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Evaluation of the Immune Response Following Exposure of Mice to Bisphenol A: Induction of Th1 Cytokine and Prolactin by BPA Exposure in the Mouse Spleen Cells

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Bisphenol A [2, 2 bis (4-hydoxyphenyl) propane; BPA] is a widely used endocrine disruptors and has estrogenic activities. Although interests on biological effect of BPA are rising, evidences of its effect on immune system are lacking. We investigated that the effect of BPA on immune parameters to postulate the mechanism, and BPA interruptions between neuroendocrine and immune system. BPA was administrated to mice by p.o. (as a drinking water) dose on 0.015, 1.5 and 30 mg/ml for 4 weeks. The BPA treatment did not result in any change in body weight, spleen weight and distribution of lymphocyte subpopulation collected from spleen. BPA induced prolactin production in spleen, and exposure of BPA increased the activity of splenocyte proliferation in response to Con A ($p<0.001$). The production of a strong Th-1 type cytokine (IFN-γ) was induced while Th-2 type (IL-4) was suppressed by BPA treatment. These were consistent with RT-PCR results of transcription factor GATA-3 and IRF-1. These findings suggested that stimulation of prolactin production by estrogenic effects of BPA would affect cytokine profiles, and lead to imbalanced cellular immune response. In addition, we could speculate that prolactin and cytokine is important mediator involved in network between neuroendocrine and immune system by BPA.

Key words: Bisphenol A (BPA), Endocrine disruptor, Prolactin, Th1cytokine

INTRODUCTION

Environmental estrogens (xenoestrogens) are diverse group of chemicals that mimic estrogenic actions and capable of acting as endocrine disruptors which can potentially interfere with the production, release, transport, metabolism, binding action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Cook *et al.*, 1997; Goloubkova *et al.*, 1997; Kavolock *et al.*, 1996). These compounds may have adverse effects on human health and wildlife (Goloubkova *et al.*, 1997).

Bisphenol A [2, 2 bis (4-hydoxyphenyl) propane; BPA], a widely used endocrine disruptor, is a monomer of plastic and epoxy resins that is used in dentistry and food packaging industry (Goloubkova *et al.*, 2000; Steinmetz *et al.*, 1997; Hanioka *et al.*, 2000; Nikula *et al.*, 1999; Stoker *et al.*, 1999; Chun and Gorski, 2000; Takao *et al.*, 1999). It has been reported that BPA may leach from plastic products due to incomplete polymerization or breakdown of the polymer during heating or autoclaving (Takahashi *et al.*, 2001). BPA has been reported to have estrogenic actions such as uterotrophic effects, decreased sperm production, promotion of cell proliferation in a breast cancer cell line and stimulation of prolactin in both *in vitro* and *in vivo* (Stoker *et al.*, 1999; Chun *et al.*, 2000; Takao *et al.*, 1999; Dogusan *et al.*, 2001).

Most studies on endocrine disruptors including BPA have been focused on reproductive toxicology and carcinogenesis (Blake and Boockfor, 1997; Goloubkova *et al.*, 2000; Ladics *et al.*, 1998) with neglecting their potential impacts on the neuroendocrine-immune axis. Kavlock *et al.* indicat-

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ed that the effects on the nervous and immune systems might be underestimated because of the incomplete characterization of the biological effects of endocrine disruptors (Ladics *et al.*, 1998; Kavlock *et al.*, 1996). Many investigators have demonstrated that there is a highly complex interrelationship between the immune and neuroendocrine systems, and the activation of the hypothalamic pituitary adrenal (HPA) axis by cytokines allowed regulation of immune system by the neuroendocrine system through a long-loop feedback mechanism (Kavlock *et al.*, 1996; Matera *et al.*, 2000). Based on evidence of an increased autoimmunity associated with prenatal diethylstilbestrol (DES) exposure, and the structural resemblance between BPA and DES (Chun and Gorski; 2000; Takahashi *et al.*, 2001), we could postulate the possibility that BPA may induce similar pathologic states (Kavlock *et al.*, 1996). In addition, the incidences of allergy and asthma are increasing and alteration of sex-hormone balance may lead to increased or accelerated onset of autoimmune syndromes (Kavlock *et al.*, 1996), it could be speculated that endocrine disruptors perceived as a threatening signal to homeostasis might lead the alterations in immune system including neuroendocrine system. Endocrine disruptors can affect the production of other sex hormone, and most of them can stimulate the prolactin production especially (McMurrary, 2001). Prolactin, an endocrine hormone from the anterior pituitary, is also synthesized and secreted by immunocyte, which is a member of the cytokine superfamily, and transduces signals to the nucleus, facilitating both direct and indirect modulation of immune system (McMurrary, 2001; Matera *et al.*, 2000). In addition, it has been well known that prolactin induce the synthesis of the helper T type 1 (Th-1) cytokines, interferon-γ (IFN-γ), and interleukine-12 (IL-12), promote cellular immunity and the increased prolactin involve in autoimmunity. Since the immune and endocrine systems are linked via various cytokine signal processes and prolactin involve in autoimmunity, it seems that investigation of effect of BPA on cytokine and prolactin production is necessary to fully understand the action mechanism of BPA on the immune system. However most studies on endocrine disruptors including BPA have been focused on reproductive toxicology and carcinogenesis (Blake and Boockfor, 1997; Goloubkova *et al.*, 2000; Ladics *et al*, 1998) and effects of BPA on immune system are scare. Therefore, we hypothesized that prolactin would be a putative mediator in mechanism that BPA affects endocrine-immune axis and investigated whether BPA stimulates prolactin release in immune cells and causes the alteration on immune parameters including cytokine profile, lymphocyte proliferation and distribution of lymphocyte subpopulations to clarify the mechanism that BPA act on neuroendocrine-immune axis.

MATERIALS AND METHODS

Animals and bisphenol A treatment

Male ICR mice (3 weeks of age; purchased from Dae Han Biolink Co., Korea) were used. All animals were housed individually in a light (12h on/off) and temperature-controlled room with food and water available ad libitum. After a quarantine period of approximately 2 weeks, mice were weighted. BPA was purchased from Sigma Chemical Co. (St Louise, USA). BPA was finally dissolved 1% ethanol. Mice (8 per group) were administrated p.o. (as drinking water) for 4 weeks with water, 1% ethanol (vehicle control), 0.015 mg/ml, 1.5 mg/ml and 30 mg/ml of BPA. Body weight and organ weight were measured at 9 weeks of age when treatment of BPA for 4 weeks was over.

Immunoblot analysis for prolactin

Mouse spleen was homogenized in 3 volume of Lysis buffer (10 mM Tris-Cl (pH 7.5), 1 mM EDTA (pH 8.0), 0.5 % Triton X-100, 2 mM PMSF, 1 μg/ml aprotinin using homogenizer. The homogenates were centrifuged at 12,000 ×g for 30 min. Whole-cell protein extracts (50 μg) were resolved on 10% acrylamide gels by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membrane (NC; Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). NC membranes were blocked overnight at 4°C in 5% skim milk in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% Tween 20). The membranes were incubated with a goat polyclonal antibody to the prolactin C-17 (Santa Cruz Biotechonology, Inc., Califonia, USA) diluted with PBST at room temperature for 2h. After washing with PBST, blots were then incubated with 1:10,000 of horseradish peroxidase-linked anti-goat IgG (Santa Cruz Biotechonology, Inc., Califonia, USA) for 2 hr. Target protein was detected by peroxidase substrate kit (DAB kit; Vector Laboratories, Inc., Burlingame, CA).

Preparation of splenocyte

ICR mice were sacrificed and spleens were placed in sterile PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Single cell suspensions were prepared by pressing the tissue through a stainless steel mesh screen. Cells were then centrifuged through Ficoll (Histopague 1119, Sigma) to obtain spleen lymphocytes. Spleen lymphocytes were maintained in phenol red free RPMI-1640 (GIBCO BRL, Rockville, USA), supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Rockville, USA), 100 mg/ml streptomycin, and 100 U/ml penicillin and 0.25 μg of amphotericin.

Cell proliferation assay

Spleen lymphocytes were cultured at 4×10^5 cells/well in 96-well tissue plate culture (BD Falcon, Co., Bedford,

USA). Cells were stimulated with 2 μg/ml of Con A at 37 °C in 5% CO_2 incubator (NAPCO; Precision Scientific, Inc.). After 48 hr of stimulation, the cultures were pulsed for 4 hr with 0.3 mg/ml XTT [sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate] in combination with 1.25 mM PMS (N-methyl dibenzopyrazine methyl sulfate) and absorbance were measured at 450 nm using ELISA Reader (SPECTRA MAX250, Molecular Devices).

Flow cytometric analysis

Spleen cells were adjusted to 2×10^7 cells/ml and incubated with blocking buffer (10% NMS, 1 μg 2.4G2 Ab (anti-FcγR))to block nonspecific binding of conjugated Abs to FcR. Cells then were stained with conjugated Abs at 4 °C for 30 min.

Following staining, cells were washed twice with staining buffer. Stained cells were fixed in 1% paraformaldehyde and analyzed on a Becton Dickinson FACS flow cytometer.

RT-PCR

Total RNAs were extracted using Trizol reagent (Gibco BRL, Rockville, USA) in accordance with the manufacture's instructions. 5 μg of total RNA were reverse transcribed in a total volume of 50 μl containing 0.5 μg of oligo dT as the primer, 1× reaction buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 500 μM dNTP mixture and 50 U of superscript II reverse transcriptase (Gibco BRL, Rockville, USA) as following step; initial step at 42°C for 2 min, incubation at 42°C for 50 min and then at 70°C for 15 min. After RT, 1.5 U of RNase H (Gibco BRL, Rockville, USA) was added to the reaction tubes and the mixture was incubated at 37°C for 20 min to remove any residual RNA from the reaction product. 5 μl of the resultant cDNA were used for PCR amplication. The final PCR mixture contained 2.5 mM $MgCl_2$, 250 μM dNTP mixture, 5 unit of *Taq* DNA polymerase (Takara Co., Tokyo, Japan), 20 pmol of forward and reverse primers. Table I is showed the primer sequences used in PCR amplification. The thermal cycling parameters as follow: initial denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 60°C for 45 sec and extension at 72°C for 1 min. A final extension of 72°C for 10 min was performed. PCR products were visualized UV light after electrophoresis through 2% agarose gel containing 0.5 μg/ml EtBr. Analysis was performed using Image Analyzer. PCR using the primer of β-actin was performed on each individual sample as an internal standard.

Table I. Primer pair sequences used in RT-PCR

	Sequence (5 → 3)[a]
β-actin	TGGAATCCTGTGGCATCCATGAAAC TAAAACGCAGCTCAGTAACAGTCCG
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG GTCACAGTTTTCAGCTGTATAGGG
IL-4	CGAAGAACACCACAGAGAGTGAGCT GACTCATTCATGGTGCAGCTTATCG
IRF-1	AGGGCTTAGGAGGCAGAGTC AAAGGCCTAGACTGGGGAGA
GATA-3	CTTATCAAGCCCAAGCGAAG TAGAAGGGGTCGGAGGAACT

[a]For each primer pair, the forward sequence is shown above the reverse sequence

Data analysis

Data were analyzed by ANOVA followed by Dunkun, Tukey and Sheffes test using SPSS 10.0 software.

RESULTS

Bisphenol A stimulate prolactin production in spleen

The average body weights and spleen weights in water only, 1% ethanol and BPA treated groups are in Table II. Although mice administrated with 30 mg/ml BPA showed more or less decreased body and spleen weight compared with other groups, there was no significant difference statistically. We performed western blotting to estimate whether BPA induce prolactin production in spleen. In case of in the absence of BPA, prolactin was not detected while increased production of the prolactin was detected in BPA treated mice. Prolactins in spleen from 0.015 and 1.5 mg/ml of BPA treated groups were increased 1.98 and 1.42 times, respectively compared to untreated group and increase of prolactin synthesis more or less reduced in group treated with 30 mg/ml of BPA (Fig. 1). These results demonstrated that BPA in concentration of 0.015-1.5 mg/ml would acts like estrogen and stimulate the prolactin synthesis in spleen, which is the second immune organ whereas BPA of high dose such as 30 mg/ml sup-

Table II. Effect of 4 weeks oral administration of bisphenol A on body weight and spleen weight in male ICR mouse

Group		Body weight (g)	Spleen weight (g)	Spleen weight/ body weight ($\times 10^3$)
Untreated	water	38.33 ± 2.001	0.12 ± 0.010	3.13 ± 0.222
BPA treated (mg/ml)	0	37.98 ± 1.279	0.13 ± 0.039	3.64 ± 0.845
	0.015	36.30 ± 2.380	0.12 ± 0.012	3.24 ± 0.485
	1.5	37.44 ± 2.487	0.11 ± 0.013	2.97 ± 0.357
	30	33.98 ± 1.179	0.11 ± 0.022	3.11 ± 0.540

There were no significant differences statistically.
Body weights were measured at 9 weeks of age for the 4 weeks bisphenol A treatment group, respectively.

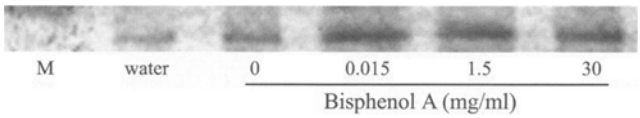

Fig. 1. Effect of bisphenol A on prolactin production in spleen. Male mice were exposed to water or various dose of BPA as indicated on the figure for 4 weeks. After treatment for 4 weeks, spleens were removed. Each lane contains equal amounts (50 μg) of total protein from spleen extract. The proteins were subjected to 10% SDS PAGE, transferred to NC membrane and incubated with a goat polyclonal antibody to the prolactin (1:200 dilution). After incubation with rabbit anti-goat secondary antibody (1:2000 dilution), the protein bends were detected using DAB kit.

press the production of prolactin by feedback inhibition rather than induce prolactin production.

Effect of bisphenol A on subpopulation and proliferation of splenocyte

In the present study, ICR mice were exposed to the BPA (0.015, 1.5 and 30 mg/ml) for 4 weeks and effects on immune parameters were analyzed. Using XTT assay, concanavalin-A (Con A; a stimulus for T-cell proliferation) induced proliferation were evaluated in all the groups. Splenocyte proliferation in response to Con A was significantly increased ($p < 0.001$) in mice exposed to BPA in comparison to non-treated mice (Fig. 2). Especially, 0.015 mg/ml of BPA treated group showed a significant increase compared with mice treated with high dose. There is no significant difference in proliferation between 1.5 mg/ml of BPA and 30 mg/ml of BPA treated case. This indicated that concentration above 1.5 mg/ml is not effective in inducing cell proliferation. This result indicated that splenocytes from mice exposed to BPA for 4 weeks obtained the enhanced proliferation activity in response to Con A and 0.015 mg/ml of BPA was the most effective. This was consistent with the result on prolactin induction and demonstrated that there is a relationship between prolactin induced by BPA treatment and acquirement of proliferative capacity in splenocyte. To determine whether the increase

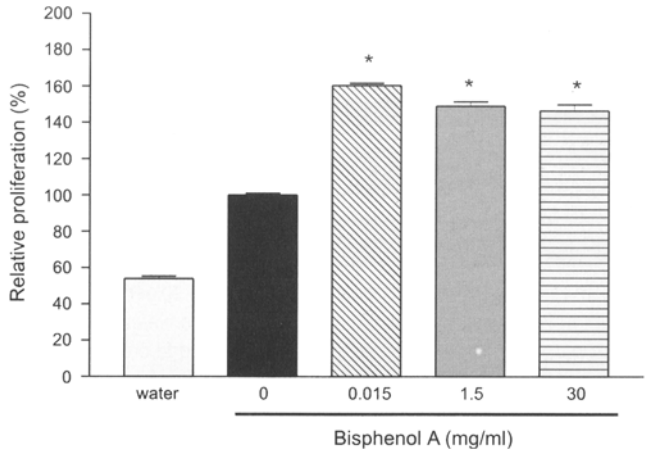

Fig. 2. Effect of bisphenol A on proliferation of splenocyte in response to Con A. Spleen lymphocytes (4×10^5) from mice treated with water or various dose of BPA as indicated on the graph for 4 weeks were cultured for 48 hr with Con A (2 μg/ml). After 48 hr stimulation, the cultures pulsed for 4 hrs with 0.3 mg/ml XTT in combination with 1.25 mM PMS for proliferation XTT assay. * $P < 0.001$.

on splenocyte proliferation shown Fig. 2 was due to a shift in the overall percentage of T cells, B cells and macrophage, these populations were analyzed using fluorescence-activated cell sorting (FACS) analysis. As demonstrated in Table III, there were no significant differences in the distribution of lymphocyte subpopulations concerning the various treatment groups. These results indicate that the alteration on splenocyte proliferation was not due to changes in the absolute numbers of splenocytes or ratios of splenocyte subpopulation.

Bisphenol A cause the alteration on cytokine profile

To investigate whether BPA causes the change on Th cell subsets and cytokine production, RT-PCR was used to quantify mRNA for the cytokines IL-4 and IFN-γ (Fig. 4). IFN-γ is associated with a Th-1 cytokine response whereas IL-4 is typically associated with the activation of Th-2 cyto-

Table III. Effect of 4 weeks oral administration of bisphenol A on relative proportions of various cell type (%) in splenocytes

Subpopulation \ Group		Cell proportion (%)				
		Untreated	BPA treated (mg/ml)			
		water	0	0.015	1.5	30
T cell	helper T ($CD3^+CD4^+$)	42.12 ± 2.66	37.20 ± 4.67	38.14 ± 3.92	37.46 ± 1.72	37.46 ± 2.57
	Cytotoxic T ($CD3^+CD8^+$)	5.38 ± 2.02	7.02 ± 2.59	5.93 ± 0.33	5.25 ± 1.10	5.20 ± 1.37
B cell, macrophage ($CD3^-CD45^+$)		39.18 ± 5.04	56.8 ± 12.21	47.63 ± 3.47	47.17 ± 6.70	53.35 ± 9.06
NK cell ($CD3^-NK1.1^+$)		7.41 ± 2.21	6.88 ± 0.75	5.17 ± 2.31	4.88 ± 0.50	5.48 ± 2.67

There were no significant differences statistically ($p > 0.05$).
Flow cytometry data are mean percent ± SD from three to four separate experiment.

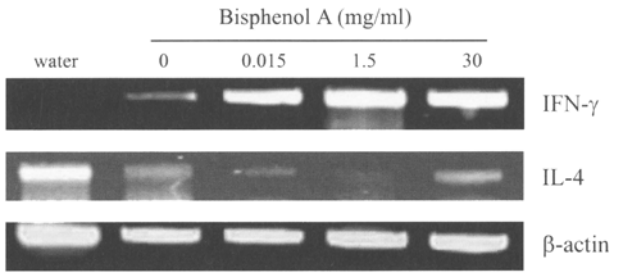

Fig. 3. Effect of bisphenol A on cytokine profile in spleen. Bisphenol A treatment for 4 weeks showed the Th1 cytokine dominant profile. Total RNAs were extracted from spleens of mice exposed to drinking water in the presence and absence of bisphenol A as described in Materials and Methods and subjected to RT-PCR. Expected sizes of products are 243, 180 and 349 bp for IFN-γ, IL-4 and β-actin, respectively.

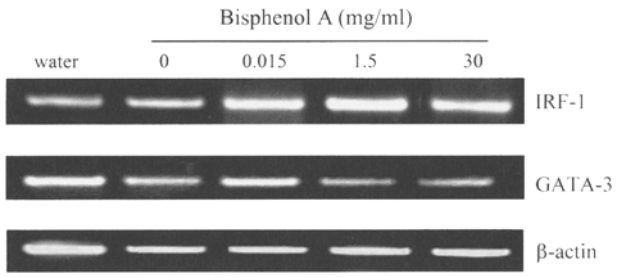

Fig. 4. Effect of bisphenol A on expression of transcription factor. Bisphenol A treatment for 4 weeks increased the mRNA expression of IRF-1, Th1 cytokine-related transcription factor. Total RNAs were extracted from spleens of mice exposed to drinking water in the presence and absence of bisphenol A as described in Materials and Methods and subjected to RT-PCR. Expected sizes of products are 500, 505 and 349 bp for IRF-1, GATA-3 and β-actin, respectively.

kine response. In the non-treated group, IFN-γ transcripts were below the limit of detection while they were present in high amounts in the BPA treated groups. From this data, we found a manifest enhancement of IFN-γ. IL-4 transcripts were present about 5 times lower amounts than IFN-γ transcripts. IL-4 transcripts were present in below detectable level among all BPA treated groups. Based on RT-PCR results for cytokine mRNA, BPA appear to be associated with dominant Th-1 cytokine response, especially IFN-γ-mediated. In Fig. 4, RT-PCR results for IRF-1 and GATA-3, which are transcription factors associated Th-1 and Th-2, respectively were shown. Transcripts of IRF-1 were increased while mRNA expression of GATA-3 was inhibited among all BPA-treated groups. This indicated that BPA induce predominantly production of Th-1 cytokines by action of transcription factors, and then may induce cellular immune response.

DISCUSSION

Recently, it has been reported that there is an interdependence of the neural, endocrine and immune systems in health and disease (Ladics *et al.*, 1998; Sakabe *et al.*, 1999). The presence of sexual dimorphisms in autoimmune diseases suggests the participation of gonadal steroid in the neuroendocrine immune crosstalk (Matera *et al.*, 2000). The high occurrence of autoimmune disease in female implicated that certain hormonal level in female affects immune system (Takahashi *et al.*, 2001). The mediators involved in hormonal production, metabolism and transport may influence the immune system. As endocrine disruptors can interfere with the production, release, transport, metabolism, binding or elimination of endogenous hormones responsible for the maintenance of homeostasis (Kavlock *et al.*, 1996), they may induce certain pathology in all fields, where hormone can act. Especially, they mimic estrogenic activity and may have adverse effects on human health. BPA is an endocrine disruptor (Goloubkova *et al.*, 2000; Kavlock *et al.*, 1996). In human, absorption of BPA through the skin has been shown to produce extensive damage to the kidney, liver, spleen, pancreas and lungs (Takahashi *et al.*, 2001) Previous reports demonstrated that maternal exposure to BPA in rodents permanently increased the size of prepubertal testis, reduced the size of epididymis, reduced sperm count and inhibited testicular function (Takao *et al.*, 1999). In recent, it has been reported that BPA have mutagenecity in RSa cells (Takahashi *et al.*, 2001).

Although some investigators have been studied that BPA can suppress the production of thymic hormone such as thymosin α-1, one of the amplicators of T-cell immunity and alter macrophage adhesion, evidences that reveal the effect of BPA on immune system, involved mechanism and key mediators is too little. Therefore, we investigated how BPA affect on immune system and which mediators involve in action of BPA. BPA have week estrogenic activity (Postel-Vinay *et al.*, 1997; Takeuchi and Tsutsumi, 2002) as well as mimicked estradiol in inducing prolactin gene expression, release, cell proliferation, and ERE activation (Steinmetz *et al.*, 1997). In general, estrogen regulates the other hormone by feedback action. Estrogen suppress FSH while stimulate the prolactin production (McMurrary, 2001). Prolactin, one of the anterior pituitary hormones, has been reported to activate cellular proliferation in non-reproductive tissue such as liver, spleen, and thymus and implicate in the development and maintenance of immune system (Feng *et al.*,1998). Prolactin is locally secreted by immune cell. In a view, BPA induce an increase of prolactin level as well as prolactin associate with abnormal immune response, we examined whether BPA cause the alteration of prolactin secretion in spleen. As shown in Fig. 1, prolactins in spleen from 0.015 and 1.5 mg/ml of BPA treated groups were increased 1.98 and 1.42 times, respectively compared to untreated group and increase of prolactin synthesis more or less reduced in group treated

with 30 mg/ml of BPA. These results demonstrated that BPA in concentration of 0.015-1.5 mg/ml would acts like estrogen and stimulate the prolactin synthesis in spleen whereas BPA of high dose such as 30 mg/ml would suppress the production of prolactin by feedback inhibition. In other *in vivo* studies using rats, BPA administrated s.c. (1 mg/rat/day) for 2 weeks increased dramatically the prolactin concentration in plasma. In vitro treatment with 10 nM BPA induces cell growth and prolactin secretion in PR1 cells, prolactin secreting cell line derived from pituitary lactotroph tumor (Chun and Gorski, 2000). There are several reports that decreased circulating prolactin by bromocriptine treatment result in significant immunosuppression in rodents (Nikula *et al.*, 1999; Masten and Shiverick, 1997; Lormee *et al.*, 2000). Vice versa, it can be suggested that increased prolactin result in hyperimmunostimulation. To investigate if BPA induce immunostimulation, we examined the effect of BPA on splenocyte proliferation, distribution of the lymphocytes and production of cytokine. At first, splenocyte proliferation in response to Con A (a stimulus for T cell proliferation) was tested. As a result, splenocyte proliferation in response to Con A was significantly increased ($p < 0.001$) in mice exposed to BPA in comparison to non-treated mice (Fig. 2). Especially, 0.015 mg/ml of BPA treated group showed a significant increase in regard to treated mice with high dose. This result indicated that splenocytes from mice exposed to BPA for 4 weeks obtained the enhanced proliferative activity in response to Con A and 0.015 mg/ml of BPA was the most effective. This is consistent with the data on prolactin induction (Fig. 1) and it is suggested that there is a relationship between prolactin induced by BPA treatment and acquirement of proliferative capacity in splenocyte. To determine whether the increase on splenocyte proliferation was due to a shift in the overall percentage of T cells, B cells and macrophage, these populations were analyzed using fluorescence-activated cell sorting (FACS) analysis. As demonstrated in Table III, there were no significant differences in the distribution of lymphocyte subpopulations concerning the various treatment groups. From these results, we could confirm that the alteration on splenocyte proliferation was not due to changes in the absolute numbers of splenocytes or ratios of splenocyte subpopulation but rather other factors related to proliferation signaling. Considering reports that the proliferation of lymphocytes in secondary lymphoid organs such as spleen, lymph nodes and mucosal lymphoid tissue depends on prolactin and growth hormone (Feng *et al.*,1998), it is possible to demonstrate that stimulation of prolactin production by BPA treatment is coupled with proliferative signals. Previous investigations have demonstrated that prolactin is a autocrine growth factor for the T cell (Matera *et al.*, 1997) and stimulate lymphocyte proliferation (Dogusan *et al.*, 2001). It has been shown that anti-prolactin antibody treatment inhibits the lymphocyte proliferation (Lormee *et al.,* 2000) and endogenous prolactin induces the proliferation response of peripheral blood T lymphocytes to the mitogen PHA, Con A (Gala and Rillema, 1995; Matera *et al.*, 2000). Also, prolactin has been shown to increase DNA synthesis and c-myc expression (Molefeysot *et al.*, 1998; Feng *et al.*, 1998). Furthermore, it has been known that prolactin activate many other signaling molecules, associated with mitogen activated protein kinase (MAPK) and cell cycle relating factors (Feng *et al.*, 1998; Dogusan *et al.*, 2001) and may regulate T cell proliferation by enhancing the expression of some genes such as IRF-1, cyclin B and histone H3 necessary to entry into S-phase. As represented in Fig. 5, prolactin binding to prolactin receptor leads to dimerization of the receptor and activation of receptor-associated protein kinase Janus tyrosine kinase (JAK)-2, which in turn phosphorylation the prolactin receptor and the signal transducers and transcription factor (STAT) (Romagnani, 1997; Imada and Leonard, 2000; Gala and Shevach, 1993). Activated STATs translocate to the nucleus and bind to the target gene such as β-casein and IRF-1 (Imada and Leonard, 2000; Gala and Shevach, 1993; Clevenger *et al.*,1990; Gunes and Mastro, 1996; Matera *et al.*, 1997; Masten and Shiverick, 1997). IRF-1 has been well known to play a key role in promoting inflammation and autoimmunity. Activation of IRF-1 stimulates the synthesis and secretion of cytokine (Yamagata *et al.*, 2000). Immune responses characterized by the production of distinct sets of cytokines are optimally protective against different types of pathogens. Helper T cells are divided into two types based on the set of cytokine they produce, i.e. IFN-γ-producing Th-1 cells and IL-4-producing Th-2 cells (Romagnani, 1997; McMurrary, 2001; Matera *et al.*, 2000). Responses dominated by the production of IFN-γ provide cellular immune response. On the other hand, responses dominated by the production of IL-4 protect against infections by extracellular pathogens by induction of humoral immunity (McMurrary, 2001). Fig. 3 showed that IFN-gamma transcripts were markedly activated in BPA-exposed groups. In addition, IL-4 transcripts were present in below detectable level among all BPA treated groups. To verify the differential regulation of Th-1/Th-2 cytokine at the transcription level, we examined the expression level of cytokine-mediated transcription factors such as GATA-3 and IRF-1 (Fig. 4). As a result, mRNA expression of IRF-1 was increased while mRNA level of GATA-3 was inhibited among all BPA-treated groups. GATA-3 regulates IL-4 transcription and plays a role in suppressing Th-1 development by inhibition IFN-γ production. Thus, inhibition of GATA-3 is able to cause the shift on cytokine profile from Th-2 to Th-1 type. IFN-γ production is directly related to a transcription factor IRF-1 induction and IRF-1 is a target gene for prolactin (MaMurrary, 2001; Matera *et al.*,

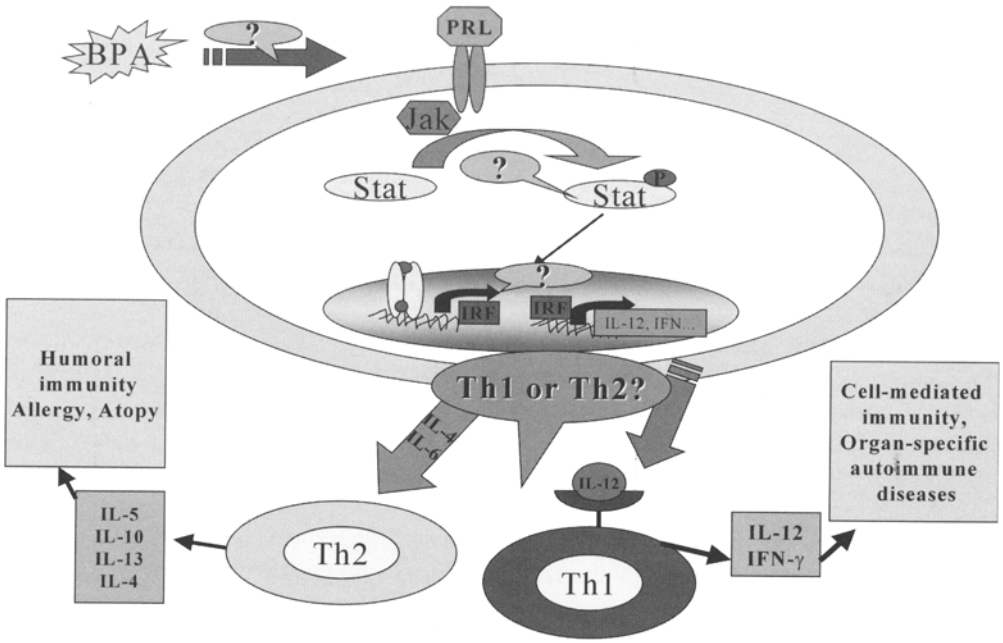

Fig. 5. Putative scheme on action of bisphenol A in immune system. Bisphenol A acts like estrogen and stimulate prolactin production in spleen as well as anterior pituitary. Increase of prolactin, which acts as an autocrine or paracrine factor, may modulate cytokine synthesis by signal transduction via surface receptor of lymphocyte. Alteration on cytokine profile may affect the immune response. Th1-dominant cytokine profile may promote cellular immunity while Th2-dominant cytokines induce humoral immunity.

2000; Mukherjee *et al.*, 1990; Dogusan *et al.*, 2001). In this study, induction of IRF-1 mRNA was accompanied by the increase of IFN-γ. This indicate that prolactin by BPA treatment is able to induce IRF-1 expression and thus to act as a modulator of signal transduction by cytokines in splenocyte. This inhibition of GATA-3 activity and enhancement of IRF-1 indicate that BPA inhibit Th-2 and enhance Th-1 response.

In summary, we investigated that BPA treatment for 4 weeks in male ICR mice induce prolactin production in spleen, proliferation of splenocyte and shift on cytokine profile from Th-2 to Th-1 type. From these results, we could establish a hypothesis on the putative mechanism, which BPA affect on neuroendocrinimmuno axis as represented in Fig. 5. Estrogenic activity of BPA makes target organ including spleen and anterior pituitary induce the prolactin production. Secreted prolactin may act in paracrine or autocrine fashion in both central and peripheral lymphoid organs. Prolactin acts via prolactin receptor, which is a member of cytokine superfamily. Prolactin stimulates the phosphorylation of the prolactin receptor-associated JAK-2 in spleen cells. This leads to the activation and subsequent binding of STATs to an IRF-1. Activation of IRF-1 allows cell to respond to soluble mediators, called cytokines. And then secreted cytokines act in autocrine or paracrine manner and induce shift from Th 2 cell to Th1 cell and lead to heperstimulated cellular immunity. Although pathological phenomenon related immune disease were not found in mice treated with BPA for 4 weeks, imbalanced Th-1 dominant immune responses may result in autoimmune disease.

As we suggested this hypothesis according to the nature of prolactin and effect of BPA, further studies should be performed to confirm the mechanism. We think that our suggestion, prolactin stimulation by BPA will mediate the imbalanced immune response, will be proved if effect of BPA on immune parameters in the absence of prolactin or prolactin receptor is investigated.

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