Effect of Bisphenol A on Murine Immune Function: Modulation of Interferon-γ, IgG2a, and Disease Symptoms in NZB × NZW F_1 Mice

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To investigate the effects of the estrogen receptor-binding molecule bisphenol A (BPA) on murine immune function *in vivo*, we fed a low dose of 2.5 μg BPA/kg body weight/day to both normal C57BL/6 and lupus-prone NZB × NZW F_1 (NZB/NZW) 5-week-old mice for 1 week. Analysis of concanavalin A (ConA)-stimulated splenic mononuclear cells by ELISA demonstrated that BPA-fed C57BL/6 males produced, on average, 40% less interferon-γ (IFN-γ; $p < 0.01$) and C57BL/6 females 28% less IFN-γ ($p < 0.05$) compared with controls. Treated female NZB/NZW mice were monitored for lupus disease symptoms, defined as proteinuria (> 100 mg/dL albumin in urine for 2 consecutive weeks). Before the development of proteinuria, BPA-fed NZB/NZW mice produced significantly less ConA-stimulated IFN-γ than did controls and displayed an average reduction of 50% in immunoglobulin G2a (IgG2a) antibody production from lipopolysaccharide (LPS)-stimulated splenocytes ($p < 0.05$). It is striking that 5-week-old female NZB/NZW mice fed a 7-day low-dose course of BPA developed proteinuria an average of 7 weeks later than did controls. Once proteinuria developed, splenocytes were stimulated with ConA for cytokine analysis. The BPA-fed mice showed a dramatic reduction of 64% in IFN-γ production and a 32% reduction in ConA-stimulated interleukin-10 ($p < 0.05$). The long-lasting effects of BPA on IFN-γ and IgG2a production likely contributed to the increased symptom-free period of the NZB/NZW mice. *Key words:* bisphenol A, C57BL/6, estrogen receptor, IgG2a, interferon-γ, lupus, NZB × NZW. *Environ Health Perspect* 111:1883–1887 (2003). doi:10.1289/ehp.6359 available via *http://dx.doi.org/* [Online 29 August 2003]

Cells of the immune system respond to sex hormones, including estrogen (McMurray 2001). Upon binding of estrogen to the estrogen receptor (ER), the ligand–receptor complex dimerizes and mediates the transcriptional activity of genes containing estrogen response elements (EREs) and/or AP-1 sites. Recent studies detected ER-α in murine splenic B and T cells by both fluorescent activated cell sorting analysis and reverse-transcriptase polymerase chain reaction (Sakazaki et al. 2002). Additionally, Grimaldi et al. (2002) identified the more recently described ER-β in murine splenic B cells. It remains unclear whether ER-β is expressed in murine splenic T cells. Estrogen has been shown to alter cytokine production and T-cell subset distribution (Ahmed et al. 1985; Correale et al. 1998). Both interferon-γ (IFN-γ) and interleukin-10 (IL-10) are modulated by estrogen (Kanda and Tamaki 1999; Karpuzoglu-Sahin et al. 2001). The IFN-γ promoter contains ERE-like sequences, and estrogen reportedly upregulates IFN-γ production (Fox et al. 1991). In addition to modulating EREs, estrogen may modulate the expression of genes through AP-1 sites, sites found in many cytokine gene promoters, including IFN-γ (Kushner et al. 2000). Both ER subtypes interact with Fos and Jun elements at AP-1 sites, but with opposite outcomes (Paech et al. 1997).

Estrogen provides a possible link between cytokines and the autoimmune disorder systemic lupus erythematosus (SLE), because both IFN-γ and IL-10 have been implicated in lupus (Csiszar et al. 2000; Gonzalez-Amaro et al. 1998). Lupus occurs at a ratio of > 8:1 in females compared with males and commonly strikes in women during the childbearing years when circulating estrogen levels are highest (Kotzin 1996). SLE is characterized by high levels of IgG autoantibodies, including anti–double-stranded DNA (anti-dsDNA) autoantibodies. Female NZB × NZW F_1 (NZB/NZW) mice spontaneously develop a disease comparable with human lupus, displaying glomerulonephritis, arthritis, and elevated levels of IgG autoantibodies, in particular the complement-activating IgG2a class (Zeng et al. 2000). The reduced average life span of female NZB/NZW mice of 40 weeks compared with 64 weeks in males implicates female sex hormones in the disease process (Walker et al. 1996). In mice, IgG2a antibodies contribute to the glomerulonephritis observed in female NZB/NZW mice, and isotype switching to IgG2a is IFN-γ dependent (Haas et al. 1997). Several studies indicate a role for IFN-γ in murine lupus: IFN-γ treatment leads to a worsening of disease in NZB/NZW mice, and glomerulonephritis is ameliorated both in IFN-γ receptor knockout mice and in NZB/NZW mice treated with soluble IFN-γ receptor (Haas et al. 1998; Hasegawa et al. 2002; Ozmen et al. 1995).

Bisphenol A (BPA) is produced by the acid-catalyzed reaction of acetone and phenol and is widely used in the manufacture of polycarbonate plastics and epoxy resins found in containers used to package food (Ben-Jonathan and Steinmetz 1998). Although structurally different from estrogen, BPA binds to both ER subtypes and with 6-fold greater affinity to ER-β (Bolger et al. 1998; Pennie et al. 1998). Luciferase reporter assays indicate that BPA may either mimic or antagonize the effects of estrogen, depending on both the cell type and the ER isotype bound (Kurosawa et al. 2002). *In vitro* BPA exposure stimulates breast cancer cell lines to proliferate, increases progesterone receptor expression in a uterine cell line, and induces c-*fos* gene expression (Bergeron et al. 1999; Schafer et al. 1999; Steinmetz et al. 1998). Several *in vivo* studies document reproductive effects of low-dose BPA exposure. Microgram quantities of BPA were shown to be estrogenic in F344 rats by increasing prolactin expression (Steinmetz et al. 1997). Male offspring of BPA-treated pregnant mice showed increased prostate size and decreased epididymal weight (Gupta 2000; Nagel et al. 1997). A review of these studies led the Endocrine Disruptors Low-Dose Peer Review Panel to cautiously confirm that exposure to very low doses of BPA can cause biologic effects (Kaiser 2000).

Few reports have appeared concerning BPA and immune function. *In vitro*, BPA has been shown to inhibit lymphocyte mitogenesis (Sakazaki et al. 2002), MCP-1 production (Inadera et al. 2000), and macrophage adhesion (Segura et al. 1999). Our study differs in that we investigated BPA modulation of immune function *in vivo*. We were interested in observing the effects of oral exposure to BPA in normal and lupus-prone mice. As recently reported, mouse strains differ in their sensitivity to estrogen (Spearow et al. 1999). For our studies, we initially used the estrogen-sensitive, nonautoimmune C57BL/6 strain. To determine the effects of BPA in a lupus model, we fed BPA to 5-week-old, female NZB/NZW mice and analyzed IFN-γ and

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IL-10 release, IgG2a antibody production, and the development of proteinuria. IFN-γ produced from stimulated cells was reduced in both C57BL/6 and NZB/NZW mice treated with BPA. In addition, BPA modulated IgG2a production and delayed the onset of lupus symptoms in NZB/NZW females.

Materials and Methods

Mice. We obtained 3- to 4-week-old C57BL/6 mice from Taconic (Germantown, NY). Initially, we purchased 3- to 4-week-old female NZB × NZW F_1 (NZB/NZW) mice from Jackson Laboratory (Bar Harbor, ME). Because of production difficulties and unavailability of female NZB/NZW mice at Jackson Labs, we also purchased 3- to 4-week-old female NZB/NZW mice from Harlan (Indianapolis, IN). All mice were housed according to guidelines of the Institutional Animal Care and Use Committee.

For the *in vivo* studies, mice were housed individually. At 5 weeks of age, either C57BL/6 or NZB/NZW mice were weighed daily and fed 2.5 μg BPA/kg body weight (bw)/day (Sigma, St. Louis, MO) diluted in phosphate-buffered saline (PBS), in a cereal treat, for 7 days. Control mice of the same age were weighed and fed PBS in a cereal treat for 7 days. Mice were observed to consume the entire dose daily. C57BL/6 mice were sacrificed by CO_2 overdose within 2–4 days of the last BPA dose, and the spleens were aseptically removed.

After *in vivo* exposure to BPA at 5 weeks of age, female NZB/NZW mice were monitored for lupus disease symptoms by measuring protein levels in urine using Albustix (Fisher Scientific, Pittsburgh, PA). Proteinuria was defined as > 100 mg/dL of protein in urine for 2 consecutive weeks (Zeng et al. 2000). Mice testing positive for proteinuria were immediately sacrificed, and splenic mononuclear cells were isolated. To assess the effects of BPA exposure in mice before the onset of proteinuria, NZB/NZW mice with < 100 mg/dL of protein in urine were sacrificed between the ages of 8 and 19 weeks.

Preparation of mononuclear cells. Mononuclear cells were isolated from spleens using aseptic technique by grinding through a mesh sieve followed by density centrifugation on Lympholyte (Accurate Chemical & Scientific Corp., Westbury, NY). Cells were counted by either trypan blue exclusion using a hemacytometer or, in some cases, using ViaCount stain (Guava Technologies, Hayward, CA) containing the intact cell–impermeant nucleic acid dye 7-AAD (Schmid et al. 1992). The viability of the mononuclear cells was typically > 95%.

Cell culture conditions. For *in vitro* exposure to BPA, splenic mononuclear cells were resuspended in complete media: RPMI-1640 supplemented with 10% fetal calf serum (Sigma), 5×10^{-5} M 2-mercaptoethanol, 0.1 mg/mL streptomycin, 100 U penicillin, and 2 mM L-glutamine (Sigma). Cells were stimulated in 24-well Costar plates at a concentration of 4×10^6 cells/mL with a final concentration of 4.0 μg/mL concanavalin A (ConA; Sigma). BPA was added at final concentrations ranging from 50 μM to 0.5 nM. Cells were incubated in a humidified chamber with 5% CO_2 at 37°C for 24 hr. Supernatants were harvested and analyzed by ELISA for IFN-γ.

After *in vivo* exposure to BPA, isolated splenic mononuclear cells were resuspended in complete media at a concentration of 4×10^6 cells/mL and stimulated with either a final concentration of 4.0 μg/mL ConA or heat-killed *Staphylococcus epidermidis*. An overnight culture of *S. epidermidis* (optical density, 0.9 at 570 nm) was incubated at 60°C for 1 hr and added to cells at a final dilution of 1:20. Cells were incubated in a humidified chamber with 5% CO_2 at 37°C. After 24 hr, supernatants were harvested and assayed for IFN-γ production by ELISA. Optimal IL-10 levels were detected in ConA-stimulated supernatants by ELISA after 72 hr incubation.

Flow cytometric analysis of subsets. To determine the percentage of B cells and T-cell subsets, mononuclear splenic cells were stained immediately upon isolation. Briefly, cells were incubated on ice with FcBlock (PharMingen, San Jose, CA) and then stained with either phycoerythrin-conjugated anti-CD4 or CD8 monoclonal antibodies (all purchased from PharMingen and used at a final concentration of 2 μg/mL), anti-CD19 biotinylated monoclonal antibody followed by streptavidin-PE (Guava Technologies), or isotype-matched control antibodies (PharMingen). Live cells were gated using 7-AAD dye exclusion (Guava Technologies) and analyzed using a Guava personal flow cytometer and CytoAnalysis software (Guava Technologies).

Cytokine analysis by ELISA. Culture supernatants were assayed for cytokine production by ELISA following the manufacturer's recommended protocol (Pierce-Endogen, Rockford, IL). In brief, 50 μL recombinant IFN-γ standards or 24-hr culture supernatants were added to each precoated well in duplicate. Plates were incubated for 2 hr at room temperature followed by the addition of biotinylated anti-IFN-γ antibody. After a 1-hr room-temperature incubation, the plate was washed, and streptavidin-horseradish peroxidase (HRP) solution was added to each well. The plate was incubated for 30 min and again washed. TMB (tetramethylbenzidine) substrate solution (Pierce-Endogen) was added, and the plates were developed in the dark for 30 min. Stop solution containing sulfuric acid was added, and absorbance was measured at 450 nm on an EL312e plate reader (Bio-Tek Instruments, Winooski, VT). Results were calculated from the standard curve using Excel (Microsoft Corporation, Redmond, WA).

To determine IL-10, we added 72-hr supernatants or recombinant IL-10 standards to each anti-mouse IL-10 precoated well in duplicate, which were incubated at room temperature for 3 hr. After a wash step, biotinylated anti-IL-10 antibody was added to each well and allowed to incubate for 1 hr at room temperature. The remainder of the procedure is identical to that outlined for IFN-γ.

IgG2a antibody production by ELISA. Splenic mononuclear cells were isolated from

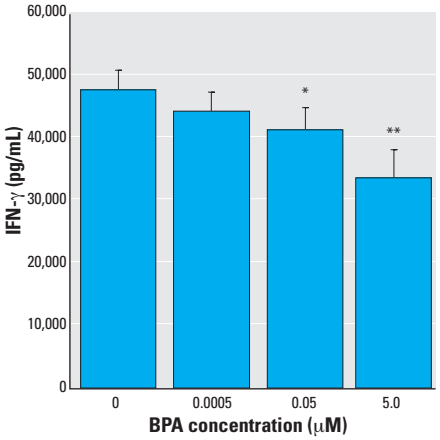

Figure 1. Inhibition of ConA-induced secretion of IFN-γ *in vitro* by BPA in a dose-dependent manner. Splenic mononuclear cells from 6-week-old C57BL/6 mice were incubated with 4.0 μg/mL ConA and BPA. IFN-γ in 24-hr culture supernatants was detected by ELISA. The data represent the mean ± SEM of seven mice.
*$p < 0.05$. **$p < 0.01$.

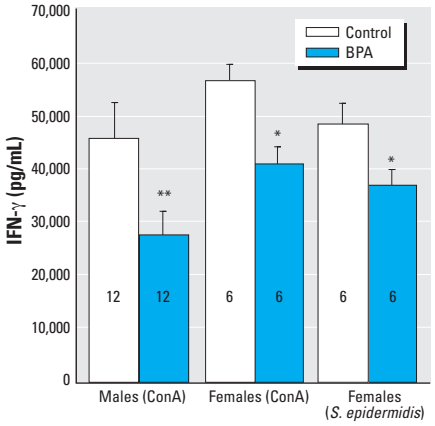

Figure 2. Decreased IFN-γ concentration in 6-week-old C57BL/6 mice after an oral dose of 2.5 μg BPA/kg bw/day for 7 days. Twenty-four-hour supernatants from splenic mononuclear cells isolated 2–4 days after the last BPA dose were stimulated with either 4.0 μg/mL ConA or heat-killed *S. epidermidis* and assayed for cytokine production by ELISA. The data represent the mean ± SEM. Numbers within the bars indicate the number of animals per treatment.
*$p < 0.05$, and **$p < 0.01$ by paired Student's *t*-test.

8- to 19-week-old female NZB/NZW mice before they displayed disease symptoms (< 100 mg/dL protein in urine) and incubated in complete media with 10.0 µg/mL lipopolysaccharide (LPS; Sigma) at 37°C in humidified air with 5% CO_2 for 72 hr. IgG2a antibody production was determined by isotype-specific ELISA. All antibodies were purchased from Southern Biotechnology (Birmingham, AL). Immulon 2 plates (Thermo Labsystems, Franklin, MA) were coated with 5.0 µg/mL goat anti-mouse IgG2a unlabeled antibody in PBS for 1 hr at 37°C. After washing, plates were blocked in PBS/0.05% Tween-20 (PBST) for 30 min at 37°C. Plates were incubated with either IgG2a standard or diluted supernatants from LPS-stimulated spleen cells for 2 hr at 37°C. After washes with PBST, 1 µg/mL biotinylated goat anti-mouse IgG2a antibody was added to each well and incubated for 1 hr at 37°C. Plates were washed with PBST, and HRP-avidin was added to each well and incubated for 1 hr at room temperature. After washing, the plates were developed using ABTS-peroxidase substrate (Bio-Rad, Hercules, CA) and read after 5 min in a Bio-tek EL312e automated plate reader set at dual wavelengths of 405 and 630 nm. The concentration of IgG2a was calculated using the IgG2a standard curve in Excel.

Statistics. Results are reported as mean ± SEM unless otherwise noted. For the *in vitro* and *in vivo* studies involving C57BL/6 mice, results were analyzed using a paired Student's *t*-test. Because of the greater variability within the untreated control NZB/NZW mice, results of studies involving NZB/NZW mice were analyzed using the nonparametric Mann-Whitney *U*-test using SPSS 11 software for Macintosh (SPSS, Chicago, IL).

Results

IFN-γ production decreases in BPA-treated mice. Initially we tested the *in vitro* effects of the ER-binding molecule BPA on immune function using the estrogen-sensitive C57BL/6 strain. BPA induced a dose-dependent decrease in mitogen-stimulated IFN-γ secretion by splenocytes from both male and female 6-week-old C57BL/6 mice (Figure 1).

To investigate the effect of *in vivo* exposure to low doses of BPA on IFN-γ production, we fed C57BL/6 mice 2.5 µg BPA/kg bw daily for 7 days. This dose was based on previously published *in vivo* studies demonstrating reproductive effects from microgram doses of BPA per kilogram body weight and the detection of microgram levels of BPA in commonly used food containers (Brotons et al. 1995; Gupta 2000). The mice were sacrificed 2–4 days after the last dose of BPA. There was a consistent and significant reduction of approximately 40% in ConA-stimulated IFN-γ production in male C57BL/6 mice treated *in vivo* with BPA (Figure 2). Control males produced 46,539 ± 6,837 pg/mL IFN-γ (n = 12) compared with 28,136 ± 4,506 pg/mL in BPA-fed males (n = 12; $p < 0.01$). Unstimulated cells produced undetectable levels of IFN-γ.

Female C57BL/6 mice in both treated and control groups consistently produced more IFN-γ than did the males in response to ConA stimulation (Figure 2). However, ConA-stimulated splenocytes from female C57BL/6 mice fed BPA for 7 days produced 28% less IFN-γ compared with untreated female controls (Figure 2; $p < 0.05$, paired Student's *t*-test). Control females produced 57,438 ± 2,955 pg/mL IFN-γ compared with 41,592 ± 6,559 pg/mL in the BPA-fed group ($p < 0.05$). To test a more physiologic stimulus, splenocytes from an additional 12 female mice were incubated with heat-killed, gram-positive *S. epidermidis.* BPA exposure significantly reduced IFN-γ production by 24% (49,212 ± 3,815 pg/ml IFN-γ vs. 37,478 ± 3,264 pg/mL IFN-γ, $p < 0.05$; Figure 2).

Lymphocyte distribution in C57BL/6 mice. To determine whether the reduction in IFN-γ observed in BPA-fed mice was due to altered lymphocyte subset distribution, we analyzed C57BL/6 splenic mononuclear cells for expression of the T-cell subset markers CD4 and CD8 and the B-cell marker CD19 by flow cytometry. Immediately upon isolation, the cells were stained with fluorescent monoclonal antibodies and analyzed by gating on live cells. Compared with control mice, both male and female BPA-treated mice showed a nonsignificant trend toward an increase in the percentage of $CD19^+$ cells (Table 1). The T-cell subsets did not differ significantly between treated or control mice for either sex.

Although subset distribution did not differ with BPA treatment, C57BL/6 mice exposed to BPA for 7 days demonstrated a significant increase in total splenic mononuclear cells after separation by density centrifugation. The mean number of splenic mononuclear cells isolated from 32 BPA-fed mice was $51.7 \times 10^6 \pm 2.9 \times 10^6$ cells compared with $40.8 \times 10^6 \pm 2.8 \times 10^6$ cells in 32 age- and sex-matched control mice ($p <$ 0.01, unpaired Student's *t*-test).

Cytokine changes in BPA-treated NZB/NZW mice. IFN-γ contributes to disease progression in lupus (Hasegawa et al. 2002). After observing modulation of IFN-γ production in C57BL/6 mice, we were interested in studying the effects of BPA in NZB/NZW mice. Ten-week-old female NZB/NZW mice fed BPA at 5 weeks of age showed a significant reduction in ConA-stimulated IFN-γ production before the onset of proteinuria symptoms (Figure 3A). BPA-fed mice with < 100 mg/dL protein in urine produced an average of 16% less IFN-γ than did the control untreated mice ($p < 0.05$, Mann-Whitney). To determine whether the reduction in IFN-γ persisted over the course of several months, we also tested

Table 1. Lymphocyte subset distribution in C57BL/6 and NZB/NZW mice.

	C57BL/6 males		C57BL/6 females		NZB/NZW females[a]	
	Control (n = 6)	BPA (n = 6)	Control (n = 6)	BPA (n = 6)	Control (n = 12)	BPA (n = 12)
$CD4^+$	26.7 ± 5.9	23.6 ± 5.3	22.4 ± 1.5	21.4 ± 2.0	32.1 ± 3.6	31.3 ± 3.4
$CD8^+$	19.9 ± 7.3	14.9 ± 2.6	15.5 ± 2.9	13.8 ± 2.8	17.9 ± 2.1	17.5 ± 1.4
$CD19^+$	33.0 ± 6.0	37.2 ± 6.5	36.8 ± 7.2	43.4 ± 4.2	34.7 ± 5.2	37.3 ± 5.2

[a]NZB/NZW mice analyzed before proteinuria developed (< 100 mg/dL protein in urine).

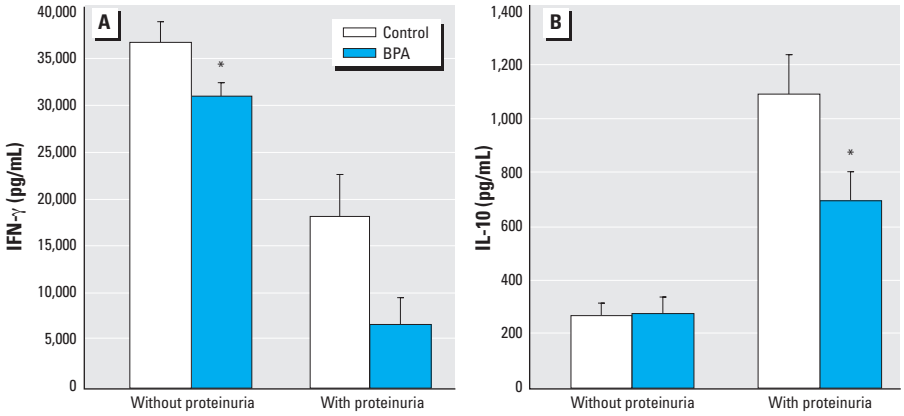

Figure 3. Release patterns of IFN-γ (*A*) and IL-10 (*B*) in 5-week-old female NZB/NZW mice fed PBS (control) or 2.5 µg BPA/kg bw/day for 7 days and monitored for proteinuria. The without-proteinuria group (n = 6 control; n = 6 BPA treated) was sacrificed at 10 weeks of age, before development of proteinuria, and ConA-stimulated splenic supernatants were analyzed for cytokine production by ELISA. The with-proteinuria group developed proteinuria before cytokine analysis [for IFN-γ, n = 5 control and n = 5 BPA treated (supernatants were not available for all mice for INF-γ); for IL-10, n = 8 control and n = 8 BPA treated].
*$p < 0.05$, Mann-Whitney.

the splenocytes of 10 female NZB/NZW mice sacrificed at the onset of proteinuria. A dramatic 64% reduction in IFN-γ was observed in ConA-stimulated splenocytes from BPA-fed mice at disease symptom onset (Figure 3A). The BPA-treated NZB/NZW mice produced an average of 6,640 pg/mL IFN-γ versus 18,186 pg/mL in the controls, a difference approaching statistical significance ($p = 0.08$, Mann-Whitney).

IL-10 is a second cytokine that is both implicated in the development of lupus and modulated by estrogen (Kanda and Tamaki 1999; Yin et al. 2002). We analyzed whether *in vivo* BPA exposure could alter IL-10 levels in female NZB/NZW mice. Before disease symptom onset, at 10 weeks of age, both control and BPA-fed mice produced similar amounts of IL-10 (Figure 3B). Control NZB/NZW mice produced 276 ± 52 pg/mL versus 288 ± 59 pg/mL IL-10 in BPA-fed animals. As disease progressed, both control and BPA-fed mice produced higher levels of IL-10. Measuring IL-10 production at the time mice developed proteinuria demonstrated that the BPA-fed mice produced 32% less IL-10 than did controls (699 ± 114 pg/mL vs. 1,097 ± 146 pg/mL, $p < 0.05$).

IgG2a production decreases in BPA-treated NZB/NZW mice. In mice, IFN-γ plays a role in isotype switching to the complement-fixing antibody class IgG2a, contributing to the glomerulonephritis associated with lupus (Zeng et al. 2000). We were interested in seeing whether the decreased release of IFN-γ observed in NZB/NZW BPA-fed mice resulted in altered IgG2a production. We measured IgG2a production in LPS-stimulated splenocytes from NZB/NZW female mice between 8 and 19 weeks of age by isotype-specific ELISA. All mice tested negative for proteinuria (< 100 mg/dL protein in urine). As shown in Figure 4, lymphocytes from BPA-treated animals ($n = 16$) produced significantly less IgG2a than age-matched controls ($n = 16$; $p < 0.05$, Mann-Whitney). The median IgG2a concentration was 10.4 ng/mL for BPA-fed mice compared with 23.0 ng/mL for untreated controls. The BPA-fed NZB/NZW mice produced a much narrower range of secreted IgG2a compared with the untreated control animals. The mean concentration of IgG2a for BPA-fed animals was 14.4 ± 3.2 ng/mL (including the one outlier; Figure 4) versus 29.0 ± 5.6 ng/mL in the untreated controls, a reduction of 50%.

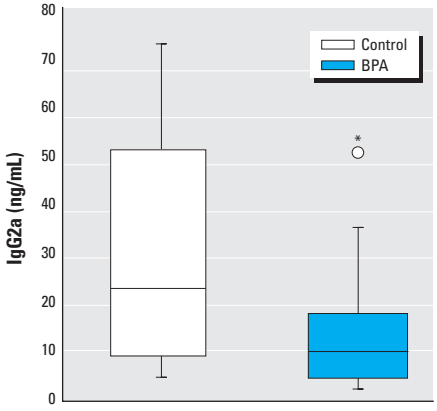

Figure 4. IgG2a production in young female NZB/NZW mice fed BPA ($n = 16$) or PBS control ($n = 16$) daily for 7 days at 5 weeks of age and sacrificed between 8 and 19 weeks of age. IgG2a was detected by isotype-specific ELISA in 72-hr LPS-stimulated splenic cell supernatants. All mice tested negative for proteinuria. The box represents the middle 50% of values; the line inside the box indicates median; bars indicate range; and the open circle represents an outlier.
*$p < 0.05$, Mann-Whitney.

To eliminate the possibility that the reduction of IgG2a in BPA-treated mice was due to a decreased percentage of B cells, we analyzed splenocytes for lymphocyte subsets by flow cytometry. The subset analysis was performed on the same NZB/NZW mice used in the IgG2a assays, with the exception of eight mice whose splenocytes were not stained. On average, BPA-fed mice had the same percentage of $CD19^+$ cells compared with untreated mice (Table 1). As observed in the C57BL/6 mice, there was a significant increase in the total number of mononuclear cells isolated from the spleens of these BPA-treated mice ($n = 16$) by density centrifugation ($43 \times 10^6 \pm 2.9 \times 10^6$ vs. $34 \times 10^6 \pm 2.8 \times 10^6$, $p < 0.05$).

Delay in disease onset observed in BPA-treated NZB/NZW mice. To analyze whether *in vivo* BPA exposure modulates the course of lupus, we fed BPA to three separate groups of 5- to 6-week-old female NZB/NZW mice for 7 days. Each group consisted of five BPA-fed mice and five control mice. In each of the three experiments, a control NZB/NZW mouse was the first to develop proteinuria. Overall, female BPA-treated NZB/NZW mice showed an average delay of 7 weeks in the onset of proteinuria compared with untreated controls (Figure 5). The earliest onset of disease symptoms was at 26 weeks in a control mouse, whereas the earliest BPA-treated mouse to develop proteinuria was 33 weeks of age. On average, the mice treated with BPA remained symptom-free for 45 weeks compared with 38 weeks in control animals. Two of the BPA-fed mice showed no signs of proteinuria at 72 weeks of age. Seven days of oral BPA exposure at a young age appears to modulate the course of disease in female NZB/NZW mice.

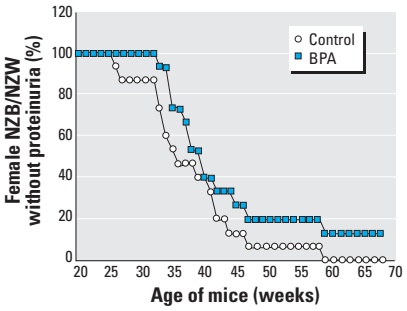

Figure 5. Development of proteinuria in 5- to 6-week-old female NZB/NZW mice fed PBS ($n = 15$) or BPA ($n = 15$) daily for 7 days.

Discussion

Although the biologic effects of BPA are not clearly understood, its ability to bind both ER isoforms makes it a potentially important modulator of immunity. Although estrogen increases IFN-γ production (Karpuzoglu-Sahin et al. 2001), we observed an inhibitory effect of BPA on IFN-γ secretion in both male and female C57BL/6 and female NZB/NZW mice. There is evidence that the outcome of transcriptional regulation at AP-1 or ERE sites is dependent both on the ER subtype involved and on the ligand (Mor et al. 2003; Paech et al. 1997). For example, when tamoxifen is bound to ER-β, it regulates AP-1 sites in a manner opposite to that of estrogen (Paech et al. 1997). Similarly, BPA may act differently than estrogen when bound to ER-α and/or ER-β, for example, by down-modulating the IFN-γ promoter.

In our studies, BPA acts in a protective manner in lupus-prone mice. It is likely that the decreased production of IFN-γ in BPA-fed mice contributed to a substantial reduction in isotype switching to IgG2a and to the prolonged symptom-free period we observed in BPA-fed NZB/NZW mice. Anti-dsDNA antibodies are a hallmark of disease in lupus-prone mice (Walker et al. 1996). Typically, anti-dsDNA antibodies of the IgG classes are not routinely detected until after 5 months of age (Yoshida et al. 2002). We were not able to detect IgG2a anti-dsDNA antibodies in the NZB/NZW mice used for antibody analysis because the mice were sacrificed between 8 and 19 weeks of age. Recently, we analyzed LPS-stimulated splenocytes isolated from 8-month-old female NZB/NZW mice that had been fed BPA for 1 week beginning at 5 weeks of age. All mice tested negative for proteinuria. BPA-fed mice demonstrated a 40% reduction in IgG2a anti-dsDNA antibodies as detected by ELISA (Sawai C. Unpublished data).

IL-10 is associated with lupus in both mice and humans (Gonzalez-Amaro et al. 1998; Llorente et al. 1995; Yin et al. 2002), yet its role in the disease appears to be complex. IL-10 may act as a regulatory cytokine that increases as a consequence of the disease process; alternatively, it may function as a contributing factor to disease (Moore et al. 2001). Knocking out the *IL-10* gene in the lupus-prone MRL-Fas^{lpr} mouse strain indicates that IL-10 plays a protective role early in disease; IL-10 is known to inhibit IFN-γ synthesis,

and the expression of IL-10 is correlated with lower IgG2a concentrations and fewer disease symptoms (Yin et al. 2002). However, in later stages of disease, high levels of IL-10 may contribute to disease progression, and anti-IL-10 antibodies delay onset of symptoms (Yin et al. 2002). A 1-week course of BPA treatment had no apparent effect on IL-10 production in young female NZB/NZW mice tested before the onset of proteinuria several weeks after treatment. Once proteinuria developed, however, BPA-fed animals produced significantly less IL-10 than did controls. To better understand the relationship between BPA and IL-10, we plan to analyze IL-10 production in female NZB/NZW mice in the days immediately after BPA treatment.

More than 8 months after treatment, BPA-fed NZB/NZW mice demonstrated a delay in proteinuria development as well as a significant reduction in IFN-γ production. The inhibition of IFN-γ by BPA appears to be long lasting and may be due to remodeling of the IFN-γ promoter. Recruitment of the histone acetylase CBP (CREB-binding protein) to a promoter contributes to increased transcription (Valapour et al. 2002). Both the nuclear factor-κB (NF-κB) and ER-α pathway use CBP (Harnish et al. 2000; Kushner et al. 2000). NF-κB is a critical transcription factor for several cytokine genes, including IFN-γ (Sica et al. 1997). BPA bound to the ER may sequester proteins such as CBP required for NF-κB–induced cytokine gene expression, thereby reducing cytokine production. Alternatively, a recent report indicates that BPA activates the transcription factor CREB (Quesada et al. 2002). Expression of the IFN-γ promoter is down-regulated by CREB-ATF1 proteins binding to both proximal and distal elements and competing for binding with Jun/ATF2 proteins (Penix et al. 1996; Zhang et al. 1998). Activation of CREB due to BPA exposure may explain the diminished production of IFN-γ. We are currently addressing the possible molecular mechanism for BPA reduction of IFN-γ production. Our studies suggest that a low-dose, short-term exposure to the ER-binding molecule BPA affects murine immune function *in vivo* and may have important implications for modulating autoimmunity.

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