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*J Immunol* 2005; 175:1415-1423; doi: 10.4049/jimmunol.175.3.1415

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Tamoxifen Blocks Estrogen-Induced B Cell Maturation but Not Survival

Elena Peeva,† Jeganathan Venkatesh,* and Betty Diamond2*†

Estrogen treatment has been shown not only to exacerbate disease activity and accelerate death in spontaneous murine models of lupus but also to induce a lupus-like phenotype in nonsparovously autoimmune mice. In mice transgenic for the H chain of an anti-DNA Ab, estrogen rescues naive autoreactive B cells that normally are deleted and causes them to mature to a marginal zone phenotype. Estrogen further leads to the activation of this population causing an elevation of serum anti-DNA Ab titers and renal disease. This study was designed to evaluate the therapeutic potential of tamoxifen, a selective estrogen receptor modulator, on estrogen-induced lupus. Mice treated with both estradiol and tamoxifen showed no elevation in anti-DNA Ab titers and consequently no glomerular IgG. The DNA-reactive B cell population that is rescued by estrogen was present in an anergic state in mice treated with both estradiol and tamoxifen. Estradiol enhances transitional B cell resistance to apoptosis and expands the population of marginal zone B cells; tamoxifen did not impede the enhanced resistance to apoptosis, but prevented the development of autoreactive cells as marginal zone B cells. Thus, estrogen-induced autoimmunity proceeds through two distinct molecular pathways, one affecting survival and the other maturation. Activation, but not survival, of autoreactive B cells can be abrogated by tamoxifen. Drugs that modulate even some of the effects of estrogen may be beneficial in patients with lupus. Eventually, understanding the pathways involved in survival and activation of autoreactive B cells will permit the development of therapies that target all relevant pathways. The Journal of Immunology, 2005, 175: 1415–1423.

For many years, it has been appreciated that women have a 10-fold higher incidence of systemic lupus erythematosus (SLE) than men (1). It has also been speculated that sex hormones contribute to the predisposition of women for SLE (2, 3). Several clinical observations are consistent with this hypothesis; the onset of lupus generally occurs in women after menarche and before menopause; the selective metabolism of estrogen to yield highly active metabolites has been reported in lupus patients (4); males with lupus have been reported to have reduced levels of testosterone and dehydroepiandrosterone (5–7). More recently, the SELENA study (Safety of Estrogen in Lupus Erythematosus National Assessment) evaluated flare rates in women on hormone replacement therapy or placebo and found a significantly higher flare rate in those patients treated with hormone (8). This study confirmed the potential for estrogen, even low dose estrogen, to exacerbate disease.

The ability of estrogen to exacerbate disease has been clearly established in murine models of lupus. In both MRL/lpr and New Zealand Black/White (NZB/W) F1 lupus-prone mouse strains, female mice have more severe disease, and altering estrogen levels alters disease expression, with more estrogen leading to high titers of autoantibodies, more severe renal disease, and earlier death (9). Recent studies have begun to delineate pathways by which estrogen affects B cell survival and signaling (10–12). We have demonstrated that estrogen breaks tolerance in BALB/c mice transgenic for the H chain of an anti-DNA Ab. These mice are not spontaneously autoimmune, but a 6-wk exposure to 75 pg/ml estradiol, which is equivalent to the serum estrogen concentration at the luteal phase of the estrus cycle, will induce survival and activation of autoreactive B cells and cause these mice to develop high titers of anti-DNA Abs, glomerular deposition of IgG, and proteinuria (10). This increase in survival and activation of autoreactive B cells is accompanied by an estrogen-mediated shift in splenic B cell populations. Although estrogen leads to a decrease in transitional B cells, there is a relative increase in T2 transitional B cells compared with T1 transitional B cells, reflecting a loss of negative selection in the T1 B cell population. Furthermore, estrogen leads to an expansion of marginal zone (MZ) B cells (11). At the molecular level, estrogen-mediated effects on B cells include up-regulation of several genes known to be important in B cell survival and activation, notably bcl-2, cd22, and shp-1 (12).

If estrogen is able to modulate disease activity in murine models of SLE and in at least some patients, understanding the immunological effects of selective estrogen receptor modulators (SERMs) becomes a clinically relevant question. The most widely used SERM is tamoxifen (13, 14), which is commonly used as an estrogen receptor (ER) antagonist in patients with breast cancer. However, tamoxifen can act not only as an ER antagonist but also as an agonist, depending on the target tissue. For example, tamoxifen is an ER antagonist in the breast, but an ER agonist in the uterus (15).

Studies performed in mouse models of SLE suggest that tamoxifen is capable of modulating estrogen effects on the immune system. Administration of tamoxifen has been shown to ameliorate disease activity in the spontaneously lupus-prone NZB/W F1 and MRL–lpr/lpr mouse strains, as well as in BALB/c mice induced to develop anti-DNA Abs following immunization with a mAb 16–6.
(16–19). Although a small scale clinical trial of 11 SLE patients treated with tamoxifen yielded no evidence that tamoxifen had a beneficial effect on clinical or laboratory indices of disease activity (20), that study was too small to be definitive. Our own studies would suggest that there is a subset of lupus patients with an estrogen-exacerbated disease, and clinical studies are needed to identify the role of SERMs in the treatment of these patients.

Here we demonstrate that tamoxifen can prevent estrogen-induced lupus in BALB/c R4A-y2b transgenic mice, causing the autoreactive B cells to enter an anergic state. The data presented demonstrate that in estrogen- and tamoxifen-treated mice, B cell tolerance induction occurs later in B cell maturation than it does in hormonally unmanipulated mice.

Materials and Methods

Transgenic mice

Wild-type BALB/c mice were purchased from The Jackson Laboratory. R4A-y2b transgenic BALB/c mice were bred at the animal facility of the Albert Einstein College of Medicine. The R4A-y2b mice are transgenic for the H chain of the nephritogenic R4A anti-DNA Ab (21, 22). Eight 16-wk-old female mice were included in the experiments. However, age-matched mice were used in each experiment, and no differences were detected between experiments using the younger (8 wk) and the older (16 wk) mice.

Estrogen and tamoxifen treatment

R4A-y2b or wild-type BALB/c female mice were implanted s.c. with 17β-estradiol, tamoxifen, estradiol plus tamoxifen, or placebo pellets (Innovative Research of America) as previously described. Both estradiol and tamoxifen were administered to mice for 5 wk. They were screened for IgG Ab (10 μg/ml) (Southern Biotechnology Associates) for 18 h at 37°C. After incubation, splenocytes were washed and stained for CD19, fixed with paraformaldehyde and incubated with a rabbit polyclonal Ab to rabbit polyclonal Ig (BD Pharmingen) and caspase expression was evaluated in CD19+ cells. Caspase expression was performed with FACS Calibur flow cytometry. The data obtained from both apoptosis assays were analyzed with FlowJo software.

Materials and Methods

Flow cytometry

Isolated splenocytes were depleted of RBC by hypotonic lysis. Spleen cells were incubated with FITC, PE, PerCP, PE-Cy7, or allophycocyanin-conjugated Abs specific for CD19, y2b, CD21, CD22, CD23, and AA4.1 (BD Pharmingm) at 4°C for 30 min, then washed, fixed in 2% paraformaldehyde, and analyzed. For intracellular staining with Abs to Bcl-2 and SPh-1 (Santa Cruz Biotechnology), cells were fixed in 2% paraformaldehyde for 10 min, and then permeabilized with 0.3% saponin/PBS.

CD19, AA4.1, CD21, and CD23 staining was used to analyze immature (transitional T1 and T2) and mature (MZ and follicular) B cell subsets. AA4.1 staining differentiated transitional from mature B cells. CD21/CD23 staining was used to determine transitional T1 (AA4.1+CD21lowCD23low and T2 (AA4.1-CD21highCD23low) B cell subsets and mature MZ AA4.1+CD21highCD23low and follicular (AA4.1+CD21intmediateCD23high) B cell subsets.

Splenic B cell subsets were also determined by staining with CD19, CD21, CD23, and CD24 and CD24 staining was used to distinguish between T1 (CD24intmediateCD23low) and T2 (CD24highCD23low) transitional B cells, whereas CD21 and CD23 were used to evaluate MZ (CD21highCD23low) and follicular (CD21intmediumCD23high) B cell subsets. Transitional B cells in R4A-y2b mice were identified as CD19+ y2b+ CD24high, MZ B cells as CD19+ y2b+CD21highCD23low and follicular B cells as CD19+ y2b+CD21intmediumCD23high.

Flow cytometry was performed with FASCalibur or LSR flow cytometer (BD Bioscience), and data were analyzed with FlowJo software (Tree Star).

Splenic immunohistochemistry

Frozen splenic sections (5 μm thick) were fixed in acetone for 5 min, blocked with 3% BSA/PBS for 3 min, and incubated with a 1/2000 dilution of Cy2-labeled anti-IgM (Jackson ImmunoResearch Laboratories) and PE-labeled anti-y2b Ab (BD Pharmingen) for 30 min.

Apoptosis assays

H2A.X phosphorylation assay. Splenocytes were incubated for 4 h at 37°C in the presence or absence of an intact anti-IgM Ab. Then, splenocytes were washed and stained for CD19, CD21, CD23, and AA4.1, fixed with paraformaldehyde, permeabilized, and incubated with either anti-phospho-histone H2A.X-FITC-conjugated Ab (Upstate Biotechnology) or the negative control mouse IgG-FITC (BD Pharmingen) for 20 min on ice. Phospho-histone H2A.X- positive cells were determined in the transitional T1 and T2 B cell subsets, as well as in the mature MZ and follicular B cell subsets. Flow cytometry was performed with LSR flow cytometer (BD Bioscience).

Activated caspase 3 assay. Splenocytes were depleted of RBC and cultured in complete RPMI medium supplemented with 10% FCS with or without anti-IgM Ab (10 μg/ml) (Southern Biotechnology Associates) for 18 h at 37°C. After incubation, splenocytes were washed and stained for CD19, fixed with paraformaldehyde and incubated with a rabbit polyclonal Ab to activated caspase 3 (clone C11, Idun Pharmaceuticals) or irrelevant rabbit polyclonal Ig (BD Pharmingen). Caspase expression was evaluated in CD19+ cells. Flow cytometry was performed with FACS Calibur flow cytometry. The data obtained from both apoptosis assays were analyzed with FlowJo software.

ELISPOT assay

Splenocytes alone or stimulated with anti-CD40 (10 μg/ml) (BD Pharmingen) and IL-4 (300 U/10^6 cells) (BD Pharmingen) or intact anti-IgM (10 μg/ml) (BD Pharmingen) were incubated in 96-well medium containing 10% FCS at 37°C. After 48 h, splenocytes were washed and added in serial dilution to Immulon-2 plates coated with calf thymus dsDNA at a concentration of 100 μg/ml. After 6 h of incubation at 37°C, biotin-conjugated goat anti-mouse γ2b (Southern Biotechnology) diluted 1/500 was added, and plates were incubated overnight at 4°C. Plates were incubated with alkaline phosphatase-conjugated streptavidin (Southern Biotechnology) at a 1:1000 dilution for 1 h at room temperature and then developed with 5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich) at room temperature for 2–4 h. Spots were counted under a dissecting microscope.

B cell hybridomas

Hybridomas were generated by conventional methodology (25) from LPS-stimulated splenocytes of R4A-y2b BALB/c mice that had been treated with estradiol and tamoxifen for 5 wk. They were screened for y2b transgene expression by RNA dot blot, as previously described (26). The S107 V γ5-positive hybridomas were further screened by RNA dot blot using a 230-bp probe for overexpression of the Vc1 gene family (21). Total RNA was extracted from all transgene-expressing clones regardless of their Vγ usage. RT-PCR was performed using a 5′ FR1 Vk primer and a 3′ κ constant region primer as described elsewhere (21). The PCR products were purified by Qiagen purification kit (Qiagen). L chain gene sequences were determined with an ABI 377 automated sequencer (PerkinElmer Life and Analytical Sciences), and sequence analysis was performed with Genetics Comparison Group software (Accelrys). Supernatants of the selected clones were normalized for γ2b concentration at 5 μg/ml, and ELISA determined their DNA reactivity.

Statistical analysis

The data were analyzed with standard statistical tests (mean value, SD, two-tailed Student t test).

Results

Tamoxifen abrogates an estrogen-induced lupus-like phenotype without decreasing the expansion of y2b-expressing B cells

To determine whether tamoxifen can antagonize estradiol-mediated effects on B cells, we compared serum titers of anti-DNA Ab in mice treated with estradiol, estradiol and tamoxifen, tamoxifen,
or placebo. Serum samples were analyzed for anti-dsDNA reactivity by ELISA. By the fourth and fifth week of treatment, the titers of anti-dsDNA Abs increased significantly only in estradiol-treated mice (Fig. 1a). Thus, tamoxifen was able to abrogate the estradiol-induced rise in anti-dsDNA Ab titers and it alone did not act as an estrogen-receptor agonist in this model.

Glomerular IgG deposition was detected only in the kidneys of estradiol-treated mice, confirming previous findings that estradiol treatment can induce production of nongenetic autoantibodies (10, 23) (Fig. 1b).

We have previously demonstrated that estrogen can break tolerance in R4A-γ2b BALB/c mice by rescuing a high affinity DNA-reactive B cell population that is normally deleted from the naive B cell repertoire and increasing the number of γ2b-expressing B cells in R4A-γ2b BALB/c mice (10). Both estradiol- and estradiol/tamoxifen-treated R4A-γ2b mice displayed an expansion of the transgene-expressing B cells in the spleen. There are no IgG-expressing cells detectable that are not γ2b expressing; thus, all IgG-expressing cells appear to express the transgene. The observation has been confirmed in our previous studies of these mice in which all IgG-expressing hybridomas expressed the transgene (10, 26).

The percentage, as well as the absolute numbers of γ2b-positive B cells, was higher in estradiol- and estradiol/tamoxifen-treated mice than in placebo or tamoxifen-treated mice. There was no difference in the number of transgene-expressing B cells between mice treated with placebo or tamoxifen alone (Fig. 1c). Thus, tamoxifen was not functioning as an agonist to expand the γ2b-expressing B cell population, but it also did not antagonize the estrogen-mediated expansion of autoreactive B cells.

**Tamoxifen influences estradiol-induced alterations of B cell subsets**

Because we had previously shown that estradiol affects B cell development, causing an alteration in the transitional B cell compartment and an expansion of MZ B cells (11), we evaluated B cell subsets in hormonally manipulated BALB/c mice. To avoid any alteration in the normal distribution of B cell subsets that may be caused by transgene expression, we analyzed wild-type mice. As previously demonstrated, estradiol-treated mice showed a shift in immature and mature B cell populations, with an inversion in the ratio of transitional T1/T2 B cell subsets and an increase in MZ B cells (Fig. 2a). The population of T1 B cells is normally larger than the population of T2 transitional B cells (27). The decrease in number of B cells in the T2 compartment is thought to reflect the impact of negative selection on T1 cells with deletion of autoreactive B cells (28). This reduction in T2 cell number is impaired by...
FIGURE 2.  
a, B cell subsets in wild-type BALB/c mice. Splenocytes from BALB/c mice treated with placebo, estradiol, estradiol/tamoxifen, and tamoxifen (n = 5 in each group) were stained for CD19, AA4.1, CD21, and CD23 to analyze immature (transitional T1 and T2) and mature (MZ and follicular) subsets. AA4.1 staining was used to distinguish between the transitional and mature B cells. CD21/CD23 staining was used to evaluate transitional T1 (CD19<sup>+</sup>AA4.1<sup>-</sup>CD21<sup>low</sup>CD23<sup>low</sup>) and T2 (CD19<sup>+</sup>AA4.1<sup>-</sup>CD21<sup>high</sup>CD23<sup>high</sup>) B cell subsets and mature MZ (CD19<sup>+</sup>AA4.1<sup>-</sup>CD21<sup>high</sup>CD23<sup>low</sup>) and follicular (CD19<sup>+</sup>AA4.1<sup>-</sup>CD21<sup>intermediate</sup>CD23<sup>high</sup>) B cell subsets. The percentage of transitional T1 B cells was decreased in both estradiol- and estradiol/tamoxifen-treated mice compared with placebo (p = 0.001 and 0.005) or tamoxifen- (p = 0.009 and 0.0001) treated mice. There was no difference in the percentage of T1 B cells between placebo- and tamoxifen-treated mice or between estradiol- and estradiol/tamoxifen-treated mice. However, the percentage of MZ B cells was increased only in estradiol-treated mice compared with placebo (p = 0.021), estradiol/tamoxifen- (p = 0.003), and tamoxifen- (p = 0.005) treated mice. There were no significant differences among the percentages of follicular B cells in the mice from all four groups. The ratio of transitional T1 and T2 B cells, which is normally greater than 1, was decreased in both estradiol- and estradiol/tamoxifen-treated mice, compared with placebo (p = 0.006 and 0.012, respectively). The absolute number of MZ B cells was higher in estradiol-treated mice than in placebo- (p = 0.021), estradiol/tamoxifen- (p = 0.008), or tamoxifen- (p = 0.015) treated mice. Splenic B cell subsets were also stained with CD19, CD21, CD23, and CD24. T1 cells were identified as CD19<sup>-</sup>CD24<sup>high</sup>CD21<sup>intermediate</sup>CD23<sup>low</sup> and follicular cells as CD19<sup>-</sup>CD24<sup>low</sup>CD21<sup>intermediate</sup>CD23<sup>high</sup>. This staining method also demonstrated an inversion of the T1:T2 ratio in both estradiol- and estradiol/tamoxifen-treated mice and an increase in MZ B cells only in estradiol-treated mice (data not shown).

b, γ2b-expressing B cell subsets in R4A-γ2b BALB/c mice. Transitional (T1 and T2) and mature (MZ and follicular Fo) B cell subsets were determined by staining for CD19, γ2b, CD21, CD23, and CD24. There was no difference in the percentage of total transitional or follicular γ2b-expressing B cells among the different treatment groups. In contrast, estradiol-treated mice displayed a higher percentage of MZ γ2b-expressing B cells (CD19<sup>+</sup>CD24<sup>-</sup>γ2b<sup>-</sup>CD21<sup>high</sup>CD23<sup>low</sup>) than placebo-, estradiol/tamoxifen-, and tamoxifen-treated mice (p = 0.02, 0.01, and 0.015, respectively). Also, the splenic MZ B cell subset from estradiol-treated mice contained a higher percentage of γ2b-expressing B cells than the MZ
Histone H2A.X (Ser139) phosphorylation in transitional T1 B cells. H2A.X phosphorylation at Ser139 was used as a marker of apoptosis. Splenocytes from estradiol/tamoxifen-treated mice than in placebo- (p = 0.038 and 0.029, respectively) or tamoxifen-treated mice (p = 0.04 and 0.034). Apoptosis assay: Histone H2A-X (Ser139) phosphorylation in transitional T1 B cells. H2A.X phosphorylation at Ser139 was used as a marker of apoptosis. Splenocytes from mice treated with placebo, estradiol, estradiol/tamoxifen, or tamoxifen (n = 4 in each group) were incubated alone or in presence of 10 µg/ml intact anti-IgM Ab at 37°C for 4 h. Phosphorylated serine-expressing cells were determined in transitional and mature B cell subsets. The percentage of phosphorylated serine-positive T1 B cells was lower in estradiol- and estradiol/tamoxifen-treated mice than in placebo- (p = 0.01 and p = 0.006, respectively) or tamoxifen- (p = 0.002 and 0.001, respectively) treated mice.

estradiol. Tamoxifen alone did not affect B cell maturation. Estradiol/tamoxifen-treated BALB/c mice, like mice treated with estradiol alone, displayed more T2 than T1 B cells, suggesting that tamoxifen does not abrogate estrogen-mediated protection from deletion at this stage of B cell maturation.

The MZ B cell population is expanded in the NZB/W lupus-prone strain and harbors autoreactive B cells (29–31). The MZ B cell population, which was also expanded in estradiol-treated BALB/c mice (11), was not increased in estradiol/tamoxifen-treated mice (Fig. 2a), demonstrating that in estradiol/tamoxifen-treated mice, the transitional cells do not preferentially mature to MZ B cells as they do in estradiol-treated mice. We confirmed this observation in R4A-γ2b mice because we demonstrated high numbers of γ2b B cells in MZ of estradiol-treated mice (Fig. 2b). In estradiol/tamoxifen-treated mice, γ2b B cells were present in the T cell zone whereas the γ2b B cells matured to become MZ B cells in estradiol-treated mice (Fig. 2c).

We have suggested that the survival and maturation of autoreactive B cells in estradiol-treated mice is mediated, in part, by up-regulation of Bcl-2, CD22, and SHP-1. Bcl-2 protects against apoptosis whereas CD22 and SHP-1 lower the strength of the BCR signal, predisposing the cells to develop into MZ B cells (11). Both estradiol- and estradiol/tamoxifen-treated mice displayed an increased expression of Bcl-2. Bcl-2 up-regulation was not seen in mice treated with tamoxifen alone (Fig. 3a). Thus, tamoxifen behaved as neither an antagonist nor an agonist with respect to Bcl-2 up-regulation.

To confirm that the transitional B cells of estradiol/tamoxifen-treated mice were resistant to BCR-signal induced apoptosis, the physiologic mediator of tolerance induction, we compared B cell apoptosis among hormonally treated BALB/c mice and placebo-treated mice using anti-IgM Ab as a surrogate Ag. Expression of phosphorylated H2A-X, with and without stimulation with anti-IgM Abs was significantly increased only in estradiol-treated mice.

Transitional B cells from estradiol- and estradiol/tamoxifen-treated mice demonstrated decreased expression of phosphorylated H2A-X after stimulation with anti-IgM compared with B cells from tamoxifen- and placebo-treated mice (Fig. 3b). Similar results were obtained assessing apoptotic cells by the expression of activated caspase 3 (data not shown). This is consistent with the increased survival of γ2b B cells in both estradiol- and estradiol/tamoxifen-treated mice. In mice that received both estradiol and tamoxifen, the up-regulation of Bcl-2 is probably of critical importance in permitting the expansion of γ2b-expressing B cells.

Tamoxifen abrogates estradiol-induced B up-regulation of CD22 and SHP-1
Estradiol up-regulates CD22 and SHP-1 (12). Up-regulation of an inhibitory coreceptor of the BCR, and its associated phosphatase, is responsible for diminished BCR signaling, which will promote the development of an MZ phenotype (11). Because we observed no increase in the MZ subset and no activation of mature autoreactive B cells, as demonstrated by the absence of serum titers of anti-DNA Ab, we analyzed the expression of CD22 and SHP-1. Both CD22 and SHP-1 were up-regulated only in B cells of estradiol-treated mice (Table I). Thus, in the presence of tamoxifen and estradiol, B cells displayed no alteration in the expression of the molecules that diminish the strength of the BCR-mediated signal and favor differentiation to the MZ subset.

Enumeration of anti-dsDNA B cells
We next sought to understand why the transgene-expressing B cell population was expanded but not secreting Ab in estradiol/tamoxifen-treated R4A-γ2b mice. We enumerated B cells secreting anti-DNA Ab without exogenous stimulation, and B cells secreting anti-DNA Ab following stimulation by agents known to activate B cells. The number of B cells spontaneously secreting anti-DNA Abs was significantly increased only in estradiol-treated mice.
compared with placebo-treated mice (Fig. 4). The lack of B cells spontaneously secreting anti-DNA Abs in estradiol/tamoxifen-treated R4A-γ2b mice was consistent with the negligible serum titers of anti-DNA Ab. Stimulation with anti-CD40 Ab and IL-4 increased the number of B cells secreting anti-dsDNA Abs in estradiol/tamoxifen-treated mice suggesting the existence of a population of autoreactive B cells. This population cannot be activated by engagement of the BCR. Thus, estradiol treatment leads to the activation of anti-DNA B cells, whereas estradiol and tamoxifen treatment leads to a population of maturation-arrested autoreactive B cells that can be activated by T cell factors to secrete autoantibody.

**B cell hybridomas from LPS-stimulated B cells from estradiol/ tamoxifen-treated mice**

In hormonally untreated mice, we have observed a population of anergic DNA-reactive B cells that fail to achieve exclusion and express endogenous μ H chain as well as the γ2b H chain. These B cells all express somatically mutated κ L chains; we have never detected anti-DNA Abs with a λ L chain associating with the R4A-H chain (26). We have previously shown that estradiol leads to the activation of anti-DNA B cells that have high affinity for DNA and express germ line encoded VK1 L chains. These cells differ from the anergic population and are normally deleted. To characterize the DNA-reactive B cell population present in estradiol/tamoxifen-treated mice, we generated hybridomas from LPS-stimulated splenic B cells. Approximately 300 wells from two fusions produced hybridomas, 40 of which secreted anti-dsDNA Abs. All of these clones expressed the γ2b transgene, in the absence of a μ H chain, and κ L chain. L chain gene usage was evaluated in ten DNA-reactive clones with high affinity binding to DNA. Six expressed Vk1 genes, 4 of which were in the germ line configuration (Table II). None expressed Vκ genes. Thus, the same B cells that are rescued from deletion in estradiol-treated mice are present in estradiol/tamoxifen-treated mice, but are anergic, and can be rescued as hybridomas following LPS stimulation. These cells differ from the anergic autoreactive B cells present in hormonally unmanipulated mice as they display allelic exclusion and, in general, no somatic mutation.

**Discussion**

The increased incidence of SLE in women of child-bearing age underscores the importance of sex and sex hormones in the development of this disease. Female reproductive hormones can exacerbate disease activity in SLE patients and animal models of lupus (1, 9, 32, 33), and also induce lupus in nonsensitively autoimmune mice (10, 24). Furthermore, a recent large scale clinical study has demonstrated that estrogen will exacerbate mild to moderate lupus flares (8). Thus, it becomes important to know how to inhibit estrogen-mediated B cell modulation.

We have previously shown that estrogen alters B cell repertoire selection and maturation in R4A-γ2b BALB/c mice by enhancing resistance to BCR-mediated apoptosis and by decreasing the strength of the BCR-mediated signal (10–12). Because tamoxifen provides a potential therapy to block the effects of estrogen and ameliorates lupus in both NZB/W F1 and MRL/lpr mice (16, 17), we have examined its impact on the production of anti-DNA Abs in the R4A-γ2b BALB/c mouse model and show here that

### Table I. CD22 and SHP-1 expression in B cells

<table>
<thead>
<tr>
<th>BCR signaling molecules</th>
<th>Placebo</th>
<th>Estradiol/Tamoxifen</th>
<th>Estradiol</th>
<th>Tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD22 (MFI)</td>
<td>217.33 (+ 6.42)</td>
<td>201.66 (+ 13.8)</td>
<td>237 (+ 2.12)</td>
<td>220.3 (+ 5.50)</td>
</tr>
<tr>
<td>SHP-1 (MFI)</td>
<td>98.13 (+ 7.68)</td>
<td>104 (+ 6.1)</td>
<td>117.75 (+ 2.08)</td>
<td>105 (+ 3.66)</td>
</tr>
</tbody>
</table>

*a Values are represented as the mean fluorescence intensity (MFI) ±SD of CD19+ B cells. MFI of CD22 surface expression was higher in estradiol than in placebo (p = 0.007), estradiol and tamoxifen (p = 0.01), or tamoxifen (p = 0.007) treated mice. Also, MFI of the intracellular expression of SHP-1 was increased in estradiol-treated mice compared with placebo (0.004), estradiol and tamoxifen (p = 0.006), or tamoxifen (p = 0.001) treated mice. Statistical significance was determined by a two-tailed paired Student t test. Three mice were included in each group.

### Table II. Light chain usage in DNA-reactive clones

<table>
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<th>Clone</th>
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<th>Jk Gene</th>
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<td>Vk1A</td>
<td>Jk1</td>
</tr>
<tr>
<td>30-1</td>
<td>Vk1A</td>
<td>Jk1</td>
</tr>
<tr>
<td>48-2</td>
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<td>10-c</td>
<td>VkRF</td>
<td>Jk5</td>
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</tbody>
</table>

**FIGURE 4.** Enumeration of naive and stimulated DNA-reactive B cells. Splenocytes from placebo- (n = 4), estradiol- (p = 5), estradiol/tamoxifen- (n = 5), and tamoxifen- (n = 3) treated R4A-γ2b mice were isolated and incubated alone or with anti-CD40 Ab and IL-4 or anti-IgG Ab for 48 h at 37°C. An ELISpot assay was performed, and anti-DNA-reactive B cells were enumerated in 105 splenocytes. There was an elevated number of spontaneously secreting DNA-reactive B cells in estradiol-treated mice compared with placebo- (p = 0.001), estradiol/tamoxifen- (p = 0.001), and tamoxifen- (p = 0.003) treated mice. Although B cells from estradiol- and estradiol/tamoxifen-treated mice displayed a higher response to anti-CD40/ IL-4 than placebo- and tamoxifen-treated mice (p = 0.006 and 0.04, and p = 0.03 and 0.045), the response to stimulation with anti-IgG Ab was increased only in estradiol-treated mice compared with placebo, estradiol/ tamoxifen, and tamoxifen (p = 0.01, p = 0.008, and p = 0.03, respectively).
anti-DNA Abs do not appear in the circulation. We had expected tamoxifen to block the estrogen effect on both BCR-mediated apoptosis and the decrease in the strength of the BCR-mediated signaling and thereby allow the normal deletion of anti-DNA B cells in this model. Instead, tamoxifen was unable to block the resistance to BCR-mediated apoptosis but was able to block B cell activation. Thus, autoreactive B cells were maturation-arrested in a nonsecreting state, and tolerance was maintained.

The clinical and murine studies that implicate estrogen as a risk factor in the pathogenesis of SLE have led to the hope that SERMs may antagonize estrogen effects and provide a therapeutic effect in the context of an unmanipulated endogenous hormone environment (16). SERMs are chemically diverse compounds that act as estrogen agonists or antagonists in a tissue-specific manner (34, 35). Like estrogen, SERMs exert their effect through ERα and ERβ (36, 37). ERα and ERβ belong to the nuclear receptor superfAMILY of ligand-dependent transcription factors and display a high degree of similarity in their size and structure (36). However, they are not functionally equivalent, because the disruption of each receptor in mice leads to a distinct phenotype (38–40), and each receptor regulates expression of a distinct set of genes (41). Because most ligands do not distinguish between ERα and ERβ, the different effects of estrogen and SERMs, and the variable effects of each SERM, cannot be explained just by their differential binding the ERs. Ligand-ER complexes recruit particular coactivators (42, 43). Tissue-specific expression of ERs and their coactivators, as well as tissue-specific differences in the uptake and metabolism of estrogen and SERMs, are all likely to help determine the effect of both estrogen and SERMs (44). In addition, a recent study demonstrated that ER can be activated even when estrogen levels are very low, suggesting the possibility of the existence of other, still not identified, ER ligands (45).

We chose to study tamoxifen because extensive clinical experience with this compound in the treatment of breast cancer has revealed a good safety profile (14), and a beneficial therapeutic effect of tamoxifen has been demonstrated in murine models of autoimmune disease (17), whereas in murine lupus induced by 16/6 MRL/lpr mice, treatment with tamoxifen selectively decreases pathogenic IgG3 anti-DNA Abs in the serum and in glomerular deposits (19). It also decreases serum levels of soluble TNF receptors 55 and 75 (16). In MRL/lpr mice, treatment with tamoxifen decreases the number of CD4+CD8- T cells (17), whereas in murine lupus induced by 16/6 Id, tamoxifen normalizes the levels of IL-1, IL-2, IL-4, and IFN-γ (18, 46). Other studies investigating the mechanism of action of tamoxifen have shown that tamoxifen decreases CD5+ B cells and inhibits dendritic cell maturation, thereby decreasing T cell activation (16).

In R4A–γ2b mice, estrogen rescues and activates a population of autoreactive B cells that would normally undergo deletion (9, 11, 12). These estrogen effects are mediated by up-regulation of the anti-apoptotic protein Bcl-2 and the BCR-associated inhibitory signaling molecules CD22 and SHP-1 (12). Tamoxifen is a partial agonist for ERα and pure antagonist for ERβ, although it displays similar affinity for both receptors (47, 48). Our data would suggest that ERα is responsible for up-regulation of Bcl-2 and that ERβ may contribute significantly to the up-regulation of CD22 and SHP-1. We are currently studying the target genes of ERα and ERβ in B cells to determine whether this hypothesis is correct. It is interesting to note that the gene encoding Bcl-2 has a classic estrogen response element whereas the genes encoding CD22 and SHP-1 have not been demonstrated to have an estrogen response element.

B cell tolerance is normally maintained by negative selection of immature autoreactive B cells in the bone marrow and the spleen. In the spleen, negative selection occurs through engagement of the BCR at the transitional stage of B cell development. Deletion occurs mainly in the T1 compartment (28), although there is some controversy about whether T2 cells may be equally susceptible to BCR-mediated deletion (49). However, it appears clear that T2 cells are more sensitive to rescue from negative selection by activated T cells. Tonically elevated estrogen levels decrease BCR-mediated apoptosis of transitional cells and lead to an expansion of the T2 subset. This resistance to apoptosis is a consequence of up-regulation of Bcl-1–2 and occurs in both estrogen- and estrogen/tamoxifen-treated mice. It has been shown that up-regulation of Bcl-2 by estrogen can protect many cell types from apoptosis (50, 51), and overexpression of Bcl-2 in B cells will lead to autoantibody production in various mouse strains (52).

Estrogen-mediated pathways also lead to an expansion of the MZ B cell subset that is the source of activated autoreactive B cells in estrogen-treated R4A–γ2b mice (11). In general, MZ B cells are expanded when BCR signaling is diminished (for review, see Ref. 53). The lack of expansion of MZ B cells in tamoxifen- and estrogen-treated mice is almost certainly a consequence of the lack of up-regulation of CD22 and SHP-1. Thus, when tamoxifen is present, BCR-mediated signals trigger anergy in B cells that have moved to the T2 stage of differentiation. These observations demonstrate that survival to the T2 stage, or resistance to apoptosis in the T1 stage, is separable from maturation to immunocompetence and suggest that different molecular pathways mediate these processes. It has been demonstrated that B cells from mice treated with TACI-Ig to mediate B cell-activating factor of the TNF family (BAFF) blockade have a developmental arrest at the T1 to T2 transition. Overexpression of Bcl-2 rescues BAFF-deprived B cells from death, but Bcl-2 cannot compensate for BAFF in delivery of a differentiation signal (54). Thus, survival signals and differentiation signals are distinct, and entry to the T2 stage appears to represent checkpoint for B cell differentiation. Mice deficient in invariant chain also display a developmental arrest at the T1 to T2 transition (55). CD45-/- mice display a B cell arrest at the T2 stage (56), similar to what is seen in estrogen- and tamoxifen-treated mice. Deletion of SHP-1 in CD45-/- mice restores B cell maturation beyond the T2 stage (57). This is consistent with the data reported here, also showing that SHP-1-regulated pathways are critical in B cell maturation from a T2 to mature phenotype.

It is well established that there are multiple checkpoints in the B cell maturation pathway. A recent study suggesting that 55–75% of transitional B cells display autoreactivity underscores the importance of these checkpoints (58). Our data demonstrate that different molecular pathways are responsible for different checkpoints. Although studies of genetically engineered or spontaneous models of SLE have demonstrated a defect in tolerance induction in naive B cells with progression through the T1 stage of cells normally deleted at that stage (59), our studies demonstrate that tolerance can be induced in cells that have matured beyond the T1 stage. The tolerance observed in our model leads to anergy and is not unlike follicular exclusion (58, 60). It will be interesting to determine whether the autoreactive cells in estrogen- and tamoxifen-treated mice also display follicular exclusion. Because T2 cells are more easily rescued from tolerance, tamoxifen may represent a possible but perhaps not optimal therapeutic strategy: autoreactive B cells that mature to the T2 stage may become fully immunocompetent and activated in a proinflammatory milieu. It will be important to determine whether aromatase inhibitors that block estrogen synthesis (61, 62) can restore normal B cell tolerance in patients with SLE with negative selection occurring within the T1 subset.
An important implication of our data is that both survival and differentiation pathways need to operate to develop immunocompetent B cells. Estrogen appears to be both a survival and a differentiation factor. Autoimmunity may be readily induced when both survival and differentiation factors combine to permit autoreactive cells to acquire immunocompetence. Our data show that even in hosts with an intrinsic resistance to B cell apoptosis, which has been shown to represent an autoimmune diathesis in mice overexpressing Bcl-2 in B cells (62), it is possible to maintain tolerance within the naive immunocompetent B cell repertoire. Further studies to understand the regulation of differentiation of T2 cells may offer new therapeutic targets in SLE.

Acknowledgments

We thank Xian Chen and Oneka Welch for technical support, Yi Bao for staining splenic sections, Matthew Scharff and Christine Grimaldi for critical reading of the manuscript, and Sylvia Jones for helping with the preparation of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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