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Estrogen Increases the Number of Plasma Cells and Enhances Their Autoantibody Production in Nonautoimmune C57BL/6 Mice

Daniela I. Verthelyi[1] and S. Ansar Ahmed[2]

Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia 24061

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The immunological consequences of chronic estrogen exposure in normal individuals are not known, particularly in relation to B cells. In this study, by employing ELIspot, image cytometry, flow cytometry, cytology, and ELISA, we show that long-term exposure of normal mice to estrogen activates B cells to produce higher numbers of not only immunoglobulin-producing cells, but also autoantibody-producing cells. Estrogen promoted a decrease in B220$^+$ splenic lymphocytes, but resulted in a 10-fold increase in plasma cells. Further, the output of immunoglobulins including autoantibodies from individual plasma cells from estrogen-exposed mice was markedly increased, suggesting B cell hyperactivity. Importantly, our findings show that treatment of normal mice, solely with estrogen, can override B cell tolerance and promote autoreactive B cells in normal individuals.

INTRODUCTION

For many years the immune system was thought to work in isolation. In recent years, as our understanding of the immune system improved, the intricate interactions between the immune and endocrine systems have been recognized. Studies on understanding the interactions of sex hormones, such as estrogens, on the immune system are becoming increasingly important for several reasons. First, women, in general, have higher levels of serum immunoglobulins and resist a variety of infections more successfully than their male counterparts (1–4). These effects are largely being attributed to sex hormones, suggesting their physiological role (1–4). Second, women have a higher incidence of autoimmune diseases (1, 2, 5). For example, women to men susceptibility ratios for systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjogren's syndrome are 10:1, 2–4:1, and 9:1, respectively (1, 5). The pathogenic role of sex hormones on autoimmune diseases is implicated by findings that the course of autoimmune diseases is modulated during pregnancy and menarche (1–4, 6) and the development of SLE-like symptoms in healthy women given inducers of ovulation, which increase levels of reproductive hormones including estrogen (5). Further, abnormal sex hormonal metabolism in SLE and RA patients has been reported (3, 7, 8). Third, the intake of estrogenic compounds for medical reasons (for, e.g., estrogen replacement by postmenopausal individuals) is on the increase. Adult exposure to estrogen (9, 10) or prenatal exposure to a synthetic estrogen like diethylstilbestrol (DES (11, 12)) has been linked to cancer. Whether estrogen-modulated effects on the immune system have contributed to cancer is a provocative possibility, which warrants investigations. Further, women prenatally exposed to DES have a higher incidence of autoimmune diseases (13). Finally, a large number of pesticides and insecticides have been found to have estrogenic effects which result in altered development as well as reproductive and immune function of a range of wild species (14).

The effects of estrogen on the regulation of the immune system have also been studied in normal and autoimmune-prone mice (15). Analogous to the human situation, normal female mice to have higher serum levels of immunoglobulins, mount a stronger response to immunization, and produce higher levels of antibodies to a variety of heteroantigens than males (1, 2, 15). Estrogens modulate B cell functions in the female reproductive tract of normal rats and are thought to play a major role in local mucosal immunity (16, 17). The majority of studies to date, however, have focused on the effects of sex hormones on strains that spontane-

[1] Present address: Division of Viral Products, CBER/FDA, 29 Lincoln Drive, Building 29A, Room 3D 02, Bethesda, MD 20892. E-mail: Verthelyi@a1.cber.fda.gov.

[2] To whom correspondence should be addressed at Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA 24061. E-mail: ansrahmd@vt.edu.

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ously develop autoimmune disorders. In general, male hormones have been shown to inhibit or ameliorate several autoimmune diseases (1, 4, 15). Female hormones, such as estrogen, have been shown to accelerate the course of B-cell-mediated autoimmune diseases (reviewed in 15, 18). We have previously shown that exposure of C57BL/6 and BALB/c mice to estrogen induces the serologic expression of autoantibodies to dsDNA and cardiolipin of IgM and IgG, but not IgA, isotypes (19–21). Importantly, the expression of anti-dsDNA and anti-cardiolipin antibodies was sustained for months after the removal of the exogenous source of estrogen, indicating that the immunomodulatory effect of this hormone is long lasting (19, 22).

The above largely *serology-based* data clearly demonstrate that estrogen boosts the levels of immunoglobulins and autoantibodies. In the present study, we investigated the effects of *in vivo* exposure to estrogen at the cellular level in nonautoimmune mice. To our knowledge, there are no comprehensive studies addressing this aspect. Our data clearly show that estrogen treatment of normal mice promotes B cell hyperactivity in the spleen and bone marrow resulting in an increase in the number of immunoglobulin-secreting plasma cells as well as the amount of antibodies secreted per cell.

MATERIALS AND METHODS

Mice. Three-week-old male C57BL/6J mice purchased from the Jackson Laboratories (Bar Harbor, ME) were housed in standard cages (three to five mice/cage) with a 14 light/10 dark h schedule and fed on a commercial diet. All experimental animals were cared for and maintained as per the VPI & SU institutional guidelines.

Sex hormonal treatment. Four-week-old male C57BL/6 mice were orchiectomized as described previously (18–22). Silastic implants (7 mm long, Dow Corning Co., Auburn, MI), containing 4–6 mg of 17 β-estradiol (E2; Sigma, St. Louis, MO), were surgically placed subcutaneously as reported earlier (18–22). Orchiectomized mice that received placebo implants served as controls. Silastic implants release hormones slowly for several months thus inducing sustained estrogen levels comparable to those found in pregnant mice (18, 19). Age-matched mice that were treated for exactly the same duration with either placebo or estrogen were terminated and examined for effects on B cell functions. Additional assessment of estrogen activity *in vivo* included: (i) osteopetrosis of the long bones and reduced bone marrow cellularity (18, 23); (ii) atrophy of the thymus and depletion of thymocytes numbers (18, 24); (iii) distention of the urinary bladder (25); and (iv) increased seminal vesicle weights (18).

Serum collection. Blood was collected retroorbitally at the time of death and sera were kept frozen at −70°C until testing for the presence of antibodies.

Isolation of lymphocytes and purification of B cells. Lymphocytes from bone marrow and spleen were isolated and enumerated as described in our earlier studies (18). Briefly, spleens were collected under sterile conditions, gently teased over a wire mesh to produce a single cell suspension. Red blood cells were eliminated by Tris ammonium chloride lysis buffer (pH 7.2). Cells were washed four times with incomplete cold RPMI medium (Mediatech, VA) and then resuspended at 5×10^6 cells/ml in RPMI medium–10% fetal calf sera (Atlanta Biologicals, Atlanta, GA) containing 1 mM nonessential amino acids (ICN Pharmaceuticals, Costa Mesa, CA), 2 mM L-glutamine (ICN), and 2-mercaptoethanol (50 mM; ICN). Bone marrow lymphocytes were obtained by flushing long bones (femur and humerus) with RPMI 1640 medium and processed in a similar fashion. Viability was above 98% as assessed by the trypan blue exclusion method. The cellularity of the bone marrow from estrogen treated mice was severely reduced.

Splenic B cells were enriched by cytolytic elimination of T cells. Splenic lymphocytes were incubated with affinity-purified rat anti-mouse Thy1.2 monoclonal antibody (10 μg/10^7 cells; ATCC cell line, 30-H12) for 45 min at 4°C, as described previously (18). Cells were then washed and resuspended at 10^7 cells/ml in low-tox baby rabbit complement (1:10 in plain RPMI; Pel-Freez, Brown Deer, WI) for 30 min at room temperature. Cells were then washed three times and resuspended in complete medium. The enrichment of B cells in the resulting cell preparations was determined by staining with phycoerythrin (PE)-conjugated rat anti-mouse B220 antibodies (1 μg/10^6 cells; PharMingen, San Diego, CA) and analyzed by flow cytometry (Epics Coulter, XL/MXL).

Quantification of plasma cells in spleen. Segments of freshly collected spleens were imprinted on a glass microscope slide, air dried, and then stained with Wright's stain. A differential count of 1000 cells was performed under 100× magnification. Samples were read blind and decoded retrospectively.

ELIspot assays. The number of B cells actively producing antibodies were enumerated utilizing the ELIspot assay performed as described (26, 27) with minor modifications. Briefly, 96-well flat-bottom well, high-binding ELISA plates (Costar, Cambridge, MA), were coated with 50 μl of either dsDNA (Sigma; 70 μg/ml in PBS), cardiolipin (Sigma; 50 μg/ml in 100% ethanol), actin (Sigma; 10 μg/ml in PBS), ovalbumin (Sigma; 0.1%), TNP–bovine γ-globulin (TNP-BGG); 10 μg/ml) or μ and γ heavy-chain-specific goat anti-mouse immunoglobulin (Southern Biotechnology Assoc., Birming-

ham, AL; 1 μg/ml in PBS). Plates were incubated for 48 h under sterile conditions at 4°C. Plates were then washed with PBS and blocked for 2 h with 125 μl per well of PBS–2% BSA. Single cell suspensions were serially diluted (100 μl/well) starting at 5×10^5 and 1×10^5 cells per well for spleen and bone marrow, respectively, and incubated for 6 h at 37°C in a 5% CO_2 incubator. Wells were thoroughly washed (five times) with PBS and alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (1:3000, Southern Biotechnology) in PBS 2% bovine serum albumin (BSA) were used as secondary antibodies. 5-Bromo-4-chloro-3-indolyl phosphate, a substrate for alkaline phosphatase, at 2.3 mM in 2-amino-2-methyl-1-propanol buffer was mixed in 4:1 ratio with 3% agarose to be used as substrate. The number of spots (each representing an antibody-producing cell) was counted under a 10× magnification. The data are shown as the number of cells actively secreting antibodies per 10^6 cells.

Flow cytometric analysis. The phenotype of B cells was determined by staining the splenic lymphocytes with optimal concentration of PE-conjugated rat anti-mouse B220 antibodies (0.5 μg/10^6 cells), PE-conjugated rat–anti-mouse CD19 (0.625 μg/ml), or PE–rat immunoglobulin isotype control (PharMingen) as reported (18, 28, 29). The cells were subjected to flow cytometry (Epics Coulter, XL/MXL) and analyzed by Immuno-4 software program.

ELISA assays. Antibodies in sera were assessed by the standard ELISA as described previously (19–21). Cardiolipin (50 μg/ml) was coated on medium-binding 96-well flat-bottom plates while high-binding plates were used for all other antigens. S1 nuclease-treated dsDNA (70 μg/ml in Tris–EDTA, pH 8.0) was coated on methylated BSA-coated plates. Other antigens used included: actin (10 μg/ml in carbonate–bicarbonate buffer), ovalbumin (100 μg/ml in PBS) (27), lysozyme (1 μg/ml in PBS), acetone-killed *Brucella abortus* strain RB51 (a gift of Dr. G. Schurig, 2 μg/ml in carbonate–bicarbonate buffer), and *Actinobacillus pleurpneumoniae* serotype 7 strain 29628 (a gift of Dr. T. Inzana, 10^9 CFU/ml in 1% formalin/PBS diluted 1:5 in carbonate–bicarbonate buffer). Antigen-coated plates were incubated overnight at 4°C. Subsequent steps were performed as previously described (19–22). Negative controls included wells with all reagents except serum ("no serum blanks") or without antigen ("no antigen blanks"). In addition, known positive controls were included to monitor the validity of the assays. Absorbancies are expressed as median specific OD which were obtained by (mean OD of sample minus mean OD of no serum blanks on the plate) (19).

Morphometric analysis. Quantification of the size and density of the spots was performed utilizing a color image analyzer (Olympus Cue 3 software; Galai Production Ltd., Israel). Nine to twelve wells per antigen and isotype were assessed for each treatment group. The image of ELIspots was recorded using a CCI single-chip camera (Autotechnica 852). Spots were digitized and analyzed for area and density. Spots that were too close to be analyzed independently were electronically gated out. The sensitivity thresholds were maintained constant for all wells. Graphs illustrate the mean density and area of spots from each well.

Statistical analysis. Two-tail nonparametric ANOVA or Mann–Whitney U tests were employed given the lack of normality of the distribution of the data.

RESULTS

Estrogen induces an increase in the number of spleen and bone marrow lymphocytes spontaneously secreting IgG and IgM immunoglobulins. The *in vivo* effects of estrogen at a cellular level on the production of antibodies by spleen and bone marrow lymphocytes was assessed by ELIspot in the absence of any obvious stimuli. The number of IgG- and IgM-secreting cells in

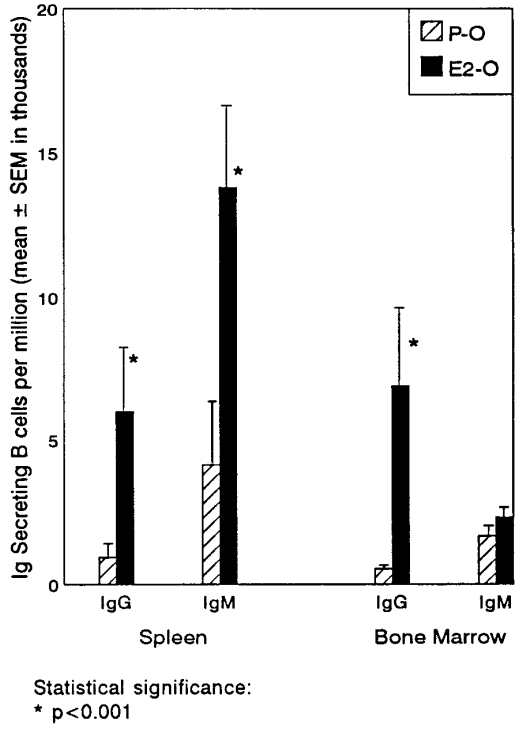

FIG. 1. Estrogen increases the number of immunoglobulin-secreting cells in the spleen and bone marrow. The number of IgG and IgM immunoglobulin-secreting cells per million lymphocytes derived from spleens (n = 14) and bone marrows (n = 12) of estrogen-treated orchiectomized (E2-O) and placebo-treated orchiectomized (P-O) mice was determined by ELIspot. Comparisons between placebo- and estrogen-treated mice were made between mice that were age matched and had undergone exactly the same duration of hormone treatment (4–6 months). Nonparametric Mann–Whitney U test was used to analyze the data.

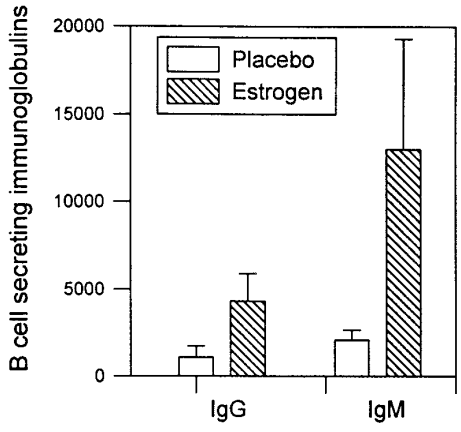

FIG. 2. Purified B cells from spleen of estrogen-treated orchiectomized mice ($n = 4$) have a higher number of cells secreting immunoglobulins than age-matched placebo-treated orchiectomized. Mice were utilized 4 months after estrogen treatment ($n = 5$). Nonparametric Mann–Whitney U test was used to analyze the data.

spleen and bone marrow from fourteen 4-month-old male C57BL/6 estrogen-treated mice (and an equal number of placebo controls) were assessed. As shown in Fig. 1, estrogen induced an increase in the number of IgG (6-fold) and IgM (3-fold)-secreting splenic lymphocytes compared to controls ($P < 0.001$). Lymphocytes from bone marrow also showed a 12-fold increase in the number of cells actively secreting IgG immunoglobulins ($P < 0.001$) but there was no significant increase in IgM secreting B cells.

Purified splenic B cells from placebo and estrogen-treated mice were also utilized to assess whether the estrogen-induced increase in the number of cells secreting immunoglobulins was dependent upon the presence of T cells and macrophages in culture. As in the case of unseparated lymphocytes, purified splenic B cells from mice treated with estrogen had increased numbers of splenic lymphocytes actively secreting immunoglobulins of both IgG and IgM isotype ($P < 0.05$) (Fig. 2). Since the pattern of increased immunoglobulin-secreting cells in the unseparated and purified B cells was similar, and given that estrogen markedly reduces the lymphocyte numbers (see below), only unseparated population of cells were used for subsequent experiments.

Estrogen does not increase the number of B cells in spleen. An increase in Ig-secreting cells in the spleen could be a result of (a) an increase in the number of lymphocytes or (b) a selective expansion of the B cell population. The total number of lymphocytes recovered from the spleen and bone marrow was consistently reduced (Table 1). The possibility of a selective expansion of B cells in the spleen was next explored. Flow cytometric analysis of a pan B cell marker B220 revealed that the exposure to estrogen did not increase the proportion of $B220^+$ cells, but rather reduced it

TABLE 1

Estrogen Decreases Lymphocyte Numbers in both Spleen and Bone Marrow

Groups	*n*	Tissue	Lymphocyte yield ($\times 10^6$)
Placebo	11	Spleen	116.0 ± 8.0
Estrogen	14	Spleen	60.5 ± 5.0
Placebo	6	Bone marrow	4.45 ± 2.5
Estrogen	10	Bone marrow	0.4 ± 0.2

Note. Prepubertal C57BL/6 mice were orchiectomized and given placebo or estrogen implants and were terminated, at the same time. Age-matched placebo and estrogen-treated mice that were treated with hormone or placebo implants (4–6 months) were compared. The total lymphocyte cellularity in the spleen and bone marrow was determined. Note decreased lymphocyte yield in bone marrow and spleen from mice treated with estrogen ($P < 0.01$ and $P < 0.05$, respectively).

(Table 2). The relative numbers of $B220^+$ were compared to the total splenic lymphocyte numbers. As shown in Table 2, the estrogen-treated mice not only had decreased relative numbers of $B220^+$ cells, but also decreased absolute numbers of $B220^+$ (per spleen). The reduction in $B220^+$ splenic cells was not due to selective downregulation of B220 surface marker on B cells, since the proportion of $CD19^+$ splenic B cells was also decreased (data not shown).

Estrogen induces an increase in the number of splenic plasma cells. The above observations suggested that estrogen did not induce a general expansion of the B cell population. The increased B cell activity may be due to an increase in plasma cells, which do not express (or have very low expression of) B220 and CD19 antigens. To address this possibility, plasma cell numbers were determined on imprints of sections of spleens (Fig. 3 and Table 3). Differential counts of spleen cells revealed a 10-fold ($P < 0.005$) increase in plasma cell

TABLE 2

Estrogen Decreases Numbers of Splenic Cells Expressing B220

Groups	*n*	Percentage of positive ± SEM	Total $B220^+$ cells/spleen ± SEM
Placebo	6	68.7 ± 4.1	68.3 ± 15.2
Estrogen	6	55.5 ± 6.9	27.2 ± 4.2

Note. The splenic lymphocytes from placebo or estrogen-treated mice were stained with PE–anti-B220 or control PE–isotype-matched antibodies and subjected to flow cytometry. Comparisons between placebo- and estrogen-treated mice were made between animals that were age matched and had undergone 6-month duration of hormone/placebo treatment. The percentage of $B220^+$ cells were related to the total lymphocyte yield per spleen to obtain total $B220^+$ population in the spleen. Spleens from estrogen-treated mice had significantly reduced number of $B220^+$ cells/spleen ($P < 0.03$).

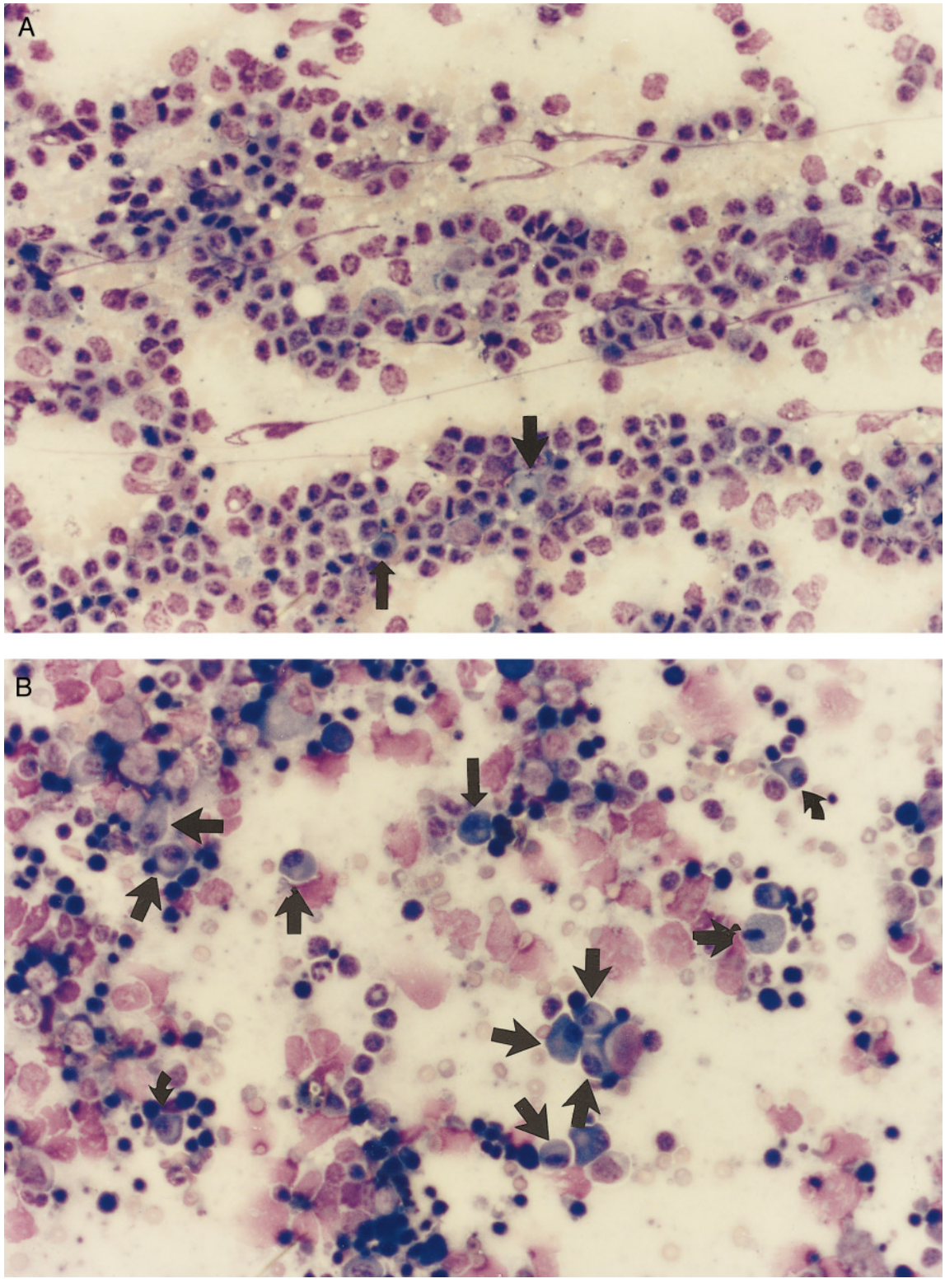

FIG. 3. Imprints of spleens from placebo and estrogen-treated orchiectomized 6-month-old C57BL/6 mice were fixed and stained with hematoxylin–eosin. (A) From placebo-treated mouse; (B) From estrogen-treated mouse. Photographs were taken under a 200× magnification. Note the increase in plasma cells (indicated with arrows) in the spleen of the mouse treated with estrogen.

TABLE 3
Estrogen Decreases Small Lymphocytes, but Increases Plasma Cells

Lymphocyte types	Placebo percentage ± SEM	Estrogen percentage ± SEM	Statistical significance
Small lymphocytes	95.88 ± 1	89.5 ± 1.4	$P < 0.01$
Prolymphocytes	1.56 ± 0.8	0.91 ± 0.25	ns
Lymphoblasts	0.72 ± 0.2	1.15 ± 0.17	ns
Plasma cells	0.36 ± 0.12	3.38 ± 0.6	$P < 0.0025$

Note. Imprints of cut splenic tissue from age-matched estrogen ($n = 9$) and placebo ($n = 9$)-treated orchiectomized mice were air dried and stained with H & E. Mice were treated for the same duration (4–6 months) with either placebo or estrogen. One-thousand cells were identified and enumerated "blind" by a clinical pathologist. The results are expressed as a percentage of the cells.

numbers in spleens from estrogen-treated orchiectomized mice than placebo-treated orchiectomized mice. Small lymphocytes were decreased in the spleens of estrogen-treated mice.

Estrogen increases the quantity of immunoglobulin yield per cell. It was observed in the ELIspot assay that the wells from lymphocytes from estrogen-treated mice not only had more spots than those from placebo-treated ones, but also that the spots appeared to be larger and more intensely stained. To quantify the size and density of the individual spots in the wells, we utilized a color image analyzer. As shown in Fig. 4, individual spots made by spleen (IgG and IgM) and bone marrow lymphocytes (IgG) from estrogen-treated mice are significantly larger and denser than those of placebo controls ($P < 0.01$).

Nonautoimmune mice treated with estrogen express antibodies to a variety of auto- and heteroantigens. In previous studies we had shown that sera of estrogen-treated mice had increased levels of IgG antibodies to self-antigens such as dsDNA, cardiolipin, and anionic phospholipids. Given our current findings that estrogen promotes plasma cell activity, we determined whether estrogen preferentially activated autoreactive B cell clones. The sera of 5-month-old male C57BL/6 mice, treated with estrogen or placebo implants, were tested by ELISA for the presence of antibodies to a panel of auto- and heteroantigens. As shown in Fig. 5, estrogen-treated nonautoimmune C57BL/6 mice expressed increased amounts of IgG antibodies to self-antigens cardiolipin, dsDNA, and actin and also to foreign proteins, such as ovalbumin and lysozyme, as well as to complex microbial antigens: acetone-killed *B. abortus* strain RB51 and *A. pleuropneumoniae.* These results appeared to indicate that estrogen promotes polyclonal B cell activation.

Estrogen increases the number of lymphocytes from spleen and bone marrow that produce antibodies to auto- and heteroantigens. The ability of estrogen to activate B cells against selected specificities was next assessed. The number of cells in the spleen and bone marrow producing antibodies to cardiolipin, ssDNA, dsDNA, actin, ovalbumin, and TNP were quantified by ELIspot. As shown in Figs. 6a and 6b, lymphocytes from placebo-treated mice had almost no detectable IgG antibody-forming cells against the antigens tested. Lymphocytes from estrogen-treated mice, however, showed a significant increase in the expression of cells spontaneously secreting IgG antibodies to self- and foreign antigens in the bone marrow and the spleen (Figs. 6a and 6b). Estrogen also induced a significant increase in the number of IgM antibody-secreting cells for all specificities tested (data not shown). To evaluate whether there is a bias toward autoreactivity in estrogen-treated mice the numbers of IgG lymphocytes secreting antibodies to self- and non-self-antigens were related to the total number of IgG-secreting cells for individual mice. The data indicate that the proportion of IgG-secreting B cells directed to self- and foreign

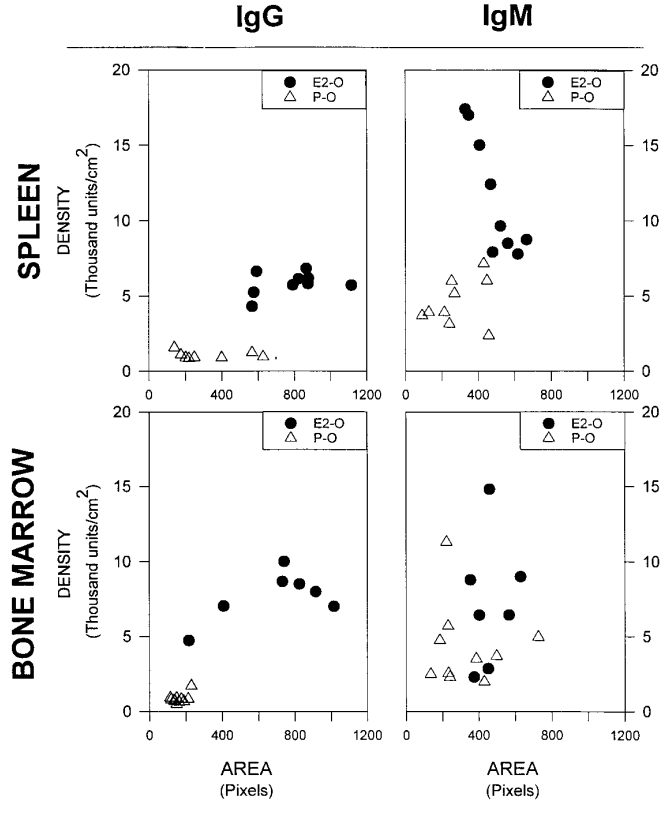

FIG. 4. Estrogen increases immunoglobulin yield per plasma cell. Morphometric analysis of the density and pixels (indicative of area) of the spots from spleen and bone marrow lymphocytes was performed. Placebo- and estrogen-treated mice that were age matched and had undergone exactly the same duration of hormone treatment (4–6 months) were compared. The mean area and density of the spots per well in an ELIspot assay are depicted. Nine to twelve mice were assessed for each group. Nonparametric Mann–Whitney U test was used to test differences among treatment groups.

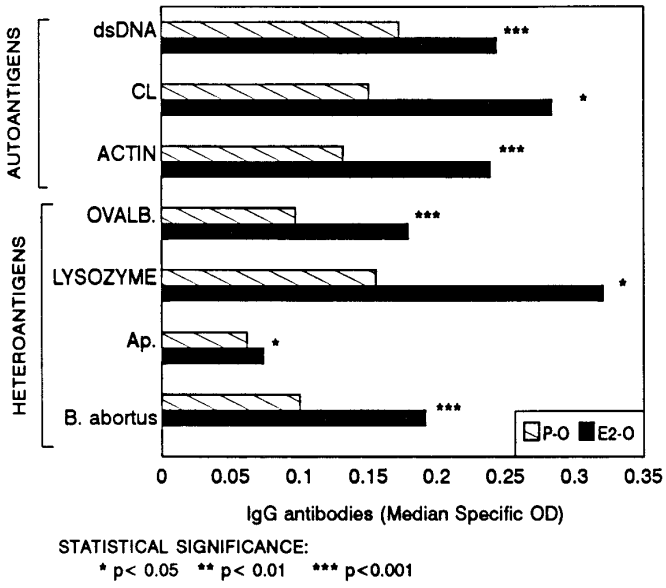

FIG. 5. Sera from orchiectomized male age-matched C57BL/6 mice treated with estrogen or placebo subcutaneous implants were tested for the expression of IgG antibodies to dsDNA, cardiolipin (CL), actin, ovalbumin (OVALB.), heat-killed *A. pneumoniae* (Ap.), and acetone-killed *B. abortus*. Age and estrogen/placebo duration (4–6 months)-matched mice were compared. Mann–Whitney U analysis was used to test for statistical differences among groups, since the absorbencies did not have a normal distribution. Accordingly, the graph shows the median value for each group.

antigens was similar in both estrogen- and placebo-treated mice (Figs. 6c and 6d). These results suggest that estrogen may act as a polyclonal B cell activator.

Antibody yield per cell in spleen and bone marrow. The mean area and density of the antigen-specific spots from nine to eleven 4- to 6-month-old mice were assessed. In general, morphometric characterization of the area and density of the antigen-specific spots from estrogen-treated mice was increased compared to those from placebo-treated mice. In the spleen, IgM antigen-specific spots from lymphocytes of estrogen-treated mice tended to be larger and denser for all specificities tested (Fig. 7a). The difference in size and density of antigen-specific IgG spots generated could not be tested due to the paucity of antigen-specific plasma cells in placebo-treated mice. Bone marrow lymphocytes from estrogen-treated mice had a significant increase in the area and density of the spots of IgM and IgG anticardiolipin-forming cells from estrogen-treated mice (Fig. 7b). Spots formed by bone marrow lymphocytes to other specificities, although larger and denser, were not statistically different from those made by the controls.

DISCUSSION

It is now well documented that sex hormones modulate the immune system of humans and animals (1, 2, 4, 30). However, to date most studies have focused on demonstrating the role of sex hormones in modulating autoimmune diseases. Sex hormone influences have been demonstrated in both experimental organ-specific and non-organ-specific autoimmune diseases (3, 24, 31–34). In general, estrogens promote the development of autoantibodies in several experimental autoimmune models. For example, administration of estrogen to mice which are genetically prone to develop autoimmune disease ((NZB × NZW)F_1 and MRL/1pr) accelerated expression of autoantibodies, development of lymphoadenopathy, arthritis, immune-complex glomerulonephritis, and death (1, 6, 32, 33). Estrogen-treated (NZB X NZW)F_1 orchiectomized mice also had increased antibodies in response to injections of non-self-antigens (35). Similarly, in nonspontaneous models for SLE, such as Balb/c mice injected with anti-DNA (16/6 id+) antibodies, estrogen treatment accelerated expression of autoantibodies (36).

The precise effects of estrogen on the immune system of normal individuals are not well studied. A better understanding of the effects of sex steroids on the normal immune system is of paramount importance

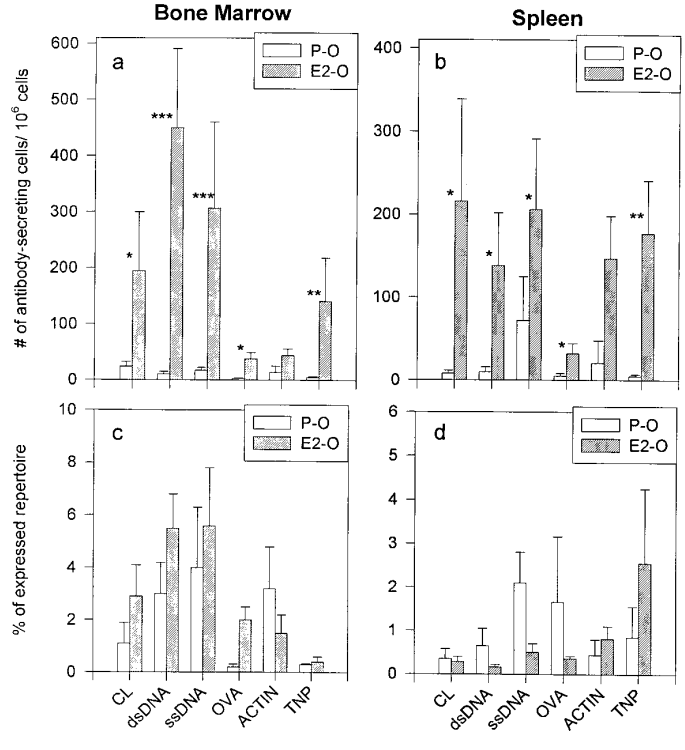

FIG. 6. Antibody-secreting cells in spleen and bone marrow. The number of antigen-specific antibody-secreting cells per million lymphocytes of IgG isotype in bone marrow (a) and spleen (b) is shown. The frequency of B cells producing antibodies reactive with each antigen, expressed as a percentage of the total number of immunoglobulin-secreting cells is shown in (c and d). Comparisons between placebo (n = 9) and estrogen (n = 12)-treated mice were made between mice that were age matched and had undergone exactly the same duration of hormone treatment (3–5 months).

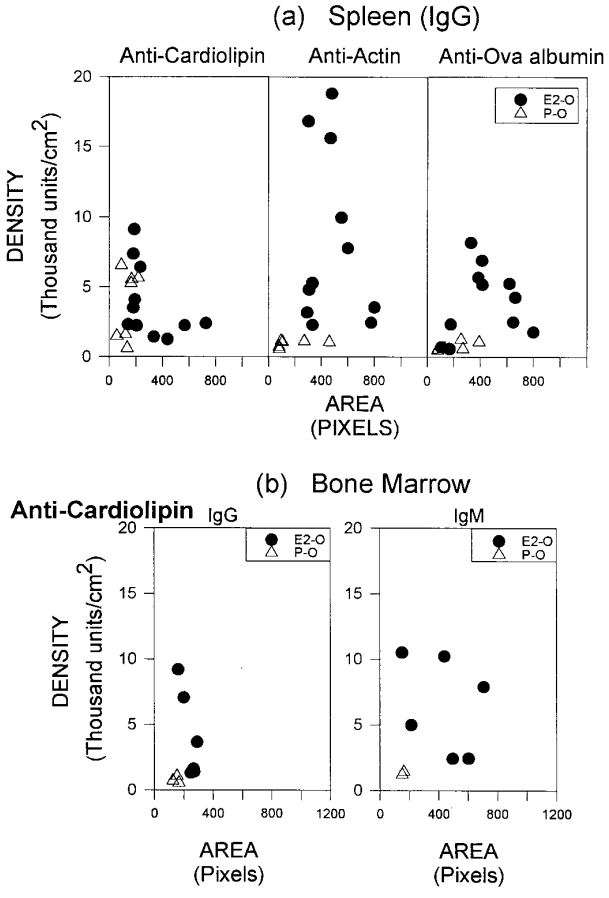

FIG. 7. Morphometric analysis of the antigen-specific IgM spots observed by ELIspot assay. (a) The mean area and density of the antibody spots of lymphocytes from spleen of estrogen-treated orchiectomized (E2-O) and placebo-treated orchiectomized (P-O) mice. Each point represents the mean value of the spots per well. Nine to twelve mice per group were assessed for each antigen. Comparisons between placebo- and estrogen-treated mice were made between mice that were age matched and had undergone exactly the same duration of hormone treatment (4–6 months). Note that of the 12 estrogen-treated orchiectomized mice, 11 had antibody-secreting cells against anti-ovalbumin, for example, while only 3 of 12 placebo-treated orchiectomized mice had cells secreting antibodies for anti-ovalbumin. Note that the spots from placebo-treated mice were smaller and less denser. (b) The area and density of IgM and IgG anticardiolipin-specific secreting cells (6 mice per group). Note that the bone marrow from estrogen-treated mice had larger and more intensely staining spots compared to those from placebo-treated mice.

since: (i) increasing numbers of women around the world take estrogen/progesterone-based oral contraceptives, sometimes for most of their life; (ii) estrogens are often prescribed to postmenopausal women (as a replacement therapy) and nulliparous women (to induce ovulation); and (iii) a large number of pesticides and insecticides have now been found to have estrogenic effects and are thought to contribute to the developmental defects of the reproductive and immune systems in a range of wild species (14).

Studies in normal individuals show that estrogen promotes B cell activity. Addition of estrogen to pokeweed mitogen-stimulated cultures of PBMC from normal humans enhanced antibody plaque-forming cells (37). Estrogen treatment of normal rats enhanced antibodies against non-self-antigens, sheep red blood cells, and polysaccharides from type II pnemococci (38). Further, we had previously shown that treatment of normal C57BL/6J (19, 21, 22), C3H (18), and Balb/c (Verthelyi, Ansar Ahmed, and Schurig, unpublished) murine strains with estrogen alone accelerates the serologic expression of antibodies to self-antigens. Further, our studies on subisotype characterization of estrogen-induced cardiolipin and dsDNA IgG autoantibodies revealed that although all IgG subisotypes were detected, IgG2b predominated and to a lesser extent so did IgG1 (19, 22). It is not clear what cytokines aid in the switch to IgG2b; however, one study has reported that TGFβ1 promotes this subisotype switch (39). Whether estrogen promotes TGFβ1 or TGFβ1-like molecules [which are found in synovial fluid of rheumatoid arthritis patients (40)] in the spleen or bone marrow needs to be examined. In this study, we expand the scope of antigens tested to show that estrogen-treated nonautoimmune C57BL/6 mice produce IgG antibodies to a wide variety of self- and non-self-antigens. More importantly, we examined long-term estrogenic effects on the B cell functions at the cellular level, an area not studied thus far. We show that although estrogen reduces the total number of splenic lymphocytes, there was an increase in the number of plasma cells. ELIspot assay revealed that estrogen-treated C57BL/6 mice show a six-fold increase in the number of IgG-producing plasma cells and a three-fold increase in IgM-producing plasma cells in the spleen. These antibodies are directed against not only non-self-antigens but also against self-antigens. Additionally, by image cytometry we show that individual plasma cells from estrogen-treated mice secrete greater quantities of immunoglobulins and specific antibodies to both non-self- and self-antigens. These findings reinforce the notion that estrogen does not selectively increase autoantibodies, rather it promotes antibodies to a broad range of specificities. The increase in numbers of plasma cells together with their higher antibody output per cell in estrogen-treated mice may contribute to increased levels of immunoglobulins and autoantibodies in the sera of these mice.

Interestingly, we did not find a selective expansion of the total B cell population, since the number of $B220^+$ splenic cells tended to be reduced. It is noteworthy that the present studies focused on long-term effects of estrogen on B cell functions 4 to 6 months after the hormonal treatment. Kinetic studies showed that a similar reduction in the number of $B220^+$ and $CD19^+$ cells was evident at 2.5, 3, 3.5, 4, 4.5, and 5 months of estrogen treatment (Hissong, Vaught, Nordyke, and

Ansar Ahmed, unpublished observations). It is likely that exposure to estrogen may have resulted in an accelerated differentiation of mature B cells into plasma cells, which lose the expression of B220 and CD19 antigens on their surface. An increase in plasma cell numbers in estrogen-treated mice may be due to delayed cell death of plasma cells. Experiments are currently being performed to assess these possibilities in our laboratory.

It is important to stress that although estrogen-mediated activation of B cells overrides tolerance and allows for the development of autoreactive plasma cells, there was no preferential expansion of autoreactive clones. Several nonexclusive mechanisms may be postulated to explain the ability of estrogen to induce B cell hyperactivity to override self-tolerance. First, estrogen may act directly on mature B cells to induce hyperactivity. However, estrogen receptors (presumably α receptors) have not been demonstrated on mature B cells (1, 6). Estrogen receptors have nevertheless been found on pro-B cells and stromal cells of the bone marrow (41). Recently a new class of estrogen receptor, $E_2R\beta$, has been identified (42, 43), which may be present on lymphoid cells and tissues, and their presence on B cells is yet to be explored. Second, estrogen may modulate cells that regulate B cell activity, for example, by downregulating T cells, macrophages, or NK cells with suppressor functions. In support of these findings, estrogen has been shown to reduce suppressor (24, 44) and NK cell (18, 31, 45) functions. Alternatively, estrogen may affect the synthesis of T-cell-derived cytokines (46, 47) which could modulate B cell functions. *In vitro* or *in vivo* studies show that estrogen or antiestrogens modulate T-cell-derived cytokines (46, 47). Cytokines such as IL-1, INF-γ, TNF-α, and TGF-β are modulated by estrogen (reviewed in 6 and 48). Preliminary evidence from our laboratory suggests that estrogen-treated mice have increased numbers of cells secreting IL-6 (Verthelyi and Ansar Ahmed, unpublished observations). Third, B cells in estrogen-treated mice may develop in an altered milieu. The estrogen-mediated reduction in the bone marrow mass could result in an increased exposure of immature B cells to self-antigens, as well as development in alternative sites which are less stringent in B cell selection. Our histopathological studies show that there are focuses of hematopoiesis in the spleen and liver of estrogen-treated mice (Verthelyi, Saunders, and Ansar Ahmed, unpublished observations). Others have shown that estrogen promoted the expansion of T cell receptor^{intermediate} lymphocytes including the forbidden T cell oligoclones in the liver (49). These include $V\beta8^+$ cells which are believed to be involved in some autoimmune diseases (50, 51). Further, female C57BL/6 mice have been shown to have increased T cell receptor^{intermediate} cells in the liver than males (52). It is speculated that overstimulation of these cells may initiate autoimmunity (52). Fourth, another mechanism may be defective elimination of plasma cells due to, for example, altered apoptotic signals. Our recent detailed studies suggest that estrogen alters LPS or anti-CD40-mediated apoptosis of B cells (Verthelyi and Ansar Ahmed, manuscript in preparation). Other studies support this possibility by showing that estrogen modulates the expression of bcl-2 in the breast tissue cancer cells (53) or the rate of apoptosis in uterine epithelial cells (54). Further our recent studies show that increased percentages of splenic B cells from estrogen-treated normal mice, even in the absence of *in vitro* stimulation by mitogens, are in the S and G2/M phases of cell cycle. (Verthelyi and Ansar Ahmed, manuscript in preparation). This suggests that estrogens promote aberrant B cell hyperactivity. Fifth, estrogens may act through the hypthalamo–hypohyseal–thymic axis (2) to modulate the neuroimmunoendocrine regulatory circuits. Finally, estrogen may activate unknown endogenous infectious agents which may in turn modulate the B cell functions.

In summary, regardless of the above conjectural possibilities, these studies show that the estrogen is a powerful immunomodulator. Exposure of normal mice to estrogen alone, in the absence of any deliberate stimulation, promotes B cell hyperactivity by enhancing the numbers and activity of plasma cells producing antibodies to both self- and non-self-antigens. These studies have significant implications in clinical medicine pertaining to female-predominant autoimmune diseases and in situations involving long-term exposure to estrogenic compounds.

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