Effects of bisphenol A on antigen-specific antibody production, proliferative responses of lymphoid cells, and TH1 and TH2 immune responses in mice

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1 We investigated the effect of bisphenol A (BPA), which binds estrogen receptors, on immune responses including production of antigen-specific antibodies, proliferative responses of lymphoid cells, and Th1 and Th2 responses.

2 For this investigation, mice were p.o. given varying doses including 3, 30, 300, and 3000 $\mu\text{g kg}^{-1}$ of BPA immediately after immunization with hen egg lysozyme (HEL) (day 0) and then daily by day 20. On day 21, anti-HEL IgG antibodies in sera and proliferative responses of spleen cells to the antigen were measured. Anti-HEL IgG2a antibodies and IFN- γ secreted from splenic lymphocytes were also measured as indicators of Th1 immune responses, while anti-HEL IgG1 antibodies and IL-4, as those of Th2 responses.

3 The results showed that treatment with 3000 $\mu\text{g kg}^{-1}$ of BPA was followed by a significant increase in anti-HEL IgG as well as the antigen-specific cell proliferation. Anti-HEL IgG2a production and IFN- γ secretion were significantly enhanced in mice treated with 300 and 30 $\mu\text{g kg}^{-1}$ of BPA, respectively, while anti-HEL IgG1 production and IL-4 secretion were augmented in animals given 3000 and 300 $\mu\text{g kg}^{-1}$ of the chemical, respectively.

4 Augmentation of these immune responses was also observed in mice exposed to 0.3–30 $\mu\text{g kg}^{-1}$ of estradiol, although Th1 responses appeared to be more sensitive to the sex hormone than Th2 responses.

5 These results suggest that BPA may play a role in augmenting immune responses, especially Th1 responses.

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Abbreviations: BPA, bisphenol A; HEL, Hen egg lysozyme

Introduction

Bisphenol A (BPA) is an intermediate in the production of polycarbonate and epoxy resins, flame retardants, and other specialty products. There are a number of final products including adhesives, building materials, optical lenses, and plastic dental sealants. BPA is moderately soluble (120–300 mg l^{-1} at pH 7) and appears to be released into the environment through its use (Staples *et al.*, 1998). Gould *et al.* (1998) demonstrated that BPA was potent in activating the estrogen receptor alpha but 26-fold less compared to estrogen, suggesting that BPA is not only an estrogen mimic but may also exhibit a mechanism of action similar to that of the sex hormone at the receptor. Therefore, considerable attention has focused on this environmental estrogen-like chemical that may affect reproductive organs. In fact, it was recently

demonstrated that exposure of mice to BPA significantly increased their prostate weight (Nagel *et al.*, 1997) and decreased sperm production (vom Saal *et al.*, 1998).

Estrogen has also been shown to play an important role in the immune system. For instance, previous studies showed that estrogen had stimulatory effects on humoral immune responses but suppressive effects on cellular responses (Abin *et al.*, 1974; Seaman *et al.*, 1978; Paavonen *et al.*, 1981; Holdstock *et al.*, 1982). More recent studies demonstrated that estrogen increased secretion of IFN- γ from splenic lymphocytes which played a major role in regulating the function of all key immune cells (Karpuzoglu-Sahin *et al.*, 2001a, b). Therefore, it is of interest to study whether BPA can influence the immune system like estrogen, although there is a report demonstrating *in vitro* that the xenoestrogen decreased substrate adherence capacity of antigen-presenting cells including macrophages (Segura *et al.*, 1999). The environmental estrogen also increases nonspecific proliferation of

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spleen cells to the mitogen concanavalin A *in vitro* (Jontell *et al.*, 1995). However, few studies showed a role for BPA in immune homeostasis *in vivo*. Therefore, we tested the hypothesis that the exposure of mice to BPA could affect antigen-specific antibody production as well as proliferative responses of lymphoid cells. In addition, we examined the effect of BPA on Th1 immune responses which play a role in cellular responses including delayed-type hypersensitivity (Fong & Mosmann, 1989; Mosmann & Coffman, 1989) and on Th2 immune responses involved in humoral responses including IgE antibody production (Snapper & Paul, 1987; Diaz-Sanchez *et al.*, 1997).

Here we show that exposure to BPA augmented antigen-specific IgG antibody production as well as proliferative responses of splenic lymphocytes in mice, although a relatively high dose of the xenoestrogen was required for the augmentation of these immune responses. Both Th1 and Th2 immune responses were also enhanced following exposure to lower doses of the environmental estrogen-like chemical, although Th1 responses appeared to be more sensitive to the chemical than Th2 responses.

Methods

Animals

Male and female DBA/1J mice, 8–9 weeks of age, were used in the experiments. The mice were bred in the animal breeding unit of Saga Medical School, Saga, Japan. They were maintained in a temperature- and light-controlled environment with free access to standard rodent chow and water.

Immunization with hen egg lysozyme (HEL)

Mice were immunized by intraperitoneal injection (i.p.) of 100 µg of HEL (Sigma Chemical Co., St Louis, MO, U.S.A.) dissolved in 100 µl of 0.9% NaCl.

Administration of BPA

Varying doses (3, 30, 300, and 3000 µg kg⁻¹) of BPA (Sigma) were dissolved in ethanol and then in corn oil (Sigma). The final concentration of ethanol in corn oil was 0.5%. A half milliliter of BPA-containing corn oil was p.o. administered immediately after immunization with HEL (day 0) and then daily by day 20. As a control, 0.5 ml of corn oil containing 0.5% ethanol alone was given. In some experiments, 0.03, 0.3, 3, and 30 µg kg⁻¹ of 17-beta estradiol (Sigma) dissolved in 0.5 ml of corn oil containing 0.5% ethanol was p.o. administered from days 0 to 20.

Measurement of HEL-specific antibodies

Blood was collected on day 21 and sera were heat inactivated at 56°C for 30 min. IgG, IgG1, and IgG2a antibodies specific for HEL were measured using an ELISA (Yoshino & Ohsawa, 1997). In brief, 96-well flat-bottomed microtiter plates were incubated with 100 µl well⁻¹ of HEL (100 µg ml⁻¹) at 37°C for 1 h and washed three times with PBS. The wells were then blocked by incubation with 100 µl of PBS containing 1% ovalbumin (Sigma) at 37°C for 1 h. After washing, the plates

were incubated with 100 µl of a 1:200, 1:1000, or 1:5000 dilution of each serum sample at 37°C for 30 min. The plates were washed, and 100 µl well⁻¹ of a 1:1,000 dilution of rat antimouse IgG, IgG1, or IgG2a labeled with alkaline phosphatase (PharMingen, San Diego, CA, U.S.A.) was added and incubated at 37°C for 1 h. After washing, 100 µl of 3 mM of *p*-nitrophenylphosphate (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was added per well and the plates were incubated in the dark at room temperature for 15 min. Absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at OD₄₀₅ ± s.e.m.

Proliferation assay

Spleens or inguinal lymph nodes were removed on day 21 and cell suspensions prepared (Yoshino, 1998). Erythrocytes in the cells were lysed with tris-NH₄Cl. A total of 5 × 10⁶ cells ml⁻¹ in 100 µl of RPMI 1640 (Flow Laboratories, Inc., McLean, VA, U.S.A.) containing 1 mM glutamine, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 5 × 10⁻⁵ M 2-mercaptoethanol, and 1% heat-inactivated autologous mouse serum were added to each microwell followed by the addition of 100 µg ml⁻¹ of HEL. The cells were cultured for 72 h. Each well was pulsed with 0.5 µCi of tritiated thymidine, and the cells were cultured for another 16 h. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques.

Cytokine measurement

Spleen or inguinal lymph node cell suspensions were prepared as described above. One milliliter of the above medium containing a total of 5 × 10⁶ cells was cultured in 24-well tissue culture plates with 100 µg ml⁻¹ of HEL. After 48 h, supernatants were harvested and stored at -70°C until assayed. Secretion of IFN-γ and IL-4 was quantified using sandwich ELISA techniques (Yoshino, 1998). The ELISA kits for these cytokines were commercially available from Funakoshi Co., Tokyo, Japan.

Statistics

To analyze data statistically, the Mann–Whitney *U*-test was used as a nonparametric statistical method because sample sizes in our experiments were small; therefore, the normality obtained was poor.

Results

Effect of BPA on production of anti-HEL IgG antibodies

To examine the effect of BPA on production of antibodies to HEL, 3, 30, 300, and 3000 µg kg⁻¹ of BPA were p.o. daily administered over a period of 3 weeks from the time of immunization with HEL (day 0) and levels of anti-HEL IgG antibodies in sera were determined on day 21. The results are shown in Table 1. No changes of levels of the antigen-specific IgG were observed in mice given 3, 30, and 300 µg kg⁻¹ of BPA. Animals given 3000 µg kg⁻¹ of BPA had 58 and 50% more anti-HEL IgG compared to controls (*P* < 0.05) when

their sera were 1/200- and 1/1000-fold diluted, respectively. HEL-specific IgG antibodies in 1/5000-fold diluted sera were not measurable because the sera were diluted out.

Effect of BPA on proliferative responses of spleen cells to HEL

When proliferative responses of spleen cells to HEL in mice exposed to BPA were measured, significant enhancement (45%) of the lymphoid cell proliferation was observed in the

Table 1 Effect of BPA on anti-HEL IgG antibody production

BPA ($\mu\text{g kg}^{-1}$)	Anti-HEL IgG (A_{405}) Serum dilution		
	1/200	1/1000	1/5000
0	0.31 $\pm$ 0.02	0.26 $\pm$ 0.02	0.11 $\pm$ 0.02
3	0.34 $\pm$ 0.03	0.26 $\pm$ 0.02	0.08 $\pm$ 0.01
30	0.32 $\pm$ 0.03	0.27 $\pm$ 0.03	0.12 $\pm$ 0.02
300	0.37 $\pm$ 0.04	0.30 $\pm$ 0.03	0.12 $\pm$ 0.02
3000	0.49 $\pm$ 0.03*	0.39 $\pm$ 0.03*	0.15 $\pm$ 0.02

Male mice were orally given the indicated doses of BPA dissolved in 0.5 ml of corn oil immediately after i.p. injection of HEL (day 0) and then daily by day 20. Anti-HEL IgG antibodies were measured on day 21 as described in Materials and methods. Values are the mean $\pm$ s.e.m. of five mice. * $P < 0.05$ versus control (0 $\mu\text{g kg}^{-1}$ BPA) (Mann–Whitney *U*-test).

Table 2 Effect of BPA on proliferative responses of spleen cells to HEL

BPA ($\mu\text{g kg}^{-1}$)	Proliferation (c.p.m)
0	25,462 $\pm$ 2035
3	22,915 $\pm$ 2638
30	24,392 $\pm$ 3067
300	29,494 $\pm$ 2573
3000	36,940 $\pm$ 3282*

Male mice were orally given the indicated doses of BPA dissolved in 0.5 ml of corn oil immediately after i.p. injection of HEL (day 0) and then daily by day 20. Proliferative responses of spleen cells to HEL were measured on day 21 as described in Materials and methods. Background counts of spleen cells cultured without HEL were between 400 and 700 c.p.m. Values are the mean $\pm$ s.e.m. of five mice. * $P < 0.05$ versus control (0 $\mu\text{g kg}^{-1}$ BPA) (Mann–Whitney *U*-test).

Table 3 Effect of BPA on anti-HEL IgG2a and IgG1 antibody production

BPA ($\mu\text{g kg}^{-1}$)	Anti-HEL (A_{405}) Serum dilution					
	1/200		1/1000		1/5000	
	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1
0	0.30 $\pm$ 0.03	0.30 $\pm$ 0.03	0.22 $\pm$ 0.02	0.32 $\pm$ 0.02	0.14 $\pm$ 0.02	0.08 $\pm$ 0.01
3	0.34 $\pm$ 0.02	0.29 $\pm$ 0.03	0.21 $\pm$ 0.03	0.34 $\pm$ 0.03	0.10 $\pm$ 0.02	0.06 $\pm$ 0.01
30	0.32 $\pm$ 0.04	0.35 $\pm$ 0.04	0.26 $\pm$ 0.02	0.30 $\pm$ 0.03	0.12 $\pm$ 0.01	0.11 $\pm$ 0.02
300	0.41 $\pm$ 0.03*	0.37 $\pm$ 0.04	0.32 $\pm$ 0.02*	0.33 $\pm$ 0.02	0.13 $\pm$ 0.02	0.08 $\pm$ 0.01
3000	0.63 $\pm$ 0.04*	0.44 $\pm$ 0.04*	0.41 $\pm$ 0.03*	0.41 $\pm$ 0.02*	0.16 $\pm$ 0.02	0.12 $\pm$ 0.02

See footnote of Table 1.

animals given 3000 but not 3, 30, or 300 $\mu\text{g kg}^{-1}$ of the environmental estrogen-like chemical (Table 2).

Effect of BPA on production of anti-HEL IgG2a and IgG1 antibodies

The effects of BPA on anti-HEL IgG2a and IgG1 antibody production, which are respectively Th1 and Th2 cell-dependent (Burnstein & Abbas, 1993; Isakson *et al.*, 1982), were examined. Table 3 shows the results. Treatment with 300 $\mu\text{g kg}^{-1}$ of BPA was followed by a significant increase in anti-HEL IgG2a. Anti-HEL IgG1 production was also augmented by BPA but by the highest dose used (3000 $\mu\text{g kg}^{-1}$).

Kinetics of proliferation of draining lymph node cells and cytokine secretion in BPA-treated mice

Kinetics of proliferative responses of inguinal lymph node cells as well as secretion of Th1 and Th2 cytokines by the draining lymphoid cells from BPA-treated mice were investigated. For this investigation, mice were daily treated with BPA from days 0–6, 20, or 48 and the immune responses were determined on days 7, 21, or 49. As shown in Table 4, no significant changes of lymphoid cell proliferation as well as IFN- γ and IL-4 secretion were observed in mice treated from days 0–6. Greater cell proliferation up to 47 and 42%, was seen in animals given 3000 $\mu\text{g kg}^{-1}$ of BPA for 21 and 49 days, respectively. On day 21, a significant increase (46%) in IFN- γ was seen in mice exposed to the dose 30 $\mu\text{g kg}^{-1}$ of BPA. Treatment with 3000 $\mu\text{g kg}^{-1}$ of BPA for 21 and 49 days resulted in more pronounced stimulation of IFN- γ secretion up to 149 and 102%, respectively. Mild but significant stimulation of IL-4 secretion up to 29 and 35%, was seen in mice treated with 3000 $\mu\text{g kg}^{-1}$ of BPA for 21 and 49 days, respectively.

Effects of BPA on cell proliferation and Th1 and Th2 immune responses in male and female mice

The effects of BPA on HEL-specific proliferation of splenocytes as well as Th1 and Th2 responses in male and female mice were studied. Both sexes exposed to BPA showed increases in all the immune responses examined (Table 5). The increases in rates of cell proliferation, IgG2a and IgG1 production, and IFN- γ and IL-4 secretion in 3000 $\mu\text{g kg}^{-1}$ BPA-treated female mice were 63, 83 and 53, and 211 and 73%, respectively, while those in the same-dose-treated male animals were 67, 92 and 48, and 243 and 56%, respectively.

Thus, there were no differences in these responses between males and females.

Effects of estradiol on cell proliferation and Th1 and Th2 immune responses

Since BPA can bind estrogen receptors and may have estrogen-like activities (Gould *et al.*, 1998), the effects of varying doses of estradiol itself on proliferative responses of splenocytes and Th1 and Th2 immune responses were investigated. Table 6 shows the results. Administration of 30 but not 0.03–3 $\mu\text{g kg}^{-1}$ of estradiol resulted in a significant

increase (53%) in proliferation of the lymphoid cells. Th1 responses including anti-HEL IgG2a production and IFN- γ secretion were significantly increased in mice given 3 and 0.3 $\mu\text{g kg}^{-1}$ of estradiol, respectively, while Th2 responses such as anti-HEL IgG1 and IL-4 production augmented in those administered with 30 $\mu\text{g kg}^{-1}$. Treatment with 30 $\mu\text{g kg}^{-1}$ of estradiol was followed by increases in anti-HEL IgG2a (91%), IFN- γ (201%), anti-HEL IgG1 (36%), and IL-4 (74%).

Discussion and conclusions

The present study demonstrates that BPA has the ability to modulate the immune system since treatment of mice with BPA augmented anti-HEL IgG antibody production, proliferative responses of splenocytes to the antigen, and Th1 and Th2 responses. There are a number of studies reporting that BPA is biologically active. For instance, treatment of rats with BPA suppresses P450-dependent mono-oxygenase activities in their liver microsomes (Hanioka *et al.*, 1998). BPA lowers serum levels of cholesterol and stimulates proliferation of human breast cancer cells (Dodge *et al.*, 1996). However, there have been few studies demonstrating the *in vivo* effect of BPA on antigen-specific responses including antibody production, although *in vitro* studies showed that BPA modulated substrate adherence capacity of antigen-presenting cells including macrophages (Segura *et al.*, 1999) and increased nonspecific proliferation of spleen cells to the mitogen concanavalin A (Jontell *et al.*, 1995).

Exposure to BPA was also associated with increases in both anti-HEL IgG2a and IgG1, although the increase in anti-HEL IgG2a appeared to be greater than that in anti-HEL IgG1. Since IgG2a and IgG1 production are dependent on Th1 and Th2 cells (Burnstein & Abbas, 1993), respectively, the increases in anti-HEL IgG2a and IgG1 in BPA-treated mice suggest that this chemical facilitates Th1 and, to a lesser extent, Th2 responses. This also appears to be supported by the result that secretion of the Th1 cytokine IFN- γ (Diamantstein *et al.*, 1988) and the Th2 cytokine IL-4 (Mu & Sewell, 1994) were augmented by BPA up to 191 and 61%, respectively.

There was also significant stimulation by BPA of proliferative responses of inguinal lymph node cells as well as

Table 4 Kinetics of the change of inguinal lymph node cell proliferation and Th1 and Th2 cytokine secretion in BPA-treated mice

Day	BPA ($\mu\text{g kg}^{-1}$)	Proliferation (c.p.m)	IFN- γ	IL-4
7	0	26,296 $\pm$ 2905	1737 $\pm$ 183	449 $\pm$ 57
	3	22,482 $\pm$ 1738	1672 $\pm$ 165	537 $\pm$ 53
	30	25,378 $\pm$ 2257	1966 $\pm$ 214	504 $\pm$ 62
	300	28,393 $\pm$ 3029	1820 $\pm$ 242	495 $\pm$ 49
	3000	31,927 $\pm$ 3417	2054 $\pm$ 235	513 $\pm$ 55
21	0	18,592 $\pm$ 1745	946 $\pm$ 58	482 $\pm$ 41
	3	20,497 $\pm$ 2466	906 $\pm$ 84	521 $\pm$ 36
	30	22,930 $\pm$ 1934	1385 $\pm$ 68*	488 $\pm$ 30
	300	24,028 $\pm$ 3450	1677 $\pm$ 142*	547 $\pm$ 51
	3000	27,368 $\pm$ 2254*	2353 $\pm$ 204*	622 $\pm$ 46*
49	0	12,582 $\pm$ 1094	635 $\pm$ 58	419 $\pm$ 27
	3	15,586 $\pm$ 2189	593 $\pm$ 64	372 $\pm$ 32
	30	13,883 $\pm$ 846	768 $\pm$ 70	440 $\pm$ 35
	300	14,771 $\pm$ 1603	1056 $\pm$ 103*	478 $\pm$ 42
	3000	17,810 $\pm$ 1352*	1280 $\pm$ 146*	565 $\pm$ 40*

Male mice were orally given the indicated doses of BPA dissolved in 0.5 ml of corn oil immediately after i.p. injection of HEL (day 0) and then daily over a period of either 6, 20, or 48 days. On day 7, 21, or 49, proliferation of inguinal lymph node cells, and secretion of IFN- γ and IL-4 from the draining lymph node cells were determined as described in Materials and methods. Values are the mean $\pm$ s.e.m. of five mice. * $P < 0.05$ versus control (0 $\mu\text{g kg}^{-1}$ BPA) (Mann–Whitney *U*-test).

Table 5 Effects of BPA on cell proliferation and Th1 and Th2 responses in male and female mice

Sex	BPA ($\mu\text{g kg}^{-1}$)	Proliferation (c.p.m)	Anti-HEL antibody (A_{405})		Cytokine (pg ml^{-1})	
			IgG2a	IgG1	IFN- γ	IL-4
Male	0	18,370 $\pm$ 2274	0.38 $\pm$ 0.03	0.27 $\pm$ 0.03	493 $\pm$ 47	304 $\pm$ 33
	3	16,938 $\pm$ 1839	0.35 $\pm$ 0.04	0.32 $\pm$ 0.04	558 $\pm$ 60	273 $\pm$ 25
	30	19,528 $\pm$ 1650	0.40 $\pm$ 0.03	0.25 $\pm$ 0.05	742 $\pm$ 82*	256 $\pm$ 30
	300	21,027 $\pm$ 2572	0.54 $\pm$ 0.04*	0.34 $\pm$ 0.04	1184 $\pm$ 77*	445 $\pm$ 36*
	3000	30,662 $\pm$ 2883*	0.73 $\pm$ 0.05*	0.40 $\pm$ 0.04*	1691 $\pm$ 146*	473 $\pm$ 41*
Female	0	22,856 $\pm$ 2125	0.52 $\pm$ 0.04	0.45 $\pm$ 0.04	793 $\pm$ 65	539 $\pm$ 47
	3	26,370 $\pm$ 2273	0.47 $\pm$ 0.05	0.47 $\pm$ 0.05	727 $\pm$ 83	530 $\pm$ 32
	30	21,602 $\pm$ 1859	0.45 $\pm$ 0.03	0.40 $\pm$ 0.05	930 $\pm$ 72	476 $\pm$ 58
	300	30,284 $\pm$ 2355*	0.74 $\pm$ 0.05*	0.51 $\pm$ 0.04	1552 $\pm$ 79*	752 $\pm$ 48*
	3000	37,292 $\pm$ 2044*	0.95 $\pm$ 0.07*	0.69 $\pm$ 0.05*	2468 $\pm$ 84*	935 $\pm$ 54*

Male and female mice were orally given the indicated doses of BPA dissolved in 0.5 ml of corn oil immediately after i.p. injection of HEL (day 0) and then daily by day 20. On day 21, proliferation of spleen cells, levels of anti-HEL IgG2a and IgG1 antibodies in sera diluted 1 to 1000, and secretion of IFN- γ and IL-4 were determined as described in Materials and methods. Values are the mean $\pm$ s.e.m. of five mice. * $P < 0.05$ versus control (0 $\mu\text{g kg}^{-1}$ BPA) (Mann–Whitney *U*-test).

Table 6 Effect of estradiol on Th1 and Th2 immune responses

Estradiol ($\mu\text{g kg}^{-1}$)	Proliferation (<i>c.p.m</i>)	Anti-HEL antibody (A_{405})		Cytokine (pg ml^{-1})	
		IgG2a	IgG1	IFN- γ	IL-4
0	16,804 $\pm$ 1485	0.34 $\pm$ 0.03	0.39 $\pm$ 0.04	814 $\pm$ 58	264 $\pm$ 27
0.03	19,572 $\pm$ 2164	0.33 $\pm$ 0.04	0.43 $\pm$ 0.03	906 $\pm$ 84	305 $\pm$ 32
0.3	14,920 $\pm$ 1732	0.39 $\pm$ 0.03	0.47 $\pm$ 0.05	1085 $\pm$ 68*	288 $\pm$ 35
3	18,469 $\pm$ 1566	0.56 $\pm$ 0.03*	0.48 $\pm$ 0.04	1877 $\pm$ 142*	331 $\pm$ 42
30	25,638 $\pm$ 2273*	0.65 $\pm$ 0.04*	0.53 $\pm$ 0.04*	2453 $\pm$ 204*	459 $\pm$ 44*

Male mice were orally given the indicated doses of estradiol dissolved in 0.5 ml of corn oil immediately after i.p. injection of HEL (day 0) and then daily by day 20. On day 21, proliferation of spleen cells, production of anti-HEL IgG2a and IgG1 antibodies, and secretion of IFN- γ and IL-4 were determined as described in Materials and methods. Values are the mean $\pm$ s.e.m. of five mice. * P < 0.05 *versus* control (0 $\mu\text{g kg}^{-1}$ estradiol) (Mann-Whitney *U*-test).

secretion of IFN- γ and IL-4 by the lymphoid cells in animals given the chemical for 21 and 49 but not 7 days, suggesting that T cells distributed to draining lymph nodes have sensitivity to BPA similar to those from spleens and that persistent exposure to BPA appears to be required before the chemical exerts its effect on the immune responses.

The precise mechanism by which exposure to BPA resulted in augmentation of HEL-specific IgG, IgG2a, and IgG1 antibody production, lymphoid cell proliferation, and secretion of IFN- γ as well as IL-4 is unknown at present. However, because BPA is potent in activating the estrogen receptor alpha, although the activation of the receptor by the environmental chemical is 26-fold less potent than that by estrogen (Gould *et al.*, 1998), the stimulatory effects of BPA on the immune responses shown above may be at least in part explained by its estrogen-like action. For instance, treatment with estradiol was also followed by enhancement of lymphoid cell proliferation and both Th1 and Th2 responses. Furthermore, it is worth noting that only 100-fold smaller doses of estradiol were required for the augmentation of such immune responses compared to those of BPA. This result may also suggest that the estrogen receptor activation by the man-made chemical is less potent than that by the sex hormone, as reported by Gould *et al.* (1998).

Estrogen was shown to have immunomodulatory effects, particularly with respect to humoral immunity, and immunosuppressive effects, particularly with respect to cellular immunity (Abin *et al.*, 1974; Seaman *et al.*, 1978; Paavonen *et al.*, 1981; Holdstock *et al.*, 1982). However, in these studies, mechanisms of action of the sex hormone on these immune responses were not clearly demonstrated, especially in terms of the role of cytokines. Recently, Karpuzoglu-Sahin *et al.* (2001a & 2001b) reported *in vitro* that estrogen increased secretion of IFN- γ from concanavalin-A activated thymocytes and splenic lymphocytes from mice, while it had no effect on secretion of IL-4, indicating that estrogen appeared to upregulate Th1 but not Th2 responses. They also showed that estrogen increased the expression of costimulatory CD80 molecules on B cells. Thus, their results were similar to ours in terms of its effect on Th1 cytokine secretion but different in terms of its effect on Th2 cytokine secretion. This discrepancy appears to be because of the difference in stimulation of splenic lymphocytes between the two experiments used. We stimulated spleen cells with the antigen HEL, while Karpuzoglu-Sahin *et al.* used the mitogen concanavalin A nonspecifically. In addition, we used spleen

cells from mice exposed to estradiol *in vivo*, while they treated the cells with the sex hormone *in vitro*.

Female mice, which should have naturally higher levels of estrogen compared to male animals, treated with BPA had also augmented immune responses similar to those in male animals given the xenoestrogen, indicating that estrogen produced naturally in females exerts no additional effects on the immune responses examined. This result suggests that regulation mechanisms of action of natural estrogen on the immune system may exist in the sex in order to prevent excessive responses that may lead to immune disorders such as autoimmune diseases.

BPA is an essential component of epoxy resins used in the lacquer lining of metal food cans and in dental sealants and has been detected in food and water (Staples *et al.*, 1998). It was previously shown that adult people (weight 50 kg) daily ingested approximately 0.15 mg (3 $\mu\text{g kg}^{-1}$ body weight) of BPA, on average, from food and water. It is also shown that nearly 1 mg (20 $\mu\text{g kg}^{-1}$ body weight) of BPA was swallowed during the first hour after application of a plastic dental sealant (Brotons *et al.*, 1995; Olea *et al.*, 1996). In the present study, mice given daily 3000 but not 300, 30, or 3 $\mu\text{g kg}^{-1}$ of BPA for 21 days had significantly higher levels of anti-HEL IgG and IgG1 antibodies and proliferative responses of lymphoid cells to the antigen compared to control animals, showing that the dosage of BPA modulating these immune responses was 1000-fold higher than that of BPA ingested daily by an average human. Anti-HEL IgG2a production as well as IL-4 secretion was greater in mice given 300 $\mu\text{g kg}^{-1}$ of BPA, which was still 100-fold higher than the dosage of this chemical fed by humans. On the other hand, significantly enhanced IFN- γ secretion was seen in animals treated with the relatively low dose 30 $\mu\text{g kg}^{-1}$ of BPA, which was only 10-fold higher than the dosage ingested daily by humans and close to that of BPA swallowed following a plastic dental sealant application. In addition, mice used in our studies had BPA only for 3 weeks, while humans ingest for a longer period. Thus, it appears to be possible that at least a part of the immune system, especially IFN- γ -mediated immune responses, in humans may be modulated by continuous exposure to the environmental estrogen-like chemical.

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