0022-1767/89/1428-2647$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 142, 2647–2653, No. 8, April 15, 1989
Printed in U.S.A.

ESTROGEN INDUCES NORMAL MURINE CD5+ B CELLS TO PRODUCE AUTOANTIBODIES[1]

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Females have better humoral immune responses and are more susceptible to autoimmune diseases than males. Normal female mice (C57BL/6J, C3H/HeJ, and NZW) have significantly increased spontaneous autoimmune plaque-forming cells (APFC) to mouse erythrocytes pretreated with bromelain (Br-ME) in spleen, peritoneal exudate cell, and bone marrow compared to their male counterparts. A minor subpopulation of B cells, CD5+ B, is thought to produce this autoantibody. As determined by dual color flow cytometry, increased APFC to Br-ME in females is not due to quantitative increase of CD5+ B cells. Rather, it is due to increased numbers (or percentages) of CD5+ B cells producing these autoantibodies, because CD5+ B cells from females produced greater numbers of APFC to Br-ME than equal numbers of cells derived from males. The increased autoantibody production in females can be attributed to the effect of estrogen on the immune response because this hormone markedly augments APFC to Br-ME in intact or orchidectomized males. Male hormone had little effect. Importantly, estrogen did not increase the numbers of B or CD5+ B cells but augmented the ability of B cells to produce this response. This was verified when a T cell-depleted B cell fraction or fluorescence-activated cell sorter purified CD5+ B cells from estrogen-treated mice proved more efficient in the production of APFC to Br-ME. These results suggest that the number of CD5+ B cells committed to produce autoantibodies to Br-ME is increased under the influence of estrogen. This is the first demonstration that estrogen can augment the production of natural autoantibodies in normal mice. The overall augmented humoral immune responses in females and the B cell hyperactivity in female predominant autoimmune diseases appears to be due to estrogen.

There is a physiologic difference in immune capabilities between the two sexes of both invertebrates and vertebrates (1–5). Females are more immunologically reactive than males as demonstrated by higher Ig levels, better immune response to heteroantigens and ability to combat infections (1–3). Females are also more susceptible to most autoimmune diseases (1, 2).

This sex-related dichotomy in immune capabilities is thought to be due to physiologic effects of sex hormone. Estrogens regulate the synthesis of serum and uterine Ig including IgM, IgA, and IgG (1, 6, 7), and augment antibodies to a variety of thymic independent (TI-1 and TI-2) Ag as well as to autoantigens (1, 8–11). Although sex hormones have major effects on T cells, their effects on B cells in general, and on CD5+ B cells in particular, are not known. CD5+ B cells in mice and humans account for much IgM autoantibody production (12–15). This minor subpopulation of B cells is relatively increased in certain autoimmune-prone mice (NZB and "viable moth-eaten") and produces autoantibodies to a variety of autoantigens including to Br-ME[4] (12, 13, 16, 17). These cells are also present in normal mice and constitute a small percentage. We have studied the effect of sex hormones on CD5+ B cell function in normal mice by 1) measuring autoantibodies to Br-ME and 2) quantitation of these cells using dual color flow cytometry.

We report that 1) lymphocytes from normal female mice produce higher numbers of APFC to Br-ME than do males, 2) normal females have greater numbers of CD5+ B cells actively producing autoantibodies to Br-ME, 3) the administration of estrogen to normal mice prepubertally augments APFC to Br-ME without a similar increase in CD5+ B cell numbers, and 4) the number of purified B cells or CD5+ B cells, derived from estrogen-treated mice are more committed to producing autoantibodies to Br-ME.

The activation of B cells by naturally occurring estrogens may account, in part, for the overall augmented immune responses in healthy females and the B cell hyperactivity seen in female predominant autoimmune diseases.

MATERIALS AND METHODS

Mice

Normal male and female [C57BL/6J; B10.D2; C3H/HeJ; NZW] mice were purchased from The Jackson Laboratories, Bar Harbor,

Received for publication June 20, 1988.
Accepted for publication January 24, 1989.
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[1] This work was supported in part by grants from National Institutes of Health San Antonio Multipurpose Arthritis Center, The Lupus Foundation of America, The American Cancer Society Institutional, University of Texas Health Science Center at San Antonio (UTHSLSA), the General Medical Research Service of the Veterans Administration and the RGK Foundation.
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[4] Abbreviations used in this paper: Br-ME, bromelain mouse E; APFC, autoimmune plaque-forming cells; orx., orchidectomized.

ME. All mice were fed on a commercial pellet diet devoid of sex hormones.

Sex hormones

Sex hormonal implants (Silastic medical grade tubing 0.062″ internal diameter × 0.125″ outer diameter and Silastic Medical Adhesive. Silicone Type A, Dow Corning Corp., Midland, MI) were prepared using powdered 17-β-estradiol (0.5 cm long), 5-α-androstan-17β-ol-3-one (5-α-dihydrotestosterone) or testosterone (both 1 to 1.5 cm long) (all obtained from Sigma Chemical Co., St. Louis, MO) (18, 19). Empty implants (sham) of required length (0.5 to 1.5 cm long) were also prepared.

Surgical procedures

Four- to five-wk-old normal, C57BL/6J and B10.D2, mice were subjected to orchidectomy by procedures described earlier (18). Some mice were sham orchidectomized and served as surgical controls. In our preliminary experiments, we noted little difference in response to estrogen between the two normal strains.

Administration of sex hormones

Four- to 5-wk-old orchidectomized, sham orchidectomized, or intact mice were implanted s.c. with sham (empty), estrogen, 5-α-dihydrotestosterone, or testosterone implants. These implants were left in situ for 2 to 3 mo. The presence of implants was checked periodically by palpation. These implants release hormones up to 4 to 5 mo (19).

In vivo sex hormone bioassays

Initially, to confirm the sex hormone effect on B cells, several known in vivo sex hormone bioassays were performed. These included the following.

Bone marrow cell volume. We and others have reported earlier that administration of estrogen to mice reduced the total bone marrow cellularity (20, 21). Individual femurs of sex hormone or sham-treated mice were flushed with one ml of cold RPMI media and the numbers of lymphocytes determined in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

NK cell activity. Estrogen has been shown to markedly reduce the splenic NK cell activity against YAC-1 target cells (21). The splenic NK cell activity was dependent on efflux of bone marrow cells. The methodology for determining NK cell activity and its analysis was as described earlier (22).

Seminal vesicular weights. The fresh gonadal and seminal vesicular weights were determined, as their weight is directly dependent on the continuous exposure to sex hormones (23).

Thymus weights. Orchidectomy is known to result in thymic hypertrophy (1, 3, 24). Conversely, administration of estrogen or testosterone induces thymic atrophy (20, 25–27). The weight of thymus glands of sex hormone treated mice were compared with controls.

Blood levels of estrogen. Serum estrogen from individual samples (40 to 200 μl) were determined as described (28). Antiserum specific for estrogen (100 μl, 1/30,000 dil., Radioassay Laboratories, Carson, CA) and 100 μl of [^{3}H]estrogen (20 pg) in diluent with 1% bovine-globulin were added to each tube. Incubation was at 4°C overnight and precipitation of bound hormone with 3 ml of 55% saturated ammonium sulfate. Cross-reaction with estrone was 0.08% and with other steroids was also insignificant. Assay sensitivity was 3 pg/tube. Intra-assay coefficient of variation was 12.9%.

Preparation and culture of splenic lymphocytes

Splenic lymphocytes were isolated by gentle separation on a wire mesh. Peritoneal cell exudates were collected with repeated peritoneal cavity lavages. Bone marrow cells were collected as described above. The erythrocytes were removed by Ficoll-Paque, pH 7.2 (Pharmacia Fine Chemicals, Piscataway, NJ) and the cells were washed in RPMI 1640 media (Hazelton, Denver, PA). The cells were adjusted to the required cell concentration in complete RPMI media which contained 10% heat-inactivated FCS (Hazelton, Denver, PA), 1 μM sodium pyruvate, 0.1 μM non-essential amino acids (GIBCO, Grand Island, NY), antibiotic (penicillin/streptomycin—234 U/ml) and antimycotic (Fungizone—10 mM), 1% HEPES buffer (GIBCO), and 2-Me (GIBCO) at a final concentration of 2.5×10^{-5} M.

In some experiments peritoneal cells or splenic lymphocytes were cultured at 10^6 cells/ml in the absence of exogenous antigens or mitogens for 3 to 5 days in complete media. This procedure is known to increase APFC to Br-ME (29). This increase was not due to FCS in the media, because similar results were noted when cells were cultured in Nutridoma, a serum-free medium, (Boehringer Mannheim, Indianapolis, IN) used as a supplement with phenol red depleted RPMI 1640.

Preparation of purified B cells

Normal C57BL/6J mice were prepubertally (4 wk of age) orchidectomized or sham operated and given empty (sham) or estrogen implants for 3 mo. The splenic lymphocytes were isolated by Ficoll-Paque and treated with anti-Thy-1.2 (Becton Dickinson, Mountain View, CA) plus baby rabbit C (Pel Freez, Roger, AK). Subsequently, cells were washed 3 times. An aliquot was stained with biotinylated anti-Thy 1.2 (Becton Dickinson) followed by fluorescein-conjugated avidin (FA-DCS, Vector Labs, Burlingame, CA) to ensure the absence of T cells. In addition, the cells were stained for B cells and CD5$^+$ B cells as described below. The remaining aliquots were plated for the production of APFC to Br-ME or FACS sorted for CD5$^+$ B and CD5$^-$ B cells as described below.

Preparation of Br-ME

The methodology for preparation of Br-ME was a slight modification of the procedure described earlier (29). Briefly, syngeneic mouse E (usually from mice less than 3 mo of age) were obtained after Ficoll-Paque separation to avoid contamination of lymphocytes and washed repeatedly. To the E pellet, an equal volume of 20 mg/ml of bromelain (Calbiochem-Behring, La Jolla, CA) was added. The cells were incubated for 45 min at 37°C and subsequently washed three times and adjusted to 20% v/v solution.

APFC to Br-ME

The APFC to Br-ME were determined as follows. Briefly, 100 μl of fresh or cultured spleen, peritoneal, or bone marrow cells at 2×10^7 cells/ml were co-cultured with 50 μl of 20% Br-ME, 20 μl of Br-ME absorbed C, and 30 μl of complete media. Controls were as follow: 1) Br-ME and C and 2) lymphocytes plus Br-ME. The final volume of controls was adjusted to 200 μl which was comparable to the test sample. Then 100 μl of the test sample mixture was loaded in the five slide chamber compartments (Spiral Scientific, Cincinnati, OH; 20 μl/compartment). Also, appropriate controls were included in other compartments of the slide chamber. The edges of the slides were sealed with paraffin-wax and the numbers of IgM APFC to Br-ME determined after 1 to 2 h incubation in a CO_2 incubator at 37°C.

Dual color analysis for CD5$^+$ B cells

A total of 10 to 20 million cells were stained for 25 to 30 min with a standardized concentration of mAb rat anti-mouse Lyt-1 IgG2a antibody (30) ((anti-CD5, 53-7.3) (a generous gift of Dr. J. T. Kung, Department of Microbiology, UTHSCA)), followed by a standardized concentration of FITC-goat antirat IgG (Boehringer Mannheim, IN) which has minimum cross-reactivity and does not bind to mouse B cells. Identical results also were obtained when FITC-mouse antirat IgG (F(ab′)$_2$) (Jackson ImmunoResearch, Avondale, PA) was used. Next, cells were stained with Texas-red-F(ab′)$_2$ fragments of goat antimouse IgM (Jackson Immunoresearch). A separate aliquot of cells was washed and stained with prestandardized Texas Red-goat antimouse IgM (F(ab′)$_2$) only or anti-CD5 followed by FITC-mouse antirat IgG F(ab′)$_2$. Cells stained with FITC-goat antirat only or an isotypic irrelevant antibody served as controls. In addition, unstained cells and Texas Red avidin stained cells were also used as controls. Cells were subjected to two color flow cytometry. The data were plotted as contour plots as well as three dimensional plots and analyzed quantitatively by a PDP/11 computer. The percent of positive cells identified by single color analysis was always compared with the corresponding fraction of double color stained cells which gave similar results. This ensured the accuracy of the data and confirmed the lack of "bleeding" between the two color channels.

Analysis of APFC to Br-ME usually was performed on whole lymphocyte preparations from various organs. The number of plated APFC/10^6 B cells or CD5$^+$ B cells was calculated based on the percentage of B cells and CD5$^+$ B cells determined from flow cytometric analysis. In certain cases, the data are represented as the percentage (%) of B or CD5$^+$ B cells plated producing APFC to Br-ME. We found that the size of plaques to Br-ME in T cell depleted cells were smaller than that seen using mixed population. It is thus possible that T cell lymphokines (IL-2, IL-4, or IL-6) may contribute to this effect.

FACS sorting

Purified splenic normal B cells, prepared as before were stained with anti-CD5 and anti-IgM antibodies as described above (for vis-

ualization of $CD5^+$ B and $CD5^-$ B cells). More than 97% of the cells stained positively with anti-IgM antibody. All macrophages and dead cells were electronically gated out. Stringent gates were established to distinguish cells that stained with anti-CD5 from those that were $CD5^-$ B. Approximately one third of the total number of cells, which were located between the $CD5^-$ B and $CD5^+$ B cells, were discarded to prevent cross-contamination. The $CD5^-$ B and $CD5^+$ B cells were sorted and individually collected. Re-analysis by flow cytometry of each sorted fraction established >97% purity of the $CD5^+$ B cells and >99% purity of the $CD5^-$ B cells.

Statistical analysis

To assess effects of sex hormones on $CD5^+$ B cells, non-parametric statistical analysis was performed using Kruskal-Wallis one way analysis of variance (31) followed by two group comparisons using the two-tailed Mann-Whitney U test (32) with the Bonferroni correction for revised alpha levels (33). Comparisons of APFC to Br-ME between groups were done using the Mann-Whitney U test. All calculations were performed on an IBM personal computer (International Business Machines, Inc., Armonk, NY) using True Epistat release 1.0 (Epistat Services, Richardson, TX).

RESULTS

Sex differences in autoantibodies to Br-ME. To demonstrate naturally occurring sex differences in the autoimmune response to Br-ME, spleen cells from several normal mouse strains were analyzed. Females have three to seven times greater numbers of APFC to Br-ME compared to age and strain matched males (Table I). Similar sex-differences in autoantibody production are noted in bone marrow and peritoneal exudate cells from these strains of mice (data not shown).The culture of splenic or peritoneal cavity lymphocytes for 3 to 5 days results in increased APFC to Br-ME (29). Cultured lymphocytes (spleen, peritoneal cavity, or bone marrow) from female mice demonstrate increased APFC to Br-ME compared with age matched males (data not shown).

Sex difference in response to Br-ME is not reflected in $CD5^+$ B cell numbers. Inasmuch as $CD5^+$ B cells are the source of these autoantibodies, we compared APFC to Br-ME with $CD5^+$ B cell numbers in spleen and peritoneal exudate. In a representative experiment, NZW females produce increased APFC to Br-ME/10^6 cells compared to males, but there are no differences in numbers of total B or $CD5^+$ B cells (Table II). The number of splenic $CD5^+$ B cells producing autoantibodies to Br-ME is greater in females than males. Similar results are noted in other strains including C57BL/6J.

This suggests that the APFC response to Br-ME can vary independently of the number of $CD5^+$ B cells. Thus, the magnitude of the response may be more dependent on the autoantibody specificity commitment in the $CD5^+$ B cell subpopulation rather than the actual numbers of these cells.

TABLE I

Females have increased APFC to Br-ME compared with males[a]

Strain	Sex	No. of Mice	APFC/10^6 Total Cells ±SEM	Statistical Significance
C57BL/6J	Male	12	38 ± 10	
	Female	12	192 ± 34	$p < 0.0005$
NZW	Male	15	139 ± 21	
	Female	20	1041 ± 209	$p < 0.0005$
C3H/HeJ	Male	7	180 ± 10	
	Female	8	543 ± 64	$p < 0.0005$

[a] The spleens of the mice (age: 12 mo) of either sex were used for APFC to Br-ME. The data were analyzed for statistical significance by Mann-Whitney U.

$CD5^+$ B cells from females produce more APFC to Br-ME than those from males. To determine which subset of B cells are primary producers of APFC to Br-ME, splenic B cells from male and female C57BL/6J mice, stained with anti-CD5, were FACS purified as $CD5^+$ B and $CD5^-$ B cells and compared for the production of autoantibodies to Br-ME. As expected, $CD5^+$ B cells are major producers of APFC to Br-ME (Fig. 1). Importantly, sorted $CD5^+$ B cells from females produced more APFC to Br-ME compared with identical numbers derived from males. This suggests that in the female environment $CD5^+$ B cells are more activated to produce APFC to Br-ME. The ratio of $CD5^+$ B to $CD5^-$ B cells producing APFC to Br-ME in females is 35 to 1 while in males it is 21 to 1.

Exogenous estrogen but not male hormone augments APFC to Br-ME. Inasmuch as there are natural sex differences in APFC to Br-ME suggesting a sex hormonal influence, we studied the effect of sex hormonal manipulation on this autoantibody response in normal (B10.D2 and C57BL/6J) mice. Depletion of male hormones by orchidectomy or augmentation by testosterone administration does not change APFC to Br-ME in either spleen or peritoneal cells (Fig. 2 *a* and *d*). In contrast, orchidectomized mice given estrogen demonstrate markedly increased APFC to Br-ME in both spleen and cultured peritoneal exudate cells (Fig. 2 *a* and *d*).

Several known sex hormone in vivo bioassays were performed to determine conclusively the effect of sex hormones. Estrogen abrogates splenic NK cell response (Fig. 2*b*), reduces bone marrow cellularity due to osteopetrosis (Fig. 2*c*) (21), and induces thymic atrophy (Fig. 2*e*) and increased weights of seminal vesicles (due to proliferation of interstitial and smooth muscle tissues) (Fig. 2*f*). In contrast, orchidectomy induces thymic hyperplasia and atrophy of hormone-dependent seminal vesicular size which is reversed by testosterone. There is little difference noted in APFC to Br-ME between mouse groups belonging to gonadal intact and sham orchidectomized (orx.)/sham implant and orx./sham implant which represents surgical controls. Similar results are obtained in C57BL/6J mice (data not shown).

As expected, male mice have lower levels of serum estrogen [(untreated $n = 5$: 67.9 ± 5.8 pg) (sham orx./sham implant $n = 5$: 82.3 ± 18.8 pg) (orx./sham implant $n = 15$: 66.8 ± 5.2 pg)] compared with females (untreated $n = 10$: 345.7 ± 152.1 pg). Estrogen administration of orchidectomized males ($n = 19$) increased estrogen serum levels similar to untreated females (425.3 ± 29.1 pg). Taken together, the above data confirms the known effects of estrogen on various systems.

The immunopotentiating effects of estrogen can be demonstrated even in the presence of male gonads. For instance, when estrogen capsules are implanted in gonadal intact normal (C57BL/6J) male mice, a large increase in APFC to Br-ME is seen. In a representative experiment, the splenic APFC/10^6 cells to Br-ME in estrogen implanted mice is 142, whereas that from sham and dihydrotestosterone implanted mice is 6 and 5, respectively.

Estrogen does not alter the number of $CD5^+$ B cells. To compare the numbers of APFC to Br-ME with $CD5^+$ B cell numbers in hormone manipulated mice, normal prepubertal C57BL/6J male mice were orchidectomized or

TABLE II

Relationship of APFC to Br-ME with CD5⁺ B cells

Sex	Tissue	APFC/10^6 B cells	%B cells	APFC/10^6 CD5⁺ B cells	%CD5⁺ B cells
Male	Spleen[a]	138	49	1037	7
Female	Spleen	1892	44	13941	8
Male	PEC[b]	28484	75	70990	30
Female	PEC	158086	79	369495	34

[a] Fresh spleens from NZW mice were pooled %B and CD5⁺ B cells (n = 4) were determined by fluorescence-activated cell sorter analysis.

[b] PEC (peritoneal exudate cells) were cultured for 3 days.

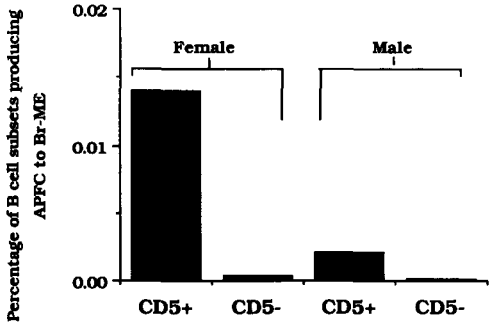


Figure 1. Purified splenic B cells from normal adult male (n = 6) and female (n = 6) C57BL/6J mice (10 mo old) were stained with anti-CD5, subjected to flow cytometry and CD5⁺ B and CD5⁻ B cells sorted. The purity of the sorted population was rechecked and the cells were used to determine APFC to Br-ME. About 5 to 10% of the unsorted population were recovered in each of the two sorted subpopulations, CD5⁺ B and CD5⁻ B cells. The data for each population are presented as the percent of B cell subsets producing APFC to Br-ME. The APFC to Br-ME was largely confined to CD5⁺ B and not CD5⁻ B subpopulation.

sham orchidectomized (surgical control), and given either sham (empty) or estrogen implants. Mice were killed 3 mo post-treatment and the estrogen effect on spontaneous autoantibody production was determined.

There is a marked increase in autoantibody response in estrogen-treated mice ((Fig. 3) ($p < 0.005$)). However, there is no comparable increase in CD5⁺ B or pan B cell numbers (Table III). When APFC to Br-ME is corrected for total B or CD5⁺ B cells (enumerated by fluorescence-activated cell sorter analysis), again there is a highly significant (5×) increase in APFC to Br-ME in estrogen-treated mice ($p < 0.005$). Similar results are obtained in peritoneal exudate cells (data not shown).

Estrogen increases commitment of CD5⁺ B cells to produce Br-ME APFC. Highly enriched splenic B cells were derived from the spleens of sham (n = 5) or prepubertally (4 wk of age) orchidectomized mice treated with (n = 8) or without (n = 5) estrogen for three months. Equal numbers of B cells were used to determine APFC to Br-ME. Again, on a per B cell basis, estrogen-treated mice had increased APFC to Br-ME (80/10^6 cells) compared with both control groups (sham/sham 8/10^6 cells; orx./sham 8/10^6 cells).

Finally, we determined whether estrogen increases the capacity of purified CD5⁺ B cells to produce APFC to Br-ME. Splenic CD5⁺ B and CD5⁻ B cell populations were sorted from C57BL/6J orchidectomized mice which had been given estrogen (n = 29) or empty (n = 18) implants for two months. The *left panel* of Figure 4 demonstrated the IgM+ cells used in this study. The *right panel* demonstrated the purity of the sorted CD5⁺ and CD5⁻ B cells upon re-analysis. As reported previously (12), CD5⁺ B cells have a greater intensity of cell surface IgM (*inset*). FACS sorted CD5⁺ B cells from estrogen-implanted mice demonstrated increased APFC to Br-ME as compared with that of CD5⁺ B cells from sham-implanted mice. The percent of CD5⁺ B cells derived from estrogen-treated mice producing APFC to Br-ME is 0.012% compared to 0.005% derived from sham-treated controls. Estrogen treatment did not increase the percentage of APFC to Br-ME in CD5⁻ B cell population (0.0007% compared to 0.0003% in orx./sham implant group). A relatively minor APFC response in CD5⁻ B cells could be due to minor contamination of CD5⁺ B cells.

DISCUSSION

Although the augmented immune responses in females is well recognized, the underlying basis for this effect is not known (1–3). Several previous studies have concentrated on T cells as the target for sex hormone action (1–3, 20, 34, 35). We now examine the relationship between

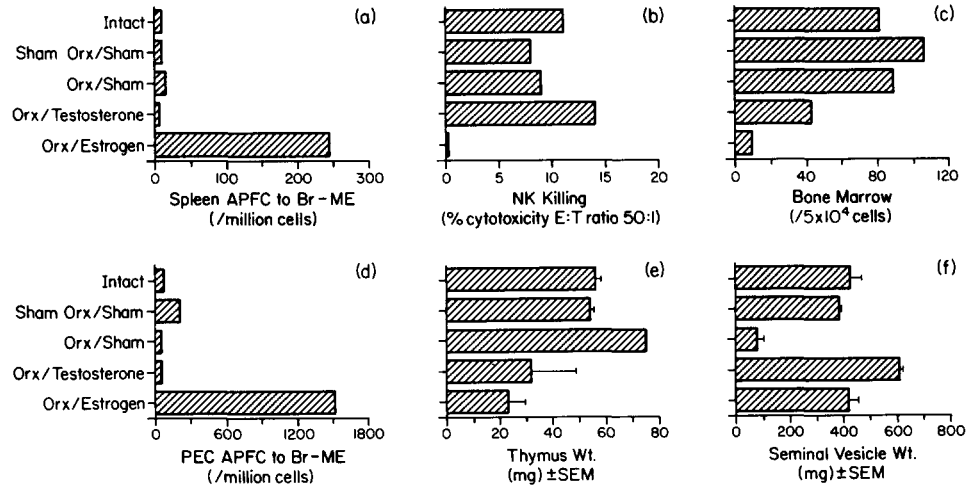
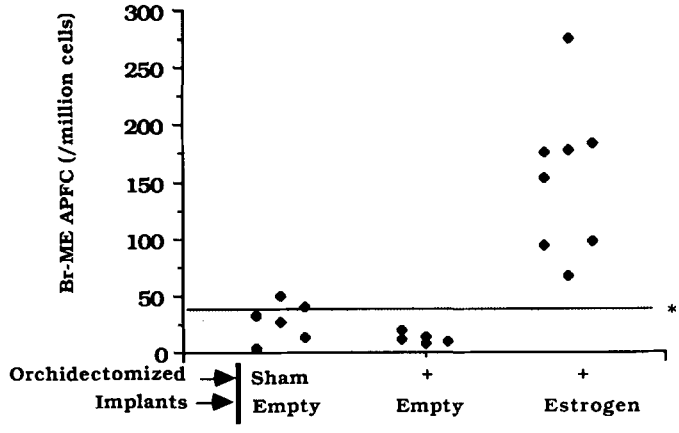


Figure 2. Normal 4-wk-old, B10.D2, mice (n = 2 to 3) were left intact, sham orchidectomized, or orchidectomized. The orchidectomized mice were given either empty, testosterone, or estrogen implants for 3 mo. Note the increased APFC to Br-ME only in mice treated with estrogen and not in male hormone manipulated mice. Several known in vivo bioassays were performed to determine conclusively in sex hormonal effects.



* **Normal range** (n=12): Mean ± SEM (38±10)

Figure 3. Four-wk-old normal C57BL/6J mice were orchidectomized or left intact, and given sham (empty) or estrogen implants which were left in situ for 3 mo. The splenic APFC to Br-ME was determined. The *bold line* and the *shaded area* represents the mean APFC to Br-ME ± SEM (38 ± 10) from 12 normal mice at the age of 12 mo. Note that all estrogen-treated normal mice had increased APFC to Br-ME compared to controls.

TABLE III

Estrogen augments APFC to Br-ME without quantitative changes in CD5⁺ B cells[a]

Sex Hormone Treatment	APFC to Br-ME/10^6 Total Cells (± SEM)	%Positive Cells (± SEM)	
		B	CD5⁺ B
Sham orchidectomy + sham implant (n = 5)	25.2 ± 7.9	50 ± 2	3.3 ± 0.6
Orchidectomized + sham implant (n = 7)	12.8 ± 2.1	59 ± 1[b]	3.1 ± 0.3
Orchidectomized + estrogen implant (n = 8)	153.0 ± 23.6[c]	49 ± 2	4.1 ± 0.9

[a] Derived from spleens of normal C57BL/6J male mice.
[b] Value differs from other two groups, $p < 0.05$.
[c] $p < 0.005$, Kruskal-Wallis one-way analysis of variation for all three group.

estrogen, which has been shown to accelerate several experimental autoimmune diseases, and CD5⁺ B cells which have been shown to produce autoantibodies in autoimmune mice and patients. We have chosen to study this relationship in normal mice because of abundant evidence that the ability to produce autoantibodies exists in these strains as well as in normal individuals.

To relate our findings of APFC to Br-ME with B cells and CD5⁺ B cells, we performed dual color flow cytometric analysis combined with sorting. We found that although normal females have increased APFC to Br-ME, the number of B or CD5⁺ B cells is not increased. In addition, we found that highly enriched splenic CD5⁺ B cells from normal female mice were more efficient producers of autoantibodies to Br-ME than equal numbers of CD5⁺ B cells from normal males. To conclude, the mechanism of this augmentation appears not to be dependent on an increase in the number of CD5⁺ B cells but on an increase in numbers of existing CD5⁺ B cells to produce autoantibodies to Br-ME. This suggests that the female environment activates these cells to express their autoimmune potential. Furthermore, the greater number of APFC to Br-ME in females compared to males suggests a physiologic effect of female hormones (e.g., estrogen).

The murine CD5⁺ B cells differ from conventional B cells in many respects including 1) anatomic location (preferential seeding in peritoneal cavity > spleen > bone marrow > lymph nodes), 2) phenotype (high surface IgM/low IgD), 3) ontogeny (in high numbers in neonatal life and subsequently diminish), 4) predominant production of IgM and IgG autoantibodies to Br-ME, ssDNA, and thymocytes and restricted response to only certain foreign Ag (12, 13). In humans, CD5⁺ B cells are increased in certain autoimmune diseases such as Sjogren's syndrome and rheumatoid arthritis (15, 35a). The CD5⁺ B cells of normal humans, when transformed by EBV infection (14) or *Staphylococcus aureus* (15), produce Ig of all major isotypes as well as rheumatoid factor, antibodies to ssDNA, thyroglobulin, and insulin. Interestingly, human CD5⁺ B cells of both normal and autoimmune individuals secrete rheumatoid factor in comparable amounts (15) whereas antibodies to IgG tetanus toxoid are restricted to CD5⁻ B cells (14).

There is very little information on the effects of sex hormones on B cells and their Ig products. In the present studies, the sex difference in APFC to Br-ME clearly has a sex hormonal basis as it can be altered profoundly by manipulating the sex hormonal milieu. Alterations in male hormone induced by orchidectomy or administration of dihydrotestosterone did not influence the APFC to Br-ME. However, administration of estrogen to normal intact or orchidectomized mice of several strains (including CBA/J and C3H/HeJ-data not shown) significantly increased autoantibodies, thus providing the basis for increased APFC to Br-ME in females. Importantly, although estrogen unequivocally increased APFC, we did not find a corresponding increase in numbers of B or CD5⁺ B cells. In two other unrelated studies, estrogen did not significantly increase the numbers of surface Ig-bearing positive (8) or antibody producing (9) cells.

Our studies suggest that the estrogen-induced increase in APFC to Br-ME is not the result of proliferation of these B cells but rather an augmentation of the activity of these cells. This fact is supported by the finding that purified B cells from estrogen-treated mice produced more autoantibodies to Br-ME compared with equal numbers of purified B cells derived from either sham-orchidectomized or orchidectomized mice with empty implants. Therefore, on a per B cell basis, there was an increased proportion of B cells committed to producing autoantibodies to Br-ME under the influence of estrogen. The relative lower degree of enrichment of APFC to Br-ME in sorted CD5⁺ B cells compared to mixed populations suggests a possible synergestic influence of other cells (T, CD5⁻ B, or macrophages). We are currently investigating this possibility.

The biologic role for CD5⁺ B cells in immune responses is currently an issue of interest. Several roles have been suggested or attributed. First CD5⁺ B cells in addition to the effector role have a physiologic immunoregulatory role (35–38). These cells, present in unprimed C57BL/6J mice, can positively regulate the antibody secreting B cells in a IgH and H-2 restricted fashion through specific recognition of idiotypic determinants (37, 38). Further, using CD5⁺ B hybridomas or CD5⁺ B cells from viable motheaten mice, it was shown that this helper effect is

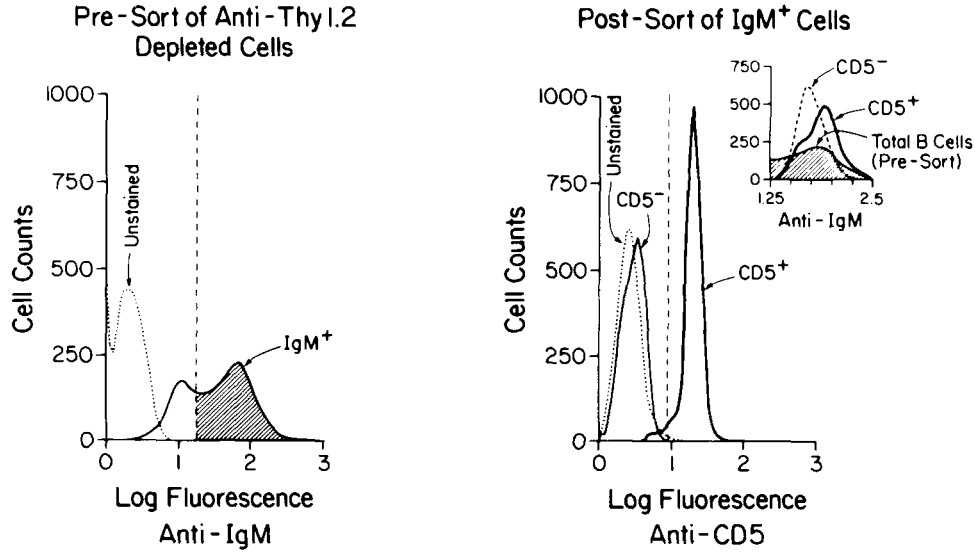


Figure 4. Thy-1.2-depleted splenic cells from prepubertal orchidectomized mice (4 to 5 wk of age) given empty (n = 18) or estrogen (n = 29) implants for 2 mo were stained with Texas-red anti-IgM. Non-B cells (macrophages) were less than 10% of the total B cell enriched fraction, and removed by electronic gating. T cells as identified by Thy-1.2 mAb were absent. The profiles of both groups were similar and the above represents data from the estrogen-treated group. The *left panel* shows only IgM^+ cells were selected by electronic gating based on appropriate controls. The IgM^+ cells (*striped area* in *left panel*) were then stained with FITC-anti-CD5 and sorted into IgM^+ $CD5^-$ and IgM^+ $CD5^+$ cell populations. The post-sorted subpopulations were reanalysed for the purity of the sort and are demonstrated (*right panel*) with only 3% contamination of the IgM^+ $CD5^+$ population IgM^+ $CD5^-$ cells. $CD5^+$ B cells derived from estrogen-treated mice produced more APFC to Br-ME compared with sham-treated controls.

mediated by soluble factors (anti-idiotypic antibody and lymphokines) secreted by these cells (39, 40). Second, it was hypothesized that human $CD5^+$ B cells express germ line Ig genes coding for receptors for non-organ specific autoantigens and their anti-idiotypes suggesting that these cells may bind to autoantigens (41). Finally, it was postulated that $CD5^+$ B cells interact via complimentary receptors with $CD3^+$ $CD4^-$ $CD8^-$ cells which also constitute a significant subset in the early stages of development. These cells may also interact with the conventional cell types to influence the adult immune repertoire (42). A disturbance of this balance during early life could contribute to the development of autoimmunity. In a separate preliminary study, we found that prenatal exposure of normal mice to estrogen augmented APFC to Br-ME in bone marrow and peritoneal cells (43). It is plausible that since naturally occurring estrogen affects the activity of $CD5^+$ B cells it, therefore, may play a biologic role in immunoregulation.

The number of $CD5^+$ B cells in humans is controlled genetically and is not modulated by environmental factors (15). Similarly, in mice the numbers of $CD5^+$ B cells depend upon the strain. Consistent with these observations our findings suggest that without alteration of numbers of $CD5^+$ B cells, the function of these cells (i.e., ability to produce APFC to Br-ME) can be increased. It has been suggested that $CD5^+$ B cells in healthy humans are in a resting state, whereas intrinsic activation of these cells may occur in certain autoimmune patients. The trigger for this cell activation could be due to a variety of factors such as viruses, oncogenes, or growth factors (14). Estrogen may be one of the direct or indirect natural biologic activators of $CD5^+$ B cells. The effects of estrogen appear to resemble that of LPS which also increases APFC to Br-ME without an increase in $CD5^+$ B cells (44). We found a sevenfold increase in APFC to Br-ME per million $CD5^+$ B cells plated in LPS injected C57BL/6J mice compared to saline-injected controls. We are currently investigating whether these effects of estrogen are expressed equally in both $CD5^+$ B and $CD5^-$ B cells in which case estrogen could qualify as a polyclonal B cell activator.

The present data does not address the issue of whether estrogen in vivo acts directly on B cells. However, we have recently developed a specialized serum free system to culture lymphocytes in the presence of sex hormones. Culturing of normal murine B cells or $CD5^+$ B tumor cells (CH 12.LX—a gift of Dr. G. Haughton, University of North Carolina, Chapel Hill, NC) with varying doses of estrogen led to a marked increase in APFC to Br-ME, thus suggesting that these cells are direct targets for sex hormones (45).

Acknowledgments. The anti-Lyt-1 (anti-CD5) antibody was a generous gift of Dr. J. Kung, Department of Microbiology, UTHSCSA. We thank Dr. J. R. Chen of Division of Clinical Immunology, for his technical assistance, Dr. David Olive of Department of Obstetrics and Gynecology, UTHSCSA, for performing statistical analysis and critical review of the paper, and Mr. Charles Thomas III for flow cytometric analysis.

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