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Endocrine Disruptors (Environmental Estrogens) Enhance Autoantibody Production by B1 Cells

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Accumulating data suggest that endocrine disruptors affect not only the reproductive system, but also the immune system. We demonstrate here that endocrine disruptors including diethylstilbestrol (DES) and bisphenol-A (BPA) enhance autoantibody production by B1 cells both *in vitro* and *in vivo*. BWF1 mice, a murine model for systemic lupus erythematosus (SLE), implanted with Silastic tubes containing DES after orchidectomy developed murine lupus characterized by immunoglobulin G (IgG) anti-DNA antibody production and IgG deposition in the glomeruli in the kidney as well as those implanted with 17β-estradiol (E2). Plaque-forming cells (PFC) producing autoantibodies specific for bromelain-treated red blood cells were significantly increased in mice implanted with DES and BPA. IgM antibody production by B1 cells *in vitro* was also enhanced in the presence of endocrine disruptors including DES and BPA. Estrogen receptor (ER) expression was upregulated in B1 cells in aged BWF1 mice that developed lupus nephritis. These results suggest that endocrine disruptors are involved in autoantibody production by B1 cells and may be an etiologic factor in the development of autoimmune diseases.

Key Words: **endocrine disruptors; environmental estrogens; autoantibody; B cell; systemic lupus erythematosus; SLE.**

It is well recognized that allergic diseases such as bronchial asthma and atopic dermatitis are markedly increased the last several decades in developed countries (Bjorksten, 1999; Burr *et al*., 1989; von Mutius, 2000). Accumulating data suggest that environmental factors contribute to the observed increase of allergic diseases (Fujimaki *et al*., 1994; Nel *et al*., 1998; Takano *et al*., 1997). It has been also reported that autoimmune diseases such as systemic lupus erythematosus (SLE), idiopathic thrombocytopenic purpura (ITP), dermatomyositis, and scleroderma are strikingly increased during several decades as well as immediate-type hypersensitivity (Ohno, 1999).

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At the same time, increasing scientific and social attention had been paid to environmentally dispersed chemicals that can enter the body by ingestion or adsorption and that mimic the actions of estrogens. These chemicals are termed *endocrine disruptors* (EDs) or *environmental estrogens* and are found in plastics (bisphenol-A, phthalates), detergents and surfactants (octylphenol, nonylphenol), pesticides (methoxychlor, dichlorodiphenyltrichloroethane or DDT, hexachlorobenzene, and dieldrin), industrial chemicals (polychlorinated biphenyls or PCBs, 2,3,7,8-tetrachlorodibenzo-p-dioxin or TCDD), phytoestrogens or natural plant estrogens (genistein, coumesterol), and mycoestrogens or fungal products from *Fusarium* sp. (zearalenone). Some of these estrogenic chemicals have also been shown to influence the immune system.

The actions of endocrine disruptors are assumed to be mediated through estrogen receptors (ERs), presumably translocate the receptor–ligand complex from the cytoplasm to the nucleus, and induce the synthesis of estrogen receptor–regulated proteins. Binding of these chemicals to novel estrogen receptors other than alpha or beta estrogen receptor is also plausible (Arcaro *et al*., 1999; Das *et al*., 1997). Endocrine disruptors mimic hormones, block or alter hormone binding to receptors, or alter the metabolism of natural estrogens (Soto *et al*., 1995).

It has been widely noted that females have stronger immune capabilities (particularly B-cell–mediated immunity) than males, as evidenced by their better immune responses to a variety of self-antigens and non-self-antigens. This phenomenon of gender-based immunecapability is largely attributed to the effects of sex hormones (Ahmed, Penhale, and Talal, 1985; Grossman, 1984; Homo-Delarche *et al*., 1991). Estrogens regulate the level of serum and uterine IgM, IgA, and IgG (Wira and Sandoe, 1987), and they augment antibody production to several non-self-antigens and self-antigens. It is possible that endocrine disruptors that mimic estrogenic activity may be involved in the increased incidence of autoimmune diseases such as SLE (Ahmed, 2000).

To test this hypothesis, we investigated effects of endocrine disruptors on autoantibody production in a popular murine model for SLE. Female BWF1 mice, a F1 hybrid between

New Zealand Black and New Zealand White mouse strains, spontaneously develop a systemic autoimmune disorder resembling SLE in humans (Kotzin, 1996; Theofilopoulos, 1985). The murine disorder is characterized by high levels of IgG autoantibodies including anti-double-stranded DNA (dsDNA) antibody and massive deposition of immune complexes in glomeruli in the kidney. Although both anti-dsDNA and ssDNA antibody are detected in the serum of SLE patients and murine models for SLE, anti-dsDNA Ab is a better marker for SLE and the disease activity (Eaton, Schneider, and Schur, 1983). There are other ways to evaluate autoantibody production including plaque-forming cell (PFC) assay for anti-RBC Ab. It has been reported that B1 cells produce autoantibody against phosphatidylcholine expressed on bromelain-treated red blood cells (Br-RBC) using PFC assay (Merucolino *et al*., 1988). This method was originally developed by Cunningham and based on sensitive RBC lysis by IgM anti-RBC antibody in the presence of complement at the single-cell level (Cunningham, 1974). More than 95% of affected mice die of renal failure caused by immune complex deposition in the kidney before 12 months of age. A marked mononuclear cell infiltration in the target organs including the kidney and lung is also observed in aged BWF1 mice.

B1 cells are a specialized cell population that are distinguished from conventional B cells (B2 cells) by their origin, cell surface phenotype, unique tissue distribution, and capacity for self-renewal (Hardy and Hayakawa, 2001; Hayakawa *et al*., 1983). The cell surface phenotype of B1 cells is $B220^{int}IgM^{high}IgD^{low}CD5^{+}IL\text{-}5R^{+}$, whereas that of B2 cells is $B220^{high}IgM^{int}IgD^{high}CD5\text{-}IL\text{-}5R^{-}$. CD5 expression is usually used for differentiating B1 cells and B2 cells. They have also been considered to be involved in autoantibody production in the development of autoimmune diseases (Murakami *et al*., 1992; 1994).

In the present study we found that 17β-estradiol (E2), a natural estrogen, and diethylstilbestrol (DES) all enhanced IgG anti-DNA antibody production and IgG immune complex deposition in the kidney. Bisphenol-A (BPA) as well as E2 and DES enhanced anti-Br-RBC autoantibody production by B1 cells *in vivo*. It was also demonstrated that the three EDs directly enhanced *in vitro* IgM antibody production, including anti-DNA antibody, by B1 cells. Possible roles of endocrine disruptors in the development of autoimmune diseases are discussed.

MATERIALS AND METHODS

Mice. Specific pathogen-free BWF1 mice, originally obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained under controlled conditions in a pathogen-free mouse facility of the Department of Molecular Preventive Medicine. Female mice aged 4 wk were used as young mice, and mice aged 8–12 mo with moderate-to-severe proteinuria were used as aged mice. All animal experiments complied with the standards set out in the guidelines of the University of Tokyo.

Silastic tube implant with endocrine disruptors. Four-week-old female BWF1 mice were ovariectomized as described previously. Silastic implants (1.5 cm in length, i.d.:o.d.; 1.57:3.18 mm, Kaneka Medix), containing 6–7 mg of the powdered form of E2, DES, or BPA (all obtained from Sigma, Chemical Co., St. Louis, MO), were surgically placed subcutaneously on the back. Ovariectomized mice that received empty Silastic tubes served as controls. The implants were left *in situ* for 3 to 4 months and blood samples were collected periodically for ELISA. Mice were killed 4 months after implantation for histological and immunological examinations.

Cell preparation. Peritoneal lavage cells were isolated by flushing the peritoneal cavity with 8–10 ml of RPMI 1640 supplemented with 10% fetal calf serum (FCS). Cell suspensions prepared from mouse spleen were passed through nylon mesh. Red blood cells in the spleen were lysed by ammonium chloride solution. Peritoneal B1 cells were purified by negative selection with MACS (Miltenyi Biotec, GmbH Bergish Gladbach, Germany) to avoid contamination of B2 cells in the peritoneal cavity. $CD5^{+}B220^{+}$ cells are usually described as B1 cells and $CD5^{-}B220^{+}$ cells as B2 cells in B cell study. However, in the *in vitro* antibody production assay, we used $Thy\text{-}1^{-}F4/80^{-}CD23^{-}$ peritoneal cells as B1 cells to avoid contamination of B2 cells in the peritoneal cavity. B2, but not B1 cells express CD23 molecules on the cell surfaces. Using this negative selection, B2 cells ($CD23^{+}$), macrophages ($F4/80^{+}$), and T cells ($Thy\text{-}1^{+}$) are effectively removed from peritoneal cells. More than 95% of residual cells are B1 cells ($CD5^{+}$ $B220^{+}$). Briefly, T lineage cells, macrophages, and B2 cells were depleted by incubating with a biotinylated mAb cocktail, including anti-Thy1.2, anti-F4/80, and anti-CD23 mAbs. The unwanted cells were then removed magnetically after incubation with streptavidin-conjugated MACS beads. In contrast, B2 cells were purified by positive selection with anti-B220 Ab coupled MACS beads (Miltenyi Biotec) because the percentage of $CD5^{+}B220^{+}$ cells is very low in the spleen of young BWF1 mice. The cell purity for B1 in each B cell fraction was more than 95% throughout the experiments.

ELISA. Anti-DNA antibody activity in the serum is usually detected using double-stranded DNA (dsDNA), whereas anti-DNA antibody activity in the culture supernatant is detected with single-stranded DNA (ssDNA). Both anti-dsDNA and ssDNA antibody are detected in the serum of SLE patients and murine models, although only anti-ssDNA antibody can be detected in the *in vitro* system, presumably because of a low concentration of anti-DNA antibody in the culture supernatant, and because of higher sensitivity of the assay for ssDNA. It is generally believed that the level of anti-dsDNA antibody is a better marker for disease activity.

Anti-DNA antibody was measured in ELISA using ssDNA for the culture supernatant and dsDNA for the serum. Double-stranded DNA was obtained by digestion of calf thymus DNA (Sigma) with S1 nuclease (Seikagaku Kogyo, Tokyo, Japan), followed by fractionation on A hydroxyapatite column. Single-stranded DNA was obtained by heat degeneration of dsDNA. Flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) treated with 0.001% protamine sulfate were coated with 50 μl of ssDNA solution (5 μg/ml), and dried overnight at 37°. Nonspecific protein binding was blocked by coating the plate with 50% FCS in PBS. After washing with 0.05% Tween 20 in phosphate-buffered saline (PBS), 50 μl of each sample was incubated for 60 min at room temperature, followed by addition of 50 μl of diluted horseradish peroxidase (HRP)–labeled goat anti-mouse IgG or anti-mouse IgM Abs (Bethyl Laboratories, Montgomery, TX). The preparations were then incubated for 60 min at room temperature, washed, and developed with 50 μl of HRP substrate (3,3′,5,5′-tetramethyl benzidine) with H_2O_2. The absorbance was read with a Emax precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 450 nm. Pooled sera of aged BWF1 mice with lupus nephritis were used as positive control at 1,000 U/ml. Serum estrogen was detected by Estradiol EIA Kit (Cayman, Ann Arbor, MI) according to the manufacturer's directions in which estradiol tracer (estradiol linked to acetylcholine esterase) is used as the competitor. The lower limit of the assay is 7.8 pg/ml. Serum BPA was detected by Bisphenol-A (BPA) ELISA Kit (TOYOBO. CO., LTD., Osaka, Japan) according to the manufacturer's directions. The lower limit of the assay is 0.01 ng/ml.

Immunofluorescence study. The kidney was embedded in Tissue-Teck OCT compound (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Six-micron cryostat sections were incubated in fluorescein (FITC)-conjugated anti-mouse IgG Ab (R19-15, PharMingen) and observed under a fluorescent

microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Normal IgG was used as control.

Plaque-forming assay. Plaque-forming cells to autologous bromelain-treated erythrocytes (Br-RBC) were enumerated in a modified Cunningham plaque assay (Cunningham, 1974).This method is based on sensitive RBC lysis by IgM anti-RBC antibody in the presence of complement at a single-cell level. It was previously reported that B1 cells produce autoantibody against phosphatidylcholine expressed on Br-RBC using the PFC assay (Merucolino *et al*., 1988). The erythrocytes were first treated with the proteolytic enzyme bromelain (Sigma) (50% erythrocyte suspension in 20 mg/ml bromelain/PBS for 60 min at 37°), then 20 μl of a 30% (v/v) suspension of the Br-RBC was mixed with 10^6 spleen cells and rabbit complement (Cedarlane Laboratories Ltd., Ontario, CA), loaded into Cunningham chambers, and incubated for 1 to 2 h in a CO_2 incubator at 37°. The plaques were counted under a phase-contrast microscope.

Flow cytometry. Flow cytometric analysis on splenic and peritoneal cells was performed with the use of an Epics Elite cell sorter (Coulter Electronics, Hialeah, FL). After Fc block with anti-CD16/32 Ab (PharMingen, San Diego, CA) for 10 min, cells were stained with FITC-conjugated anti-CD4 (GK1.5, PharMingen), phycoerythrin (PE)-conjugated anti-CD8α (53–6.7, PharMingen) and allophycocyanin (APC)-conjugated anti-B220 (RA3-6B2, PharMingen). Cells were also stained with FITC-conjugated anti-CD5 (53–7.3RRH) and APC-conjugated anti-B220 Abs. $CD5^+B220^+$ cells were detected as B1 cells, and CD5-$B220^+$ cells were detected as B2 cells (shown in Figure 1 of the Supplementary Material online). As reported by Hayakawa *et al*. (1983), B1 cells express a lower level of B220 than B2 cells do. The expression level of CD5 molecules is also lower than on T cells. Twenty thousand cells were analyzed on live cells.

B cell culture. B1 or B2 cells (1×10^6 cells/ml) were cultured in triplicate in 96 well plates with or without EDs at indicated doses in 200 μl of RMPI 1640 medium (ICN Biomedical, Inc., Aurora, OH) at 37° in an atmosphere of 5% CO_2 in air for 4 days. Phenol red–free medium containing 10% FCS treated with dextran-coated charcoal was used to exclude the effect of endogenous estrogens. The culture supernatants were harvested 4 days after culture and stored at −80° until use. The amount of total IgM and IgM anti-DNA Ab in the supernatant was measured by ELISA as described. Stock solutions (10^{-2} M) of E2, BPA, DES, and nonylphenol (NP) (Aldrich Chemical Co., St. Louis, MO) were prepared in ethanol and diluted to appropriate concentration so that the final ethanol concentration was less than 0.1%. No suppressive activity of ethanol on cell culture was observed at this concentration.

Reverse transcription and real-time quantitative polymerase chain reaction (PCR) analysis. Total cellular RNA was prepared from purified B1 and B2 cells using RNeasy Mini Kit (Qiagen, Hilden, Germany). Five μg total cellular RNA was reversely transcribed into cDNA using Superscript preamplification kit (Life Technologies, Gaithersburg, MD) and amplified with specific oligonucleotide primers. The sense primer for ER-α was 5′-GGCCT-GACTCTGCAGCAGCAG-3′, and the antisense primer was 5′-GTTGGGGA-AGCCCTCTGCTTC-3′. The sense primer for ER-β was 5′-GGCATTCTACAGTCCTGCTG-3′, and the antisense primer was 5′-TCTGCA-TAGAGAAGCGATGA-3′. The PCR was performed by thermal cycle for 36 cycles of 95° for 60 s, 60° for 60 s, and 72° for 120 s, and the final extension was done at 72° for 10 min. The PCR products of β-actin, ERα, and ERβ were examined by 1.5% agarose gel electrophoresis. After ethidium bromide staining, bands were visible only at the expected size for each mRNA product. Real-time quantitative PCR analysis was performed by means of the ABI7700 sequence detector system (PE Applied Biosystems, Foster City, CA). FAM (6-carboxyl-fluorescein)-labeled primers were used as target hybridization probes for ER-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ER-α; 5′-GCAT-GATGAAAGGCGGCATACGGAA-3′, GAPDH; 5′-AAGGGACACAGT-CAAGGCCGAGAAT-3′). The thermal cycling condition included 50° for 2 min and 95° for 10 min, followed by 45 cycles of amplification at 95° for 15 s and 55° for 1.5 min for denaturing and annealing, respectively. The PCR reactions were run in duplicate; ER-α quantity was normalized by the level of GAPDH.

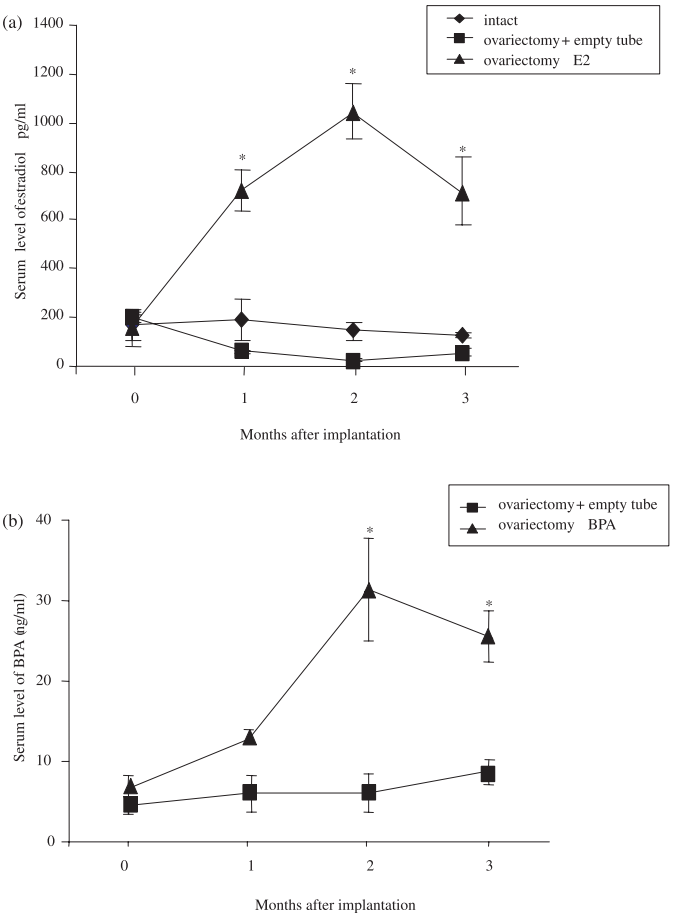

FIG. 1. Long-lasting release of 17β-estradiol (E2) or bisphenol-A (BPA) in mice implanted with Silastic tubes containing E2 or BPA. A. Prepubertal BWF1 mice (4 weeks old, $n = 4$) were ovariectomized and implanted subcutaneously on the back with Silastic tubes containing (a) E2 or (b) BPA. Empty tubes (square) were implanted after ovariectomy in control mice. Intact mice (diamond) were also used as controls in (a). The serum levels of E2 and BPA were detected by ELISA. The results are presented as the mean ± SE. Statistical analysis was performed by Student's *t*-test. * $p < 0.01$.

Statistics. Comparisons between two groups were performed using the Student's *t*-test. Results are presented as mean ± SD. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Long-Lasting Release of Estrogen or BPA into the Circulation in Mice Implanted with EDs-Containing Silastic Tubes

Prepubertal female BWF1 mice that were ovariectomized and implanted with Silastic tubes containing 6–8 mg of 17β-estradiol (E2) showed an elevated level of E2 in the serum for more than 3 months. In contrast, in mice implanted with empty Silastic tubes the serum level of E2 was decreased after ovariectomy when compared to the level in intact mice, where a low, but significant level of E2 was detected (Fig. 1a). The estrogen level in diestrous

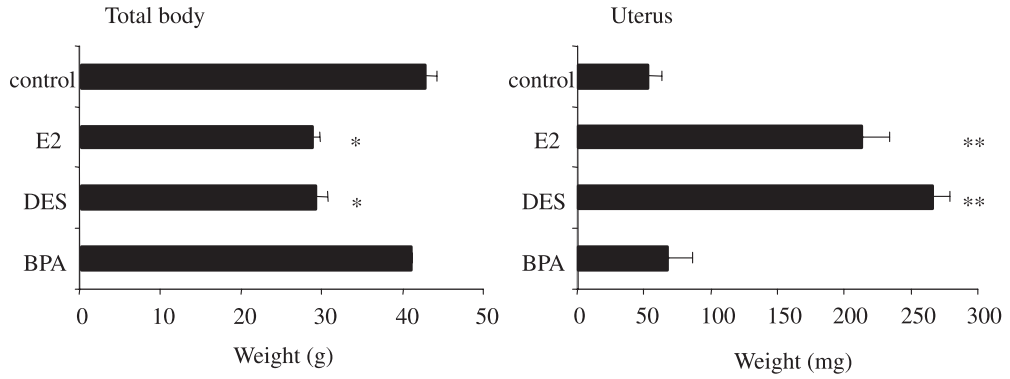

FIG. 2. The effects of a Silastic tube implant on total body and uterus weight. The 4-wk-old mice were ovariectomized and implanted with Silastic tubes containing E2, diethylstilbestrol (DES), or bisphenol-A (BPA). The weight was measured 4 months after implantation. The results are presented as the mean ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.001$.

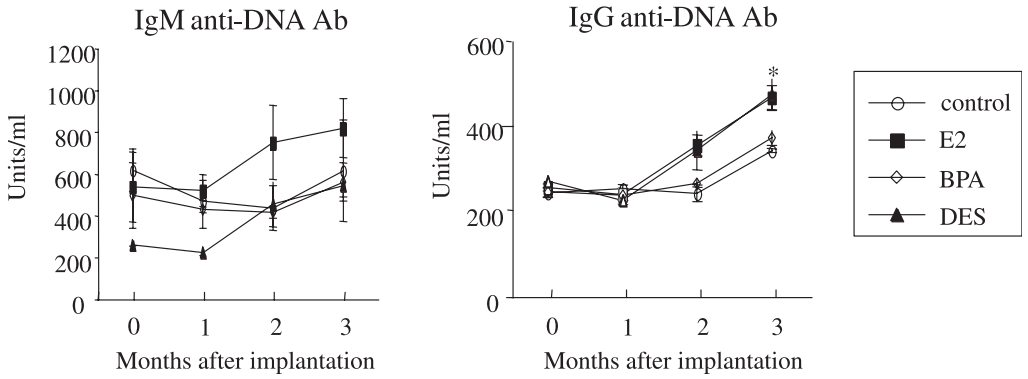

FIG. 3. Enhanced IgG anti-DNA Ab production in mice implanted with E2 and DES. The serum level of anti-DNA antibody was examined using the same mice as described in Figure 1. The weight was measured 4 months after implantation. The results are presented as the mean ± SEM ($n = 4$). * $p < 0.05$.

female mice is around 20–30 pg/ml and increases to 100–200 pg/ml during estrus. It reaches to 5,000–10,000 pg/ml during pregnancy. So, the serum levels of E2 in mice implanted with E2 Silastic tubes are in the middle of those in estrus and pregnancy. Long-lasting release of BPA from implanted tubes was also observed (Fig. 1b). Consistent with the effective release of E2, total body weight was decreased whereas uterus weight was increased in mice that received the Silastic tube implants, demonstrating the estrogenic activity *in vivo* (Fig. 2). Mice that received Silastic tube implants containing DES also showed a similar uterine change to those with E2 implants, whereas mice that received bisphenol-A (BPA) showed no uterine change (Fig. 2). The uterus of mice that received E2 or DES showed a marked hypertrophic change morphologically (data not shown).

Induction of Lupus Nephritis in Mice Implanted with DES-Containing Silastic Tubes

The development of anti-DNA Antibody (Ab) has been a hallmark of autoimmunity. Mice implanted with E2 showed an increased serum level of anti-DNA Ab as early as 3 months after implantation, a finding that is consistent with a previous report (Roubinian *et al*., 1978) (Fig. 3). Mice that received DES showed a similar level of IgG anti-DNA Ab production (Fig. 3), but there was no significant difference in IgM anti-DNA Ab production (Fig. 3). Immune complex deposition in the glomeruli in the kidney was prominent in both the mice that received E2 and those that were given DES (Fig. 4). Mice with the BPA implant showed no sign of immune complex deposition at this point in the study.

DES-Enhanced and BPA-Enhanced Anti-Br-RBC Autoanitbody Production by B1 Cells

To examine the effect of EDs on autoantibody production by B1 cells, a PFC assay using Br-RBC was conducted. A previous study had demonstrated that anti-Br-RBC was mainly produced by B1 cells (Ahmed *et al*., 1989). It should be noted that mice implanted with BPA, as well as those that received E2 and DES, showed an increased PFC against Br-RBC (Fig. 5).

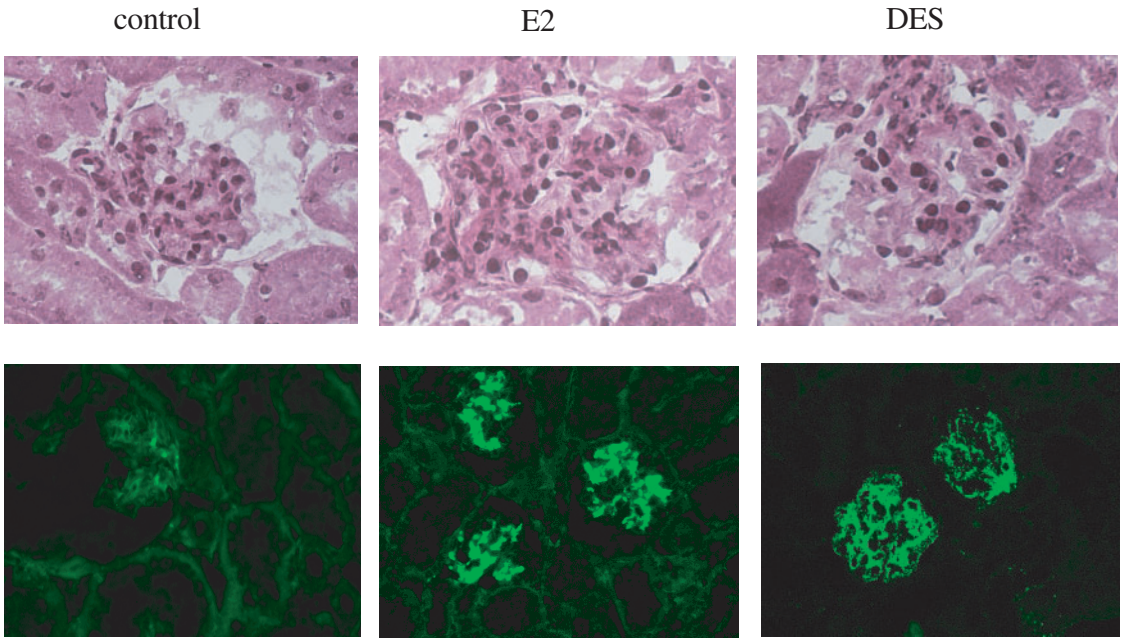

FIG. 4. E2 implants and DES implants result in IgG deposition in the glomeruli in the kidney in BWF1 mice. Cryosections of kidneys were prepared 4 months after implantation and were stained with FITC-labeled anti-mouse IgG antibody (lower panel, magnification ×100). Mice implanted with empty Silastic tubes were used as control. H&E stainings of the sections were also presented (upper panel, magnification ×100).

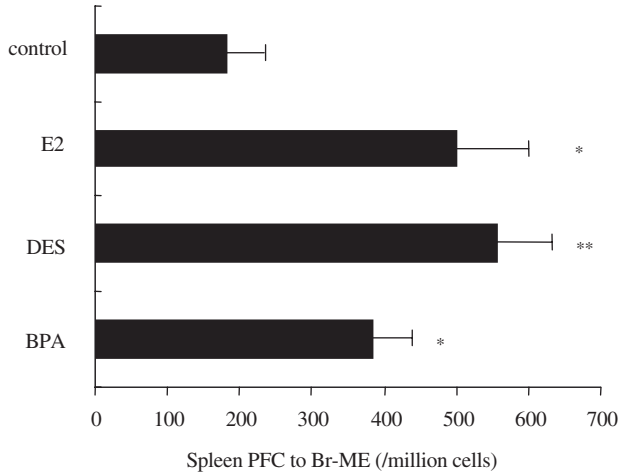

FIG. 5. Enhanced anti-Br-RBC autoantibody production in mice implanted with endocrine disruptors (EDs). The 4-wk-old mice ($n = 4$) were implanted with E2, DES, or BPA after ovariectomy and anti-Br-RBC autoantibody–producing cells in the spleen were detected by a modified Cunningham plaque-forming assay. Untreated BWF1 mice were used as control. Each spleen was assayed in triplicate for plaque-forming cells (PFC), and the mean number of PFC per 10^6 spleen cells was calculated. Data are presented as the mean ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$.

No Significant Effect of Endocrine Disruptors on Splenic Lymphocytes

FACS analysis revealed that the percentage of B1, B2, CD4, and CD8 cells in the spleen was not significantly changed in mice that had received these endocrine disruptors compared to control mice (Fig. 6). Absolute cell number of each cellular component also remained unchanged (Fig. 6). No significant change was observed in the peritoneal cells either (data not shown).

Endocrine Disruptors Directly Enhance IgM Antibody Production by B1 Cells In Vitro

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 cells, B1 cells were cultured in the presence of EDs. IgM production by B1 cells in the presence of E2 (100 nM), DES (100 nM), BPA (1 μM), was significantly enhanced, whereas no direct effect on B2 cells was observed (Fig. 7). The concentration of each endocrine disruptor was pre-determined by dose response experiments. IgM production by B1 cells in the presence of EDs was more prominent on aged BWF1 mice developing lupus nephritis. Nonylphenol (1 μM) also enhanced IgM production by B1 cells obtained from aged BWF1 mice. Phytoestrogens with weak estrogenic activity such as daidzein and genistein also showed a direct enhancing effect on IgM production by B1 cells (data not shown). IgM anti-DNA antibody activity in the culture supernatants was also higher in those from aged BWF1 mice than in those from young mice (in the presence of E2; 155.37 ± 2.81 U/ml vs. 126.30 ± 0.86 U/ml, $p < 0.05$, in the presence of DES; 152.60 ± 0.65 U/ml vs. 128.05 ± 1.96 U/ml; $p < 0.05$). In contrast, immunoglobulin production by conventional B cells (B2 cells) was not enhanced in the presence of endocrine disruptors used in the present study.

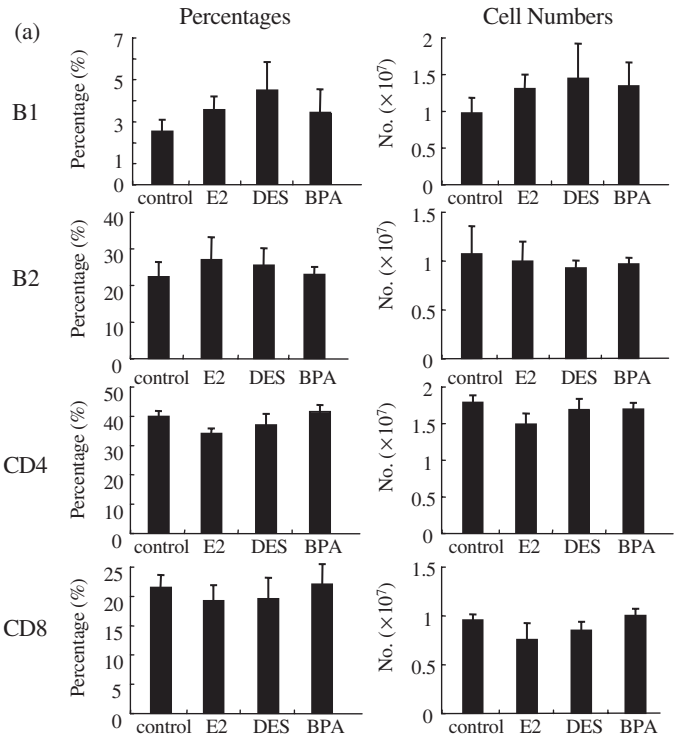

FIG. 6. No significant effect of ED implants on splenic lymphocytes. Spleen cells from mice implanted with EDs were stained for B1 ($CD5^+B220^+$), B2 ($CD5^-B220^+$), CD4, and CD8 cells as described in *Materials and Methods* and were analyzed on a flow cytometer. Twenty thousand live cells were counted. The absolute number of each cellular compartment was also presented. Data are presented as the mean ± SEM ($n = 4$).

Estrogen Receptor Expression in B Cells

To investigate whether the direct enhancing effect of EDs on B1 cells is attributed to higher expression of ER genes in B1 cells, ER mRNA expression was examined in RT-PCR and real-time quantitative PCR analysis. Similar levels of ER-α and ER-β mRNA were expressed in B1 and B2 cells from young mice were used throughout the present study. Interestingly, however, B1 cells from aged mice exhibited increased expression of ER-α mRNA compared to young mice (Fig. 8).

DISCUSSION

In this study, we demonstrated that EDs including DES and BPA enhanced autoantibody production by B1 cells both *in vitro* and *in vivo* in a murine model for SLE. Roubinian *et al*. (1978) previously reported the essential role of estrogen in the development of lupus nephritis in BWF1 mice, a murine model for SLE. It was also reported that $CD5^+$ B cells obtained from aged female C57BL/6 mice or B cells from B10.D2 mice implanted with estrogen after ovariectomy produced autoantibody to Br-RBC (Murakami *et al*., 1994). It is well documented that B1 cells in the peritoneal cavity and spleen are increased in certain murine autoimmune models including NZB, NZB.H2bm12, moth-eaten mice, and BWF1 mice (Hayakawa *et al*., 1983). Likewise, elevated levels of B1 cells have been documented in patients with autoimmune disorders, notably in Sjögren syndrome and rheumatoid arthritis (Dauphinee, Tovar, and Talal., 1988; Plater-Zyberk *et al*., 1985). Furthermore, downregulation/elimination of B1 cells either by administration of anti-IL-10 antibody (Ishida *et al*., 1992; 1994) or by intrapertoneal injection of distilled water (Murakami *et al*., 1995) delays the onset and severity of lupus nephritis in BWF1 mice.

Diethylstilbestrol, a synthetic estrogen with strong estrogenic activity, once had been prescribed to pregnant women to ameliorate problems during pregnancy for a certain period of time. It caused adverse effects on the female offspring known as "DES daughter syndrome" (Wingard and Turiel, 1988). Limited studies in humans have suggested that DES-exposed women developed a variety of autoimmune diseases (Noller *et al*., 1988). Animal studies have confirmed that DES has profound immunomodulatory effects, including the induction of autoantibodies to cardiolipin (Forsberg, 2000). These results are consistent with our findings that a DES implant resulted in increased plaque-forming cells (PFC) producing anti-Br-RBC autoantibody, IgG anti-DNA antibody production, and immune complex deposition in castrated BWF1 mice. Diethystilbestrol also directly enhanced IgM production by purified B1 cells *in vitro* as effectively as E2. It should be noted that BPA also showed a significant enhancement of anti-Br-RBC autoantibody production *in vivo* and increased IgM antibody production by B1 cells *in vitro*, in spite of weak estrogenic activity. These findings suggest a possibility that B1 cells are sensitive target cells for endocrine disruptors with weak estrogenic activity. In contrast, BPA failed to enhance IgG anti-DNA production, whereas it promoted IgM autoantibody production by B1 cells both *in vivo* and *in vitro*. This is presumably due to weak estrogenic activity of BPA (10,000-fold weaker than E2) level in mice implanted with BPA, as shown in Figure 1. It has been reported that BPA and NP express their activity at concentrations of 10^{-6} to 10^{-7} M in the human breast cancer cell line MCF-7, whereas 17β-estradiol is still effective at 10^{-7} to 10^{-11} M (Inadera *et al*., 2000). It is well established that IgG, but not IgM, anti-DNA antibody production and subsequent IgG immune complex deposition in the kidney are well correlated with the development of lupus nephritis, as reviewed by Shirai *et al*. (1991). Because IgG anti-DNA antibody production needs T cell help, including cytokine production, it is possible that the serum level of BPA is enough to activate anti-Br-RBC autoantibody by B1 cells, but not enough to activate helper T cells to provide T cell help essential for IgG class switching. It has been reported that E2 can modulate both pro-inflammatory and anti-inflammatory cytokine syntheses by CD4 T cells depending the dose of hormone (Gilmore, Weiner, and Correale, 1997). Nevertheless, many other articles describing the effect of estrogens on T cells indicate that the effect varies depending on the experimental systems employed, and therefore the precise mechanism

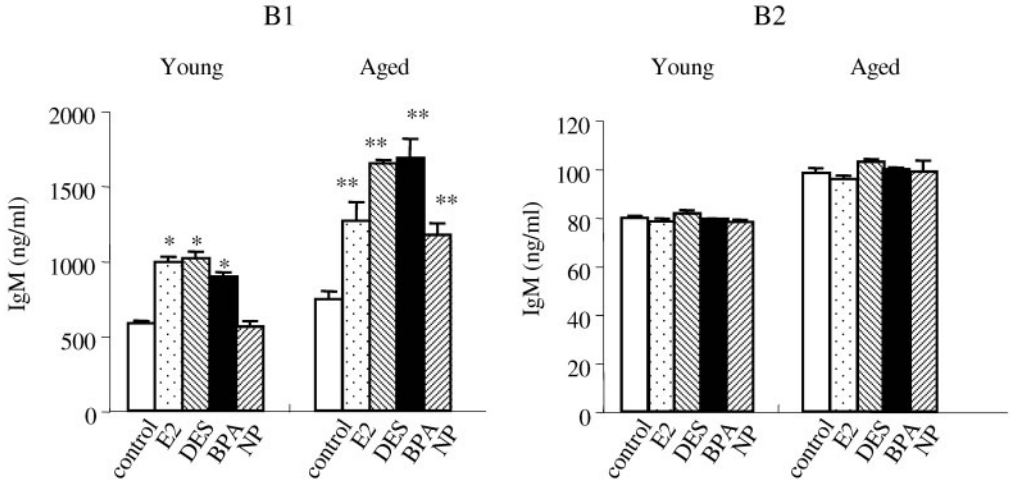

FIG. 7. Direct effect of EDs on IgM antibody production by B1 cells. B1 or B2 cells were cultured in the presence of E2 (100 nM), DES (100 nM), BPA (1 μM), or NP (1 μM) for 4 days, and the amounts of total IgM in the culture supernatants were determined by ELISA as described in *Materials and Methods*. The optimal concentration of each endocrine disruptor was pre-determined by dose-response experiments. The results are presented as the mean ± SEM. A representative result from three independent experiments is presented. $*p < 0.05$, $**p < 0.01$.

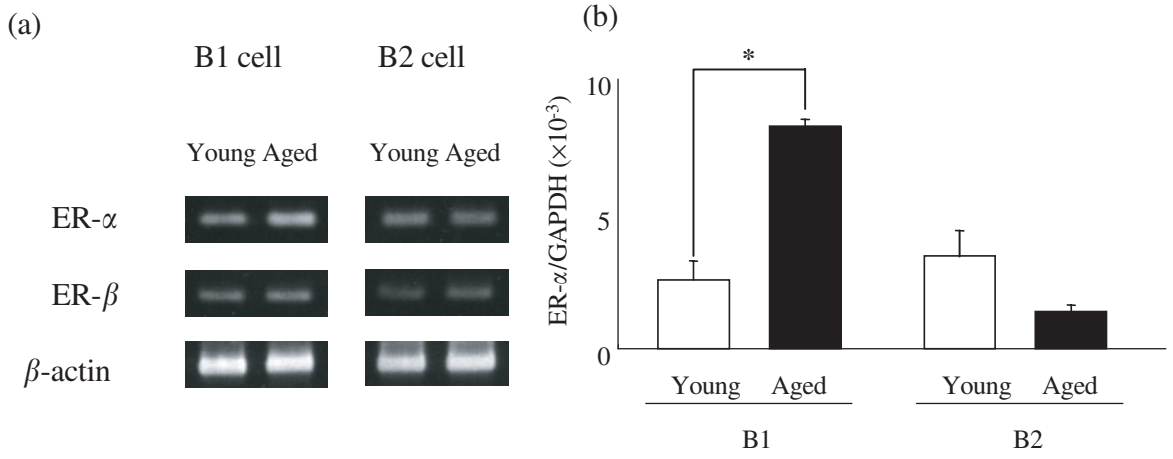

FIG. 8. Increased expression of estrogen receptor (ER)-alpha gene in B1 cells in aged BWF1 mice. Expression level of ER-α and ER-β genes in B cells was examined by RT-PCR (a) and quantitative real-time PCR (b) analysis. B1 cells were prepared from peritoneal cells and B2 cells from spleen. Total cellular RNA prepared from B1 or B2 cells of young or aged BWF1 mice were used for RT-PCR and quantitative real-time PCR analysis as described in *Materials and Methods*. A representative result of three experiments is presented $*p < 0.01$.

of action remains largely unknown (Ahmed, 2000). It remains to be elucidated whether Ig class-switching from IgM to IgG occurs in B1 cells in the development of autoimmune diseases. Preliminary data suggest that B1 cells produce IgG antibody in the presence of CD4 T cells obtained from aged, but not young BWF1 mice (unpublished data). It is possible that T cells as well as B2 cells have a higher threshold for endocrine disruptors than B1 cells do. In other words, B1 cells are immunocompetent cells highly sensitive to endocrine disruptors in the immune system. In the present study, mice were implanted with 300–350 mg/kg of BPA; yet the safe daily intake for BPA is 50 mg/kg/day. On the other hand, the permissible elution limit of BPA from polycarbonate tableware is 2.5 ppm, whereas actual elution from tableware is at most several parts per billion. It is, therefore, very unlikely that people are exposed to the high dose of BPA used in the present study. However, because B1 cells appeared to be more sensitive to EDs in the immune system, the effect of lower doses and longer exposures of BPA should be investigated in the future.

Many EDs bind to ERs, and then the estrogen–ER complex recognizes the estrogen response element (ERE) located in the 5′-flanking region of a particular gene to be expressed (Nishikawa *et al*., 1999). However, it remains unknown which genes in B1 cells are activated or suppressed by stimulation with EDs. Comprehensive gene expression analysis on B1 cells by SAGE (serial analysis of gene expression) or by DNA

microarray will be useful to identify the responsible genes for enhanced autoantibody production by B1 cells. It should be noted that ERα mRNA in B1 cells was increased in aged BWF1 mice, whereas that in B2 cells was rather decreased. Erlandsson *et al*. (2003) reported that ER-α, but not ER-β, is necessary for the E2-mediated elevation of immunoglobulin production by B cells in young mice. It was also demonstrated that E2 treatment suppresses B-cell development in the bone marrow (Medina, Stasser, and Kincade, 2000) and stimulates antibody production (Carlsten *et al*., 1989). Enhanced expression of ER-α gene in B1 cells in aged BWF1 mice may possibly facilitate autoantibody production by B1 cells by EDs during the development of lupus nephritis.

In conclusion, we demonstrated that endocrine disruptors directly enhanced autoantibody production by B1 cells. The increased incidence of autoimmune diseases such as SLE and ITP for several decades may possibly be attributed to accumulated endocrine disruptors. B1 cells, because of their high sensitivity, may also provide a good tool for evaluating biological significance of endocrine disruptors for the development of autoimmune diseases.

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