-dependent manner. The levels of BSA-specific IgM, IgA, IgG, IgG1, IgG2a and IgG2b in animals treated with 10 and 15 mg E_3 were only 20-30% of the levels in control animals.

E_3 stimulates the production of PPS-14-induced specific Abs. In order to assess the effect of E_3 on the production of specific Abs against PPS-14, a component of the bacterial wall, female BALB/c mice were similarly implanted with a pellet containing different doses of E_3 followed by i.p. injections of PPS-14. As shown in Fig. 2, treatment of animals with a low dose of E_3 (0.5 mg) + PPS-14 significantly increased PPS-14-specific Ab levels for all assessed Ig classes and subclasses (IgM, IgA, IgG, IgG1, IgG2a and IgG2b) compared with the control animals treated with the vehicle + PPS-14. IgM, the dominant class among the induced PPS-14-specific Abs, was increased by ~60% over the control. The serum levels of PPS-14-specific IgA were also elevated by E_3 treatment at a dose of 0.5 mg and the increase was increased by 90% compared with the control. Specific total IgG was increased by 100%, IgG2a by 150%, IgG2b by 130% and IgG1 by 70%.

With increasing doses, the stimulatory effect of E_3 on PPS-14-specific Ab production was gradually reversed in a dose-dependent manner. Although E_3 at a dose of 2.5 mg also increased PPS-14-specific Ab production, the extent of the increase was less than that observed at a dose of 0.5 mg. The stimulatory effect of E_3 was further weakened when E_3 was increased to 5 and 10 mg. Animals treated with E_3 at 15 mg had comparable levels of PPS-14-specific IgG1 and IgG2b compared with the control animals (without E_3 treatment), and levels of specific IgM, IgA, IgG and IgG2a were partially lower than the corresponding controls.

Effect of different doses of E_3 on the static status of the immune system. For comparison, the effect of different doses of E_3 on the total serum Ab levels (specific + nonspecific Abs) as well as on the weight of central (thymus) and peripheral (spleen) lymph organs in animals without antigen

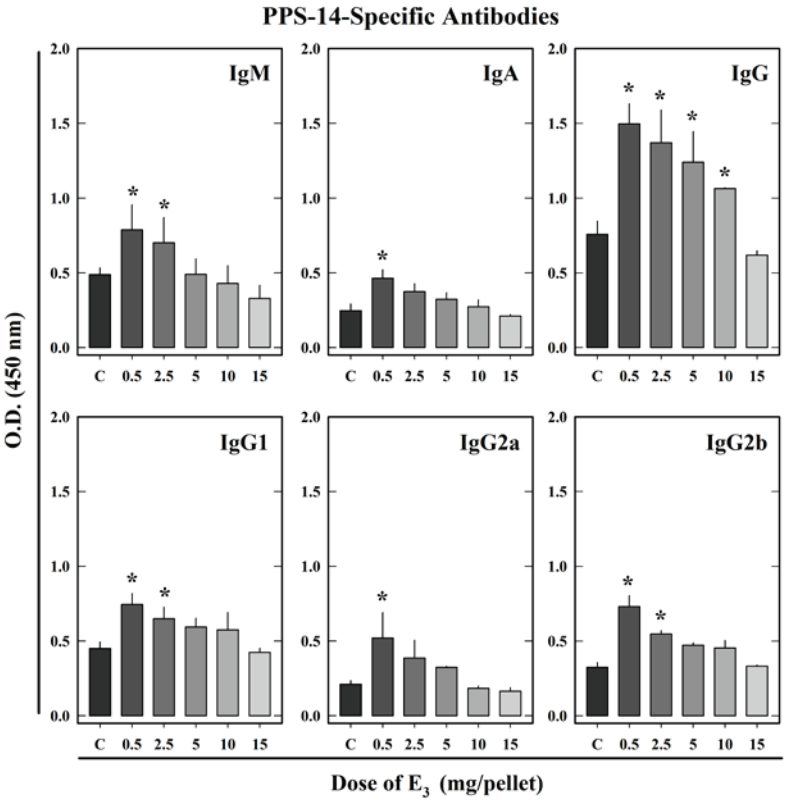

Figure 2. Dose-dependent effect of E_3 on PPS-14-induced specific Ab production. Female 7-8-week-old BALB/c mice (n=6 per group) were implanted with a vehicle pellet (control) or a pellet containing different doses of E_3 plus immunization with intraperitoneal injections of PPS-14 as described in the Materials and methods. The levels of PPS-14-specific Abs in the E_3-treated animals are expressed as a percentage of the corresponding levels in vehicle-treated control animals. Each value shown in the figure is the mean ± standard deviation of six animals. Note that the serum sample of each animal was determined at several dilutions and then the average value was used to represent that individual animal. *P<0.05, compared with the corresponding control. E_3, estriol; PPS-14, pneumococcal polysaccharide serotype-14; Ab, antibody; OD, optical density; Ig, immunoglobulin.

challenge was also determined. The total Ab levels in control and E_3-treated animals are summarized in Fig. 3. In the same groups of animals, alterations in total Ab levels were markedly less pronounced compared with changes in specific Ab levels. Levels of total IgM, IgA and IgG2a in animals immunized with BSA or PPS-14 were partially decreased by E_3 treatment in a dose-dependent manner. By contrast, levels of total IgG, IgG1 and IgG2b in animals immunized with BSA or PPS-14 were not considerably affected by E_3 treatment.

Wet weights of the thymus and spleen of each animal were determined and the ratios of the organ weights to body weights (organ weight indices) are summarized in Fig. 4. Treatment with E_3 caused a dose-dependent decrease of thymus weight index in animals either immunized with BSA or PPS-14 (Fig. 4A). By contrast, the spleen weight was reduced by E_3 treatment only in animals immunized with BSA, but not in animals immunized with PPS-14 (Fig. 4B). In addition, the weight index of the heart, which is known to be unaffected by estrogen treatment, was also determined for comparison. As expected, no significant difference was observed for heart weight indices among animals of different treatment groups (data not shown).

Discussion

Hormonal alterations during pregnancy have been suggested to have a contributing role in the changes observed in the maternal immune system. Progesterone, estrogens (particularly E_2) and chorionic gonadotropin have all been indicated to be important in the suppression of maternal immunity for the full acceptance of the allogeneic fetus (14,16). By contrast, few studies have examined the role of E_3, an estrogen produced in a large quantity only during human pregnancy, in the induction of immunotolerance during pregnancy. Jansson *et al* reported that E_3 has a more potent therapeutic effect on experimental autoimmune encephalomyelitis than does E_2 (22). However, Bebo *et al* indicated that an ameliorating effect of E_3, which was observed at circulating levels similar to pregnancy, was not significantly different from E_2 (23). The present study reported a unique modulating effect of E_3 on antigen-induced Ab responses *in vivo*. It is apparent that this effect depends on the dose of E_3 used. E_3 at doses of 0.5 and 2.5 mg did not have a significant effect on BSA-specific Ab production. However, at higher doses (>5 mg), it markedly reduced the serum levels of BSA-specific Abs in a dose-dependent manner. By contrast, E_3 had a stimulatory effect on PPS-14-induced specific Ab production. The stimulation was strongest at a low dose (0.5 mg) and weakened at higher doses (up to 15 mg). Notably, a similar phenomenon was reported by Salazar *et al* (24). By injecting heat-killed *Streptococcus pneumoniae* to mice, it was demonstrated that propanil (an herbicide with endocrine-disrupting activity) significantly increased the number of phosphatidylcholine (a TI-2 antigen of the bacterium)-specific Ab-secreting B cells in the spleen whereas the Ab response

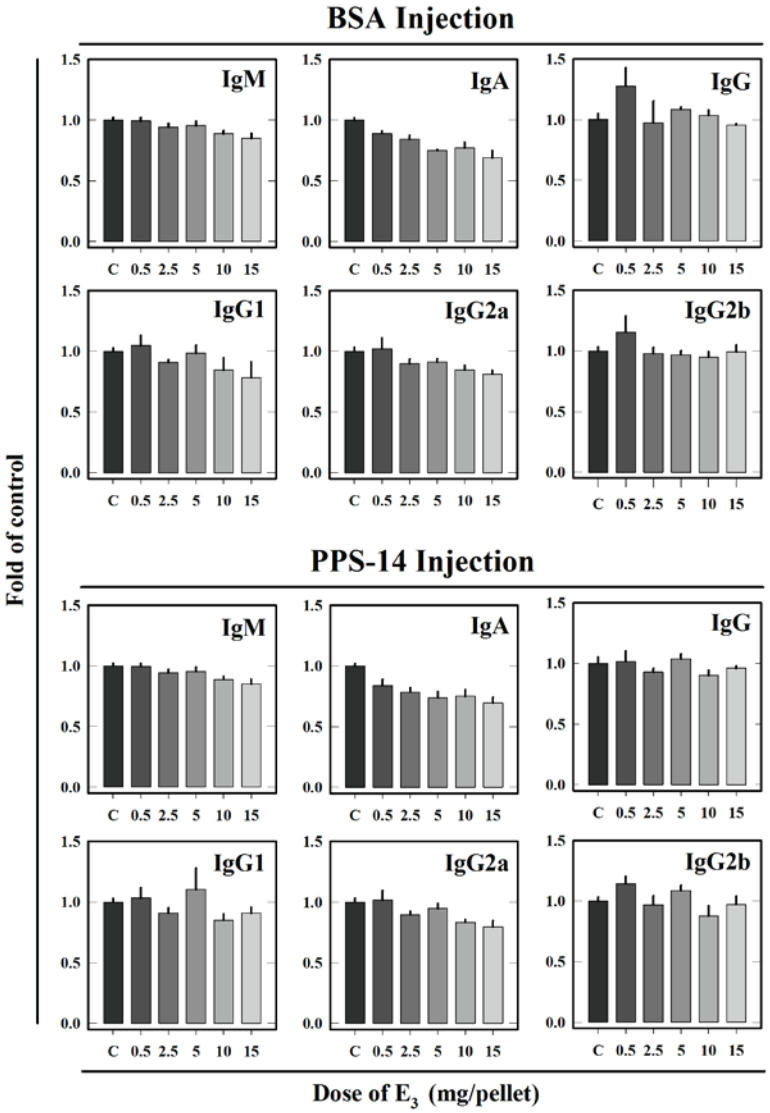

Figure 3. Effect of E_3 on total serum Ig levels. The total Ab levels of different Ig classes in animals treated with E_3 + BSA (top six panels), or E_3 + PPS-14 (bottom six panels) are shown as the fold of the control. Several serum dilutions were determined for each serum sample and the data shown in this figure are presented as the average values for several serum dilutions. No significant difference was observed between the E3-treated group and with the control group. Ab, antibody; E_3, estriol; Ig, immunoglobulin; BSA, bovine serum albumin; PPS-14, pneumococcal polysaccharide serotype-14.

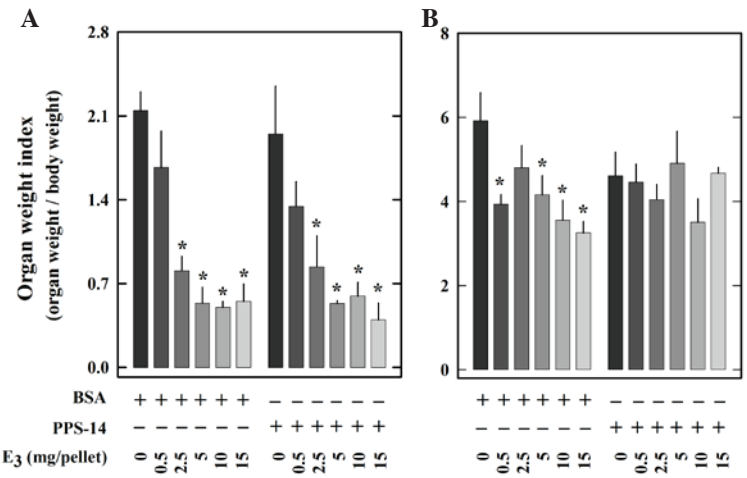

Figure 4. Effect of E_3 on the weight of immune organs. The (A) thymus and (B) spleen were harvested from each animal at the end of the experiments. Their wet weights were measured and the weight indices (organ weight/body weight) were calculated. *P<0.05, compared with the corresponding control. E_3, estriol; BSA, bovine serum albumin; PPS-14, pneumococcal polysaccharide serotype-14.

to pneumococcal surface protein A (a bacterial TD antigen) was not affected (24). A possible explanation for these results is that Ab production induced by TI-2 and TD antigens is regulated differently. While cytokine production is important for both, direct interaction between T and B cells may have a critical role in TD antigen-induced Ab response. E_3 may function differently in these two pathways. It is of note that since E_3 is known to promote the production of tolerogenic dendritic cells *in vivo*, which would be protective against autoimmunity (25), the effect of E_3 as observed in the present study may also contribute to the unique modulating effect of E_3 on antigen-induced Ab response *in vivo*.

Similar to E_2, E_3 was found to have a pronounced effect on the thymus. Atrophy induced by estrogen treatment was as high as 75% when the *in vivo* estrogen dose was ≥5 mg/pellet (regardless of the antigens used). In the spleen, marked changes were only observed for BSA-injected mice but not for PPS-14-injected mice. These observations are in line with the differential modulating effects of E_3 on BSA-specific and PPS-14-specific Ab production. The reduction in spleen weight following E_3 treatment in BSA-immunized mice possibly indicates a decrease in the total cell population, as flow cytometric analysis demonstrated that the percentages of splenic Th, Tc and B cells are hardly affected by E_3. A non-selective suppression of all splenic lymphocytes by E_3 may account for the observed decreases in all BSA-specific Abs.

In conclusion, the data presented in the present study suggest that the immunological alterations observed during pregnancy may be partly associated with the immune-modulating effect of E_3, which is a quantitatively-predominant estrogen produced during pregnancy. When E_3 was used at a dose of 5 mg/pellet, which would result in *in vivo* estrogen levels relevant to those observed during human pregnancy (20,21), it downregulated the production of BSA-specific Abs, whereas it upregulated the production of PPS-14-specific Abs. This is considered to be beneficial for reducing the risk of developing Ab-mediated autoimmune attacks against the maternal and fetal components during pregnancy while enhancing the ability of the maternal body to ward off bacterial infections. Therefore, it is suggested that E_3 may partially contribute to the development of fetal tolerance, although other maternal factors, including other hormones, T regulatory cells and suppressive dendritic cells, may also contribute to the maintenance of a successful pregnancy.

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