Immune response to ovalbumin following bisphenol A administration in mice fed with a low level of dietary protein

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Background and Purpose: We have previously shown that bisphenol A (BPA) augments T-helper (Th) 1 activity with no significant effects on an established oral tolerance to ovalbumin (OVA) in mice fed with a normal protein diet. The present study aimed to examine the effect of BPA on the immune response in a mouse model maintained on a very low protein diet (5% casein).

Methods: Mice were fed on a 5% protein diet, together with either OVA (OVA-fed) or water (water-fed), immunized intraperitoneally with OVA at 3-week intervals and administered BPA between the 2 immunizations. A week after the last immunization, animals were sacrificed and examined by enzyme-linked immunosorbent assay for serum titers of total immunoglobulin E (IgE), OVA-specific IgE, immunoglobulin G (IgG), IgG1, IgG2a, and the production of interferon-gamma, interleukin (IL)-4, and IL-12.

Results: In both BPA-treated and non-treated animals, OVA feeding resulted in lower titers of total and OVA-specific IgE, and OVA-specific IgG ($p<0.05$). There were higher levels of interferon-gamma ($p<0.05$), IL-4, and IL-12 ($p<0.05$) in animals with OVA tolerance following BPA treatment. However, IL-12 production was augmented only in BPA-treated water-fed animals ($p<0.01$).

Conclusion: BPA administration in mice fed with a low level of dietary protein augmented Th1 cytokines more profoundly in the animals with OVA tolerance than in the non-tolerant animals.

Key words: Bisphenol A; Food hypersensitivity; Immunoglobulin E; Ovalbumin

Introduction

Allergic diseases are hypersensitivity disorders, some of which are known to be associated with the production of specific immunoglobulin E (IgE) to allergens of environmental sources. Levels of higher serum IgE than normal are often found in patients with type I allergic diseases [1,2]. Prevalence of these diseases has increased significantly during the last few decades. However, there is a considerably lower prevalence of allergic diseases in developing countries [3] as compared to developed ones. Environmental factors, such as increased exposure to allergens and air pollution, have received much attention from researchers as possible grounds for the escalation of such diseases. Besides, the nutritional status of the subject is thought to play an important role in the increasing prevalence of these allergic diseases.

Recent reports suggest that endocrine disruptors (environmental hormones) affect not only the reproductive system but also the immune system [4-6]. Bisphenol A (BPA) is an environmental disruptor and a chemical widely used in the manufacture of polycarbonate plastics and epoxy resins for food packaging containers [7]. It is also commonly used in products like food cans, adhesives, and plastic dental sealants. It has been proposed that BPA, due to its estrogen-like activity, may possess an immunoregulatory role on the T-helper (Th) 1/Th2 balance.

Despite the accumulative data on the effect of environmental factors on immune responses, it is still

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Table 1. Composition of experimental diet

Constituent	Amount (g/kg of diet)
Casein	50
Carbohydrate[a]	820
Cellulose	20
Mineral mixture[b]	50
Vitamin mixture[c]	10
Corn oil	50
Total energy (kcal/kg)	3930

[a]Starch:sucrose, 2:1 ratio.

[b]Mixture (mg/kg diet): Calcium hydrogen orthophosphate dihydrate, 7280; potassium dihydrogen orthophosphate, 12,860; sodium dihydrogen orthophosphate, 4680; sodium chloride, 2330; calcium lactate, 17,550; ferrous citrate, 1590; magnesium sulfate, 3590; zinc carbonate, 55; $MnSO_4 \cdot 4\text{-}6H_2O$, 60; copper sulfate pentahydrate, 15; potassium iodide, 5.

[c]Composition (vitamin units or mg/kg diet): thiamine-hydrochloride, 12; riboflavin, 40; pyridoxine-hydrochloride, 8; vitamin B_{12}, 0.005; ascorbic acid, 300; D-biotin, 0.2; folic acid, 2; calcium pantothenate, 50; p-aminobenzoic acid, 50; niacin, 60; inositol, 60; choline chloride, 2000; retinol acetate, 5000; ergocalciferol, 1000 IU; tocopherol acetate, 50; menadione, 52.

unclear as to how and to what extent environmental factors are involved in the increased prevalence of allergic diseases, in particular food allergy. In developing countries, there is a lesser prevalence of the allergic diseases in comparison to that in the developed countries [8,9], suggesting a possible role of malnutrition.

We have previously shown that dietary protein intake affects induction of immune tolerance to ovalbumin (OVA) [10-12]. Also, we have further reported the effect of BPA on the cellular and humoral immune responses to OVA either with or without an induced oral tolerance to the antigen in animals maintained on a normal protein diet [13]. Nevertheless, there have been no reports on the immunomodulatory effects of BPA with concurrent protein deficiency. Thus, the current study was conducted to clarify its effect on immune responses to OVA in mice maintained on a low level of dietary protein.

Methods

Experimental design

Specific pathogen-free BALB/c mice aged 6 weeks (Japan SLC, Inc., Shizoka, Japan) were randomly divided into 2 groups, water-fed and OVA-fed (Fig. 1). The mice in each group were further subdivided into 2 groups, BPA-treated and non-treated. The animals were housed in a room with temperatures maintained at $22 \pm 2°C$ on a 12-h light/dark cycle and fed with an experimental diet containing 5% protein (Table 1) throughout. The Animal Research Ethics Committee at the University of Tokushima, Japan approved the study.

Immunization, induction of oral tolerance, and BPA administration

All groups of mice were immunized intraperitoneally with 100 μg of alum-precipitated OVA (5X crystallized; Seikagaku Corp., Tokyo, Japan) under sterile conditions on days 28 and 49, as previously described [10]. BPA (Sigma, St. Louis, MO, USA) was dissolved in corn oil to give a concentration of 200 mg/mL (Sigma, Japan). From day 41 onwards, each group of mice was injected intraperitoneally with BPA solution (0.1 mg/g of animal weight) 4 times every second day. Mice were given 5 mg of OVA (5X crystallized; Seikagaku Corp.) dissolved in 0.5 mL of sterile distilled water orally for 4 consecutive days after 3 weeks of starting the experimental diet, in order to induce oral tolerance.

Preparation of serum and cytokine production

One week after the last immunization, mice were sacrificed under anesthesia with Nembatul (Dainippon and Pharmaceutical Co. Ltd., Japan). Blood was collected and sera was separated by centrifugation and kept at –70°C until further use. Spleen cell suspension was prepared following the procedure described previously [9]. Briefly, spleens were removed and pressed by plunger of syringe in RPMI-1640 media (Sigma)
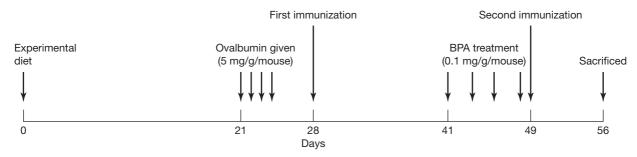

Fig. 1. Experimental design to examine the effects of bisphenol A (BPA) on immune function in mice fed with a low-protein diet.

containing antibiotic-antimycotic (Gibco-BRL Life Technologies, MD, USA). The splenocytes were washed by centrifugation in the media, followed by treatment with 0.83% ammonium chloride in Tris-hydrochloride (pH 7.4) at 37°C for 5 min to remove the red blood cells. Finally, a spleen cell suspension (5×10^6 cells/well) was prepared in RPMI-1640 medium containing antibiotic-antimycotic, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5×10^5 M 2-mercaptoethanol, and 10% fetal bovine serum and cultured with or without concanavalin A (Con A) under air containing 5% carbon dioxide. After 72 h, the supernatants of 3 cultures from each mouse were collected and pooled for enzyme-linked immunosorbent assay (ELISA) analysis of interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-12.

Total and OVA-specific IgE

A quantitative ELISA kit (Bethyl Laboratories Inc., TX, USA) was used to determine IgE by the direct ELISA method as described earlier [13]. Briefly, immunotitre plates were coated with affinity-purified monoclonal goat anti-mouse IgE diluted in a buffer containing 0.05 M carbonate-bicarbonate, pH 9.6. Then, serum samples were added and the microtitre plates were incubated for 90 min after treatment with a blocking solution (pH 8.0) containing 50 mM Tris, 0.14 M sodium chloride, 0.05% Tween 20 and 1% bovine serum. After washings with buffer solution, the microtitre plates were dispensed with goat anti-mouse IgE conjugated with horseradish peroxidase. Absorbance was measured at 450 nm in a microplate reader (Model 680; Bio-Rad Laboratories, Inc., USA) after addition of tetramethylbenzidine as substrate. For calibration, a serially diluted mouse IgE calibrator (Bethyl Laboratories, Inc.) was used.

The OVA-specific IgE was measured in a manner similar to that of total IgE measurement, except that the wells were coated with 200 μL (100 μg/mL) of OVA dissolved in 50 mM carbonate buffer, pH 9.6, and incubated overnight at 4°C.

OVA-specific immunoglobulin G, immunoglobulin G1 and immunoglobulin G2a

OVA-specific immunoglobulin G (IgG) and its subclasses were also measured using ELISA. Briefly, 96-well plates were coated with OVA as described above and incubated overnight at 4°C. They were then treated with the blocking solution. After 4 washings with washing buffer, the samples, diluted 1000-fold

for IgG and IgG1 and 50-fold for IgG2a, were added to the plates and incubated at room temperature for 90 min. Then, 2000-fold diluted goat anti-mouse IgG (H and L chain-specific), IgG1 (γ_1 chain-specific) or IgG2a ($\gamma_2$alpha chain-specific) conjugated to alkaline phosphatase (Southern Biotechnology Associates, Inc., UK) were used as capture antibodies and incubated. Finally, p-nitrophenyl phosphate was added and absorbance was finally measured at 415 nm after reaction was stopped using 150 mM ethylenediamine tetra-acetic acid.

Assessment of cytokines in culture supernatants

Levels of IFN-γ, IL-4, and IL-12 in the culture supernatants of splenocytes were measured following the instructions from manufacturers using a commercially available kit (eBioscience, CA, USA). In brief, microtiter plates (Nunc Maxisorp, NY, USA) were coated with anti-mouse IFN-γ, IL-4, or IL-12 rat monoclonal antibodies and incubated overnight at 4°C. The wells were washed 4 times with phosphate-buffered saline containing 0.05% Tween-20. After blocking the non-specific binding sites and 3 subsequent washings, the wells were incubated for 90 min at 4ºC with samples of culture supernatants. For standard curves, the plates were incubated in the same way with serially diluted recombinant mouse cytokines. The wells were washed again 5 times and incubated at room temperature for 90 min with anti-mouse IFN-γ, IL-4, or IL-12 capture antibodies. Finally, the plates were washed and incubated for 30 min with horseradish peroxidase-conjugated to Avidin. They were washed again 7 times with the washing buffer before addition of the substrate (eBioscience). The microtitre plates were measured for absorbance at 450 nm in an ELISA reader.

Statistical analysis

Data were analyzed with the Statistical Package for the Social Sciences for Windows (Version 11; SPSS Chicago, IL, USA). Statistical analysis was done by the Mann-Whitney U test. p Values <0.05 were considered statistically significant.

Results

Nutritional parameters

As shown in Table 2, feeding animals with the low-protein diets resulted in a negative body weight change in all groups at the end of the experimental period. Treatment with the compound led to a larger spleen size both in water-fed ($p<0.05$) and OVA-fed ($p<0.05$)

Table 2. Body and spleen weights of water-fed and ovalbumin (OVA)-fed mice with or without bisphenol A (BPA) treatment[a]

| | Animal group | | | |
| | Water-fed | | OVA-fed | |
	BPA (–) [n = 7]	BPA (+) [n = 6]	BPA (–) [n = 5]	BPA (+) [n = 6]
Initial body weight (g)	20.25 ± 1.31	20.71 ± 0.98	20.29 ± 0.75	20.68 ± 1.46
Final body weight (g)	16.92 ± 2.8	18.88 ± 1.69	17.39 ± 0.42	18.32 ± 2.42
Spleen weight (mg)	142 ± 47[b,c]	257 ± 62	173 ± 15[d]	336 ± 27

Abbreviations: (–) = animals not treated with BPA; (+) = animals treated with BPA
[a]Data were taken from 5-7 mice in each group and expressed as mean ± standard deviation.
[b]$p<0.05$ vs BPA (+) water-fed group.
[c]$p<0.01$ vs BPA (–) OVA-fed group.
[d]$p<0.01$ vs BPA (+) OVA-fed group.

animals. The group of OVA-fed animals not treated with BPA showed significantly higher spleen weights ($p<0.01$). In the BPA treated groups, OVA feeding did not result in a statistically significant difference in spleen weight ($p=0.065$).

Total IgE, OVA-specific IgE, IgG, IgG1, and IgG2a

As shown in Fig. 2, OVA feeding resulted in significant suppression of total and OVA-specific IgE in both BPA-treated and non-treated animals ($p<0.05$). In both water-fed and OVA-fed groups, BPA treatment resulted in slightly suppressed total and OVA-specific IgE, which show no statistical significance, except for total IgE among the OVA-fed animals.

When effects of treatment with BPA on OVA-specific IgG, IgG1, and IgG2a were examined, titers of IgG were found to be lower in OVA-fed animals compared to the corresponding water-fed animals with or without the treatment ($p<0.05$) [Fig. 3A]. However, neither the administration of BPA nor OVA feeding caused any statistically significant change in the levels of serum OVA-specific IgG1 in both the water-fed and OVA-tolerant animals (Fig. 3B). Although not statistically significant, the level of IgG2a was generally higher in BPA-treated water-fed animals than in the non-BPA-treated OVA-fed group.

Levels of IFN-γ, IL-4, and IL-12

Cytokines, such as IFN-γ, IL-4, and IL-12, are known to affect IgE antibody production in mice. All animal groups were stimulated with Con A and the culture supernatants of their splenocytes examined by ELISA for cytokines. As shown in Table 3, IL-4 production was suppressed after OVA feeding in animals not treated with BPA, although the difference was not statistically
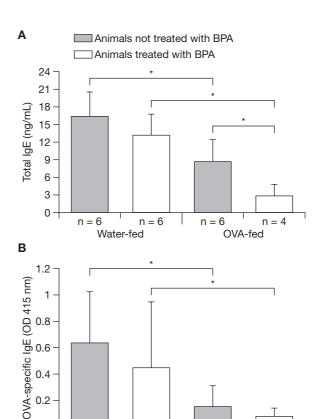

Fig. 2. Serum immunoglobulin E (IgE) response in water-fed and ovalbumin (OVA)-fed mice, with and without bisphenol A (BPA) treatment showing: (A) total IgE; and (B) OVA-specific IgE. Results are expressed as mean ± standard deviation (bars) calculated from data taken from 4-6 mice in each group. *$p<0.05$. OD = optical density.

significant ($p=0.073$). Also, significantly higher IL-4 production was observed in OVA-fed animals following BPA administration ($p<0.05$). On the other hand, IFN-γ production was found to be suppressed in non-BPA treated animals after OVA feeding ($p<0.05$), but not in

A

Legend:
- ▨ Animals not treated with BPA
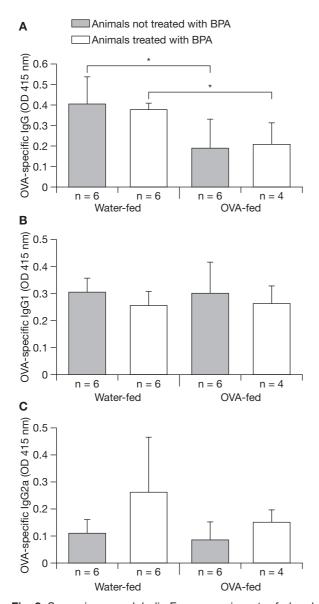- ☐ Animals treated with BPA

B

C

Fig. 3. Serum immunoglobulin E response in water-fed and ovalbumin (OVA)-fed mice, with and without bisphenol A (BPA) treatment showing: (A) OVA-specific immunoglobulin G (IgG); (B) IgG1; and (C) IgG2a. Results are expressed as mean ± standard deviation (bars) calculated from data taken from 4-6 mice in each group. *$p < 0.05$. OD = optical density.

those treated with the compound (Table 3). In addition, the production of IFN-γ was higher following BPA administration in both OVA-non-tolerant and tolerant animals, with the levels being statistically significant for the latter group ($p < 0.05$). Finally, there were higher titers of IL-12 following BPA treatment within both non-tolerant ($p < 0.01$) and tolerant mice ($p < 0.05$). Also, OVA feeding was followed by a significantly lower production of IL-12 in animals not treated with BPA ($p < 0.05$).

Discussion

Although there are many reports regarding the effects of BPA on endocrine systems, especially reproductive organs, less is known about its effect on the immune system. In an earlier study, we examined the effects of BPA exposure on the immune response in mice fed with a normal protein diet and challenged with OVA [13]. The current work was designed to look into the modulatory effects of BPA on the immune response in mice when a concurrent protein deficiency existed. To induce a state of protein insufficiency, animals were fed with a low protein (5%) casein-based diet (Table 1). This was to mimic the poor nutritional status seen in many parts of the world, where low protein intake remains one of the major public health problems.

BPA has been reported to reduce body weight or body weight gain, and to increase organ weights, such as liver, kidneys, adrenals, spleen, pituitary, and brain in weaning and adult animals [4]. The present study also found that administration of BPA resulted in an increase in the weight of spleen in all BPA-treated animals with or without an induced oral tolerance. However, we observed an increase in body weight following BPA administration, unlike previous reports which found that BPA administration resulted in reduced body weight and body weight gain [4]. This discrepancy in observations regarding weight gain might be due to the different strain of mice used. Recent reports have shown that mouse strains differ in their sensitivity to estrogen [7,14]; since BPA has an estrogen-like activity [4], this might account for the different weight gain tendencies observed. In addition, physical parameters of the animal may be affected by the dose and method of BPA treatment.

The dose of BPA used in the present study was extremely high as compared to the levels found in the environment [7]. However, as microgram amounts of BPA are detectable in liquids from canned foods [14], the level of human exposure to BPA remains unknown and may very well be significant.

OVA feeding in both BPA-treated and non-treated animals resulted in a substantial decrease in the production of total and OVA-specific IgEs. We have previously reported that BPA administration resulted in significant suppression of the antibody [13]. This may indicate that mice fed with low-protein diets had relatively higher levels of IgE antibody when compared to those fed with normal dietary protein content. Interestingly, in line with our previous report [15], OVA-specific IgE was

Table 3. Levels of interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-12 in water-fed and ovalbumin (OVA)-fed mice with or without bisphenol A (BPA) treatment[a]

Cytokine	Animal group			
	Water-fed		OVA-fed	
	BPA (–) [n = 7]	BPA (+) [n = 6]	BPA (–) [n = 5]	BPA (+) [n = 6]
IFN-γ (ng/mL)	101.78 ± 90.86[b]	161.42 ± 64.20	9.85 ± 17.01[e]	107.71 ± 91.33
IL-4 (pg/mL)	43.5 ± 32.02	60.26 ± 19.84	12.95 ± 7.89[f]	50.16 ± 31.34
IL-12 (pg/mL)	31.32 ± 17.65[c,d]	136.48 ± 94.16	9.14 ± 20.45[g]	99.50 ± 105.65

Abbreviations: (–) = animals not treated with BPA; (+) = animals treated with BPA

[a]Data were taken from 5-7 mice in each group and expressed as mean ± standard deviation.

[b,c]$p<0.05$ vs BPA (–) OVA-fed group.

[d]$p<0.05$ vs BPA (+) water-fed group.

[e,f,g]$p<0.05$ vs BPA (+) OVA-fed group.

not affected in tolerant groups with low protein diets. In the current study, OVA-fed animals had remarkably lower production of OVA-specific IgG, IgG1, and IgG2a. The suppressed production of antibodies in response to OVA after induction of tolerance was greater when compared to our previous report [13] using animals fed on a normal protein diet. This further supports our previous observations that mice fed with a low protein diet are more susceptible to the induction of oral tolerance [10-12]. In addition, it is known that IgG2a production is dependent on the Th1 immune response [16]. Augmented production of OVA-specific IgG2a, found in this study, appeared to be greater in non-tolerant animals than those fed with OVA. This may indicate the facilitatory effect of BPA on the Th1 immune response in water-fed groups and to a lesser extent in tolerant animals. Also, suppression of OVA-specific IgG after the induction of oral tolerance was comparable between BPA-treated and non-treated animals. Therefore, it is speculated that oral tolerance was not reversed following BPA treatment.

The particular mechanism by which BPA affects the immune system remains unclear. However, it is known that cells of the immune system respond to sex hormones, including estrogens [17]. Like many estrogens, BPA can activate the estrogen receptor alpha [18], although this effect is reported to be 26-fold weaker than that of estrogen [19]. It is has also been reported that a 100-fold lower dose of estradiol is sufficient for the augmentation of immune responses, in particular antibody production, when compared to BPA.

The cytokine microenvironment has several immunoregulatory roles, whi ch makes these molecules important modulators and effectors of the immune response. Production of IL-4, a proinflammatory cytokine closely associated with allergic immune responses, increased by 1.38- and 3.87-fold, respectively, following BPA exposure in water-fed and OVA-fed animals. However, OVA feeding did not lead to a lower production of IL-4 in BPA-treated animals as compared to those not treated with the compound. Besides, the production of IFN-γ, which is an early response to dietary antigen in intestinal tissues, also increased following BPA treatment by 1.58- and 10.93-fold, respectively, in water-fed and OVA-fed animals. These findings are in good agreement with previous findings that production of the Th1 cytokine IFN-γ [20], and the Th2 cytokine, IL-4 [21], was augmented on BPA exposure, but different from our previous reports [10,11]. This difference can be attributed to the different stimulants used for cytokine production. There was a generally lower production of the 2 cytokines in the current study as compared to our previous report, and this can be attributed to the low protein content in the experimental diet.

IL-12 is known to be a key cytokine, which directs naive T cells towards Th1 polarization. This role is partly attributed to its ability to induce IFN-γ production by natural killer and Th1 cells, which in turn promotes Th1 development. In line with our previous report [13], there was an increased production of IL-12 following BPA administration in both tolerant and non-tolerant animals.

While protein restriction is known to induce a state of immunodeficiency, the mechanisms responsible for this remain obscure. It is speculated that low levels of dietary protein selectively compromise several components of the cellular immune response, in particular related to the cytokine microenvironment, which are critical elements involved in the regulation of immune responses involved in the induction of oral tolerance. However, better understanding of the mechanisms

underlying the deficiencies in humoral and cell-mediated immunity warrants further studies.

In summary, mice were examined for the effect of BPA on the immune response to OVA when fed with low dietary proteins with or without an induced oral tolerance. The present results suggest that induced tolerance to OVA is maintained even following BPA treatment. Also, BPA administration increased Th1 cytokines more profoundly in tolerant than in non-tolerant animals. In conclusion, the present study suggests that protein insufficiency may affect the immunological effects of BPA, an environmental disrupter. However, further exhaustive studies that examine different doses, strains of animals, and environmental compounds and the effects of these in assorted combinations are warranted.

Acknowledgments

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