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Antibodies to Cardiolipin in Normal C57BL/6J Mice: Induction by Estrogen but not Dihydrotestosterone

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Autoantibodies against cardiolipin, a phospholipid, have been demonstrated in a variety of pathological states including several autoimmune conditions in humans and in certain lupus-prone mice. In this study we detected antibodies reactive to cardiolipin in normal C57BL/6J mice by ELISA. The autoantibodies are detected less frequently in the serum of male than in female mice, suggesting the influence of sex hormones. The relative refractoriness of normal male mice to the induction of anticardiolipin antibodies is not due to the suppressive effects of male hormones, since prepubertal orchiectomy has little influence on this autoantibody. Further, dihydrotestosterone treatment of orchiectomized mice has minimal effect on anticardiolipin antibodies. However, orchiectomized mice when given estrogen develop a marked increase in the incidence as well as the levels of these autoantibodies. Similarly, estrogen treatment of female mice further augments the incidence and the levels of these autoantibodies. Estrogen-treated mice also have antibodies reactive against other membrane phospholipids including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The intensity of binding of autoantibodies to the above phospholipids varies among individual mice. To our knowledge, this is the first report on the demonstration of antiphospholipid autoantibodies in normal mice and induction of these antibodies by estrogen.

Introduction

Autoantibodies against membrane phospholipids such as cardiolipin have been detected in a subset of patients with systemic lupus erythematosus (SLE) [1, 2] and

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primary Sjögren's Syndrome [3]. These autoantibodies are frequently detected in patients with antiphospholipid syndrome (APS) [4, 5] who manifest diverse clinical conditions such as arterial and venous thrombosis of large vessels, thrombocytopenia and repeated fetal loss. In general, moderate to high levels of IgG rather than IgM anticardiolipin antibodies have been closely associated with thrombocytopenia, fetal distress and/or fetal loss [6, 7]. Although, as yet there is no definitive evidence to support a causal role of anticardiolipin antibodies in fetal loss in humans, there is strong evidence, in animal studies, for a possible role of anticardiolipin antibodies in the outcome of pregnancy [8]. Passive transfer of anticardiolipin antibodies (mouse monoclonal or human polyclonal) to naive mice, either before breeding or during pregnancy, resulted in diminished fecundity rate, increased resorption index of embryos per pregnancy, and reduced embryo and placenta weights [8]. Elevated anticardiolipin antibodies have also been detected in human patients with neurological complications including focal cerebral ischemia, myelopathy, chorea, migraines and seizures [9]. Anticardiolipin antibodies are also present in a number of acute infections (e.g. chicken pox, mumps) in humans [10]. In Epstein-Barr Virus infected humans mainly IgM anticardiolipin antibodies are present, while in SLE patients the predominant isotype is IgG [11]. Administration of chlorpromazine to psychiatric patients has been shown in induce anticardiolipin antibodies [12].

In contrast to the human situation, the existence of anticardiolipin antibodies in animal models has not been well documented. The presence of these antibodies has been reported in SLE-prone MRL/MP/lpr/lpr (MRL/lpr) and MRL/+/+ mice [13] as well as NZW × BXSB F1 mice [14]. Anticardiolipin antibodies are not evident in all SLE-prone mice. For example, NZB/NZW (F1) mice, a classical murine model of SLE, do not develop these autoantibodies [13]. In this study we report that normal C57BL/6J mice spontaneously produce anticardiolipin antibodies.

Autoantibodies in immunopathologic conditions have been shown to be potentiated by estrogen [15, 16]. For example, administration of estrogen to autoimmune-prone NZB/NZW F_1 mice markedly accelerated the disease process characterized by increased autoantibodies to DNA, severe immune complex glomerulonephritis, and earlier death compared to controls [16–18]. In SLE human patients, alteration of sex hormone levels including menopause, pregnancy or intake of estrogen containing contraceptives affects the course of the disease [19, 20]. SLE patients also manifest abnormalities in the metabolism of sex hormones (hyperestrogenic/hypoandrogenic states) which could influence the disease process [21]. Estrogen has been shown to enhance the binding of autoantibodies against nuclear autoantigens (Ro and La) to cultured human keratinocytes [22]. It has thus been suggested that estrogen may be an in-vivo trigger factor for immunologic damage in cutaneous and neonatal lupus [22].

While the effects of estrogen on the regulation of autoantibodies in autoimmune individuals is relatively well documented [15, 16], there is little information on the effects of this hormone on the induction or regulation of autoantibodies in normal (non-autoimmune) animals. In previous study we reported that estrogen treatment of normal mice markedly promoted autoantibodies to bromelin-treated mouse erythrocytes (BR-ME) [23]. In the present study, we have investigated the role of sex hormones on the expression of IgG antibodies to cardiolipin and other phospholipids

Table 1

Groups	Sex	Hormonal treatment	Orchiectomy
S-S	M	Empty (Sham) implants	No (Sham surgery)
S-O	M	Empty (Sham) implants	Yes
DHT	M	5α Dihydrotestosterone	Yes
E_2-0	M	17β-Estradiol	Yes
E_2-S	M	17β-Estradiol	No (Sham surgery)
UN	M	None	None
E2	F	17β-Estradiol	N/A
UN	F	None	N/A

in normal mice. Together, our observations suggest that estrogen can promote the induction of autoantibodies in normal mice.

Materials and methods

Mice

Male and female C57BL/6J mice were initially purchase from the Jackson laboratories. They were subsequently bred in our laboratory animal facility. Mice were housed in standard cages (3–5 mice/cage) and fed on a commercial diet devoid of sex hormones. They were maintained in 14 light/10 dark hours in temperature and humidity controlled conditions. The health of mice was routinely monitored by the veterinarian-in-charge and laboratory animal resources technicians. These mice remained clinically healthy throughout the study.

Sex hormonal treatment

Four to five-week-old male mice were subjected to sham orchiectomy or orchiectomy procedures under pentobarbital anesthesia as described previously [24]. These mice were given one of the following treatments, estrogen [17-β-estradiol (as prepared implants [24] or commercial pellets, Innovative Research of America, Toledo, OH)], 5-α-dihydrotestosterone, or empty (sham) implants (Table 1). Estrogen and 5-α-dihydrotestosterone subcutaneous implants have shown to release sex hormones over a period of approximately 3–4 months [17, 24]. Estrogen pellets were surgically placed subcutaneously by a specially designed trocar. These pellets are designed as timed (2-month) release capsules. In unrelated experiments we found no significant differences in the biological effects of estrogen (e.g. on seminal vesicles, bladder or thymus) between the two types of preparations of hormones (Ansar Ahmed, unpublished observations). Sham orchiectomized mice that were recipients of empty (sham) implants served as internal surgical stress controls. Four-week-old female mice with intact ovaries were also given estrogen pellets or empty implants.

Serum collection

Serum from mice was collected individually at various time periods after treatment with sex hormones and aliquots were kept frozen at −70°C until use.

Antiphospholipid antibodies

Antibodies to cardiolipin were detected by a previously reported standardized method [13, 25] with minor modifications. Briefly, each of 96-well flat bottom plates (Costar, EIA/RIA plate) were coated with 30 µl of Cardiolipin (Sigma, MO, USA) in ethanol at a concentration of 50 µg/ml. Plates were left open at 4°C overnight to evaporate the diluent. The PBS containing 2% BSA (90 µl/well) was added for 1 hour and plates were then washed 3 times with excess volume of PBS. Sera, derived from mice of differing sex hormone treatment, were diluted (starting at 1:100 dilution; 50 µl/well) in PBS containing 10% adult bovine serum (ABS, JRH Bioscience, KS, USA). Ten percent ABS in PBS has been commonly employed to block non-specific binding [13, 25]. The diluted sera was added to cardiolipin-coated wells in duplicates and serial double dilutions were performed. Plates were incubated for 3 hours at room temperature. After washing, 50 µl of alkaline phosphatase conjugated Goat anti-mouse IgG (Gamma chain specific, Caltag, CA, USA), in an optimal (1:3000) dilutions in PBS-10% ABS was added and the plates were further incubated for 1 hour. Plates were washed with PBS, developed with *p*-nitrophenylphosphate (Sigma, MO, USA) in diethanolamine buffer (pH 9.8) and read after 60 minutes. The optical density (OD) was determined at 405 nm using a kinetic microplate reader (Molecular Devices). Negative controls included wells with all reagents except serum (no serum blanks) or antigen (no-antigen blanks). The cut-off point for positivity (1:100 dilution) or titer was determined as 3SD above the mean OD of eight 'no serum' blanks included in each plate. In our experience a few mouse sera had low non-specific binding to wells which lacked antigen (but ethanol added). Also 'no serum' blanks were comparable with 'known negative sera' blanks suggesting lack of non-specific binding. In addition, eight wells of each plate always included a high titer anticardiolipin positive serum from MRL/lpr mouse which served as a positive control. Further, as reported earlier [13], we found that sera from many NZB/NZW (F1) mice were negative for anticardiolipin antibodies (but positive for anti-DNA), which further validates the specificity of the assay. This stringent approach allowed us to conservatively but definitively determine the anticardiolipin levels.

In order to evaluate the influence of ABS containing B_2-glycoprotein 1 (B_2-GPI) on the binding of antibodies to cardiolipin, we diluted selected serum samples (five known strong positives, five known borderline positives, and five known negatives) in PBS containing different concentrations of ABS (1, 10 and 20%) or PBS-1% BSA. Appropriate controls (no serum) for each type of buffer were included. No differences were found in the no-serum blanks that utilized buffer containing (β_2-GPI) (PBS with 1, 10 or 20% ABS) or devoid of β_2-GPI (PBS-1% BSA). The assay was otherwise performed as before.

Inhibition studies

To access the specificity of the assay, anticardiolipin antibody inhibition studies were performed as described earlier [27]. Serum samples (1:100 dilution) from five estrogen-treated mice with high titers of anticardiolipin antibodies (1:1600 and above) were preincubated with cardiolipin, either as a solution of micelles in PBS (1 mg/ml) or bound to polystyrene plates (4.9 mg/ml in ethanol) for 1 hour at room

temperature and then overnight at 4°C. Cardiolipin micelles were prepared by evaporating cardiolipin under a nitrogen stream and then resuspended in PBS by vigorous vortexing [27]. The preincubated sera was assayed for cardiolipin by ELISA as before. Appropriate controls (included in the same plate) were: serum preincubated in PBS devoid of Cardiolipin; micelle solution alone (no-serum); no-serum and no-micelle blanks; and serum that was not preincubated. The controls for the inhibition studies that utilized bound cardiolipin included sera preincubated in ethanol coated plates. Preincubation of serum with cardiolipin markedly inhibited the binding of anticardiolipin antibodies to cardiolipin (% inhibition range: 72 to 93%). Similar findings have been reported in autoimmune-prone MRL mice [13].

Antibodies to other phospholipids

Antibodies to phospholipids [phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine or phophatidylserine (Sigma, MO)] were determined by the above procedures. The phospholipids were, however, dissolved in a chloroform and methanol mixture (1:3 v/v) as reported by Gharavi *et al.* [13].

Thrombocyte count

Fresh heparinized blood from mice was added to Unopette units (Becton-Dickson). The instructions of the manufacturer were followed to enumerate the platelets in a Neubauer hematocytometer. The platelet numbers in unmanipulated mice were comparable to previous reports [28]. Importantly, in order to correlate platelet numbers with antibodies to cardiolipin, paired blood and serum samples, respectively, were collected from individual mice.

Statistical analysis

Non-parametric tests were employed, given the lack of normality of data distribution. The Mann-Whitney test was employed as an appropriate statistical tool for the evaluation of serum titers. The χ^2 test was used to determine the differences in incidence of antibodies between groups. The relationship between age and anticardiolipin antibodies was analyzed by the non-parametric χ^2 test and linear regression. Spearman's Rho was used to correlate the level of anticardiolipin antibodies and estrogen levels as well as antibody levels and platelet numbers.

Results

Sex differences in anticardiolipin antibodies

Only four out of eighteen male mice had detectable levels of spontaneous anticardiolipin antibodies, compared to 11 out of 19 female mice (Table 2). The mean titer (natural log scale) of these autoantibodies was 1.13 in the males compared to 3.31 in females ($P = < 0.05$).

Table 2. *Normal male mice have decreased incidence and levels of anticardiolipin antibodies compared to females*

Group	*n*	Incidence (%)	Mean titer ± SEM	Median titer
Male	18	22.2	1.13 ± 0.52	0
Female	19	57.8	3.31 ± 0.64	4.6

Statistical significance: incidence $P = < 0.05$; titers $P = < 0.05$.

Serum of normal male and female mice was used to determine anticardiolipin antibodies by ELISA. The age of mice is as follows. Females: mean = 10.4 months; median = 8.8 months; range = 3–22 months. Males: mean = 10.8 months; median = 9.8 months; range = 3–22 months.

Table 3. *Depletion of male hormones does not increase anticardiolipin antibodies*

Group	*n*	Incidence (%)	Mean titer ± SEM	Median titer
Sham orchiectomized	9	33.3	1.68 ± 0.85	0
Orchiectomized	9	22.2	1.17 ± 0.78	0

Statistical Significance: incidence, P = NS; titers, P = NS.

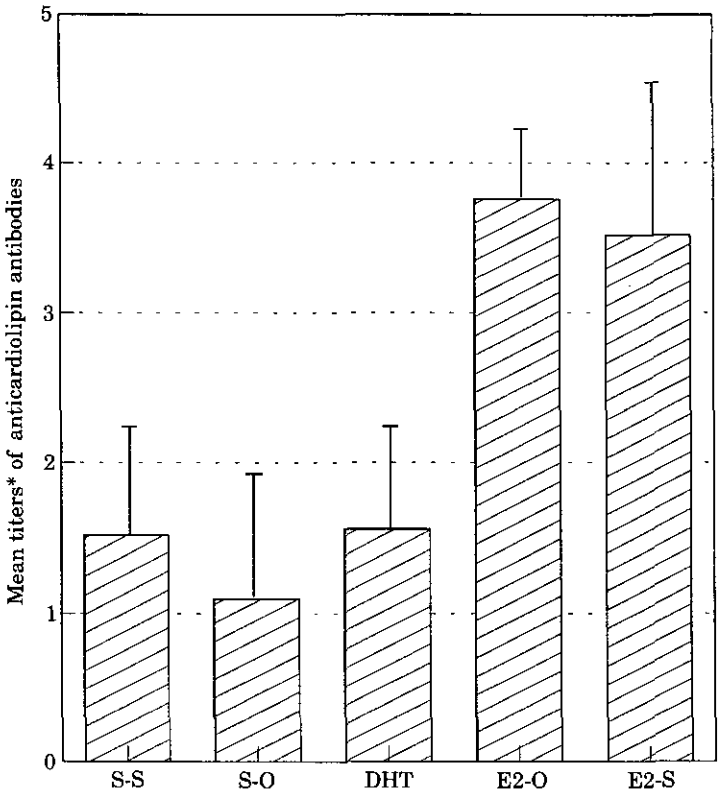
Sera from age-matched sham orchiectomized and orchiectomized mice were analysed for anticardiolipin antibodies by ELISA. Anticardiolipin antibodies were analysed by ELISA in the sera of sham orchiectomized and orchiectomized mice that were matched relative to age (mean = 7.8 months; median = 9 months; range = 3–11 months).

Orchiectomy does not augment anticardiolipin antibodies

Since male mice have a lower incidence and titer of anticardiolipin antibodies than females, we investigated whether this is due to suppression by male sex hormones. Prepubertal male mice were orchiectomized to deplete male sex hormones and their sera were examined for anticardiolipin antibodies several months after surgery. As shown in Table 3, orchiectomy had little effect on augmenting this autoantibody when compared to sham orchiectomized controls (statistically not significant; incidence, $P = 0.74$; titers, $P = 0.78$).

Estrogen but not dihydrotestosterone markedly augments anticardiolipin antibodies in normal mice

In order to investigate whether anticardiolipin antibodies are regulated by sex hormones, prepubertal male mice were orchiectomized and given either estrogen or dihydrotestosterone treatment. Mice belonging to control groups included: sham orchiectomized and orchiectomized mice that received empty implants. Dihydrotestosterone did not significantly increase either the incidence or the levels of these



	S-S	S-O	DHT	E2-O	E2-S
Median*:	0	0	0	4.6	4.95
Incidence: (%)	30.0	20.0	33.3	62.7	60.0

Statistical significance

	Incidence	Titers
S-O vs DHT	NS	NS
S-O vs E2-O	$P < 0.002$	$P < 0.005$
S-O vs S-S	NS	NS
E2-S vs S-S	$P < 0.005$	$P < 0.005$
E2-O vs E2-S	NS	NS

* In natural log. scale.

Figure 1. Normal male C57BL/6J mice were prepubertally orchiectomized or sham orchiectomized at 4–5 weeks of age and given empty (Sham), dihydrotestosterone (DHT), or estrogen (E_2) implants.

autoantibodies compared to controls (Figure 1). Estrogen treatment of orchiectomized mice markedly increased the incidence ($P<0.002$) and titers ($P<0.005$) of anticardiolipin. Importantly, both types of estrogen preparation (pellet and implant) induced anticardiolipin autoantibodies to a similar degree (i.e., no statistical difference). Treatment of gonadal intact male mice with estrogen also increased the incidence and titers of antibodies reactive with cardiolipin. Surgical stress did not influence anticardiolipin antibodies as there is no significant difference in either the incidence or level of anticardiolipin antibodies between mice belonging to S-S (Figure 1) and gonadal intact groups (UN) (Table 2).

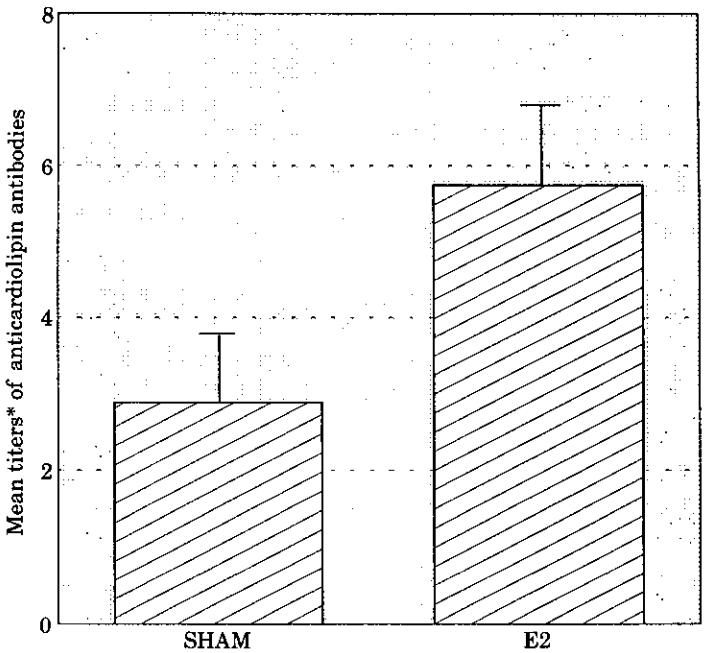


Figure 2. Four to five-week-old female mice were given either empty (sham) or estrogen implants. Sera from mice were analysed for anticardiolipin antibodies.

Estrogen promotes anticardiolipin antibodies in normal female mice

Since estrogen treatment of male (orchiectomized) mice clearly enhanced anticardiolipin antibodies to levels above those in unmanipulated female mice, we next determined whether this hormone would further increase the incidence and/or the titers of this autoantibody in gonadal intact female mice. As shown in Figure 2, estrogen treatment of female mice markedly increased these autoantibodies both in terms of incidence ($P<0.02$) and titers ($P<0.05$). The lack of significant difference in the incidence or titers between unmanipulated (UN) (Table 3) and sham implanted female mice (SHAM) (Figure 2) further indicates that the surgical stress did not influence these autoantibodies.

Anticardiolipin antibodies are increased in estrogen-treated mice at all ages studied

The possible influence of age on anticardiolipin antibodies was examined since mice of different ages were employed in this study. Anticardiolipin antibodies (incidence and titers) were analyzed with respect to age for each treatment group independently.

Table 4. *Estrogen increases anticardiolipin antibodies in normal mice examined at 4 and 9 months of age*

	4 months				9 months			
	n	Incidence (%)	Mean ± SEM titer	Median	*n*	Incidence (%)	Mean ± SEM titer	Median
Controls	7	0	0 ± 0	0	11	36	1.94 ± 0.67	0
Estrogen	12	50	2.93 ± 0.91	2.3	15	67	4.11 ± 0.68	5.3

Titers are expressed in a natural log scale.

Statistical significance:	Incidence	Titers
Estrogen 4 months vs 9 months	P = NS (P = 0.39)	P = NS (P = 0.42)
Controls 4 months vs 9 months	P = NS (P = 0.13)	P = NS (P = 0.18)
Estrogen vs Controls at 4 months	$P < 0.05$	$P < 0.05$
Estrogen vs Controls at 9 months	$P < 0.05$	$P < 0.05$

We found that age, *per se*, had minimal influence (statistically non-significant) on anticardiolipin antibodies within each treatment group. As seen in Table 4, which compares 4 and 9-month-old mice, the titers and incidence of anticardiolipin antibodies for estrogen-treated mice were significantly higher than their age-matched controls.

Binding of anticardiolipin antibodies is not increased by ABS

ABS contains β_2-GPI [29] which has been proposed as a necessary cofactor for the binding of anticardiolipin antibodies to cardiolipin-coated polystyrene plates [26]. We investigated whether increasing concentrations of ABS in the buffer (used to dilute sera) would increase the binding of the anticardiolipin antibodies. This was not the case as 14 out of 15 sera had lower binding (mean reduction in the ODs at 1:100 dilution: 47%) when diluted in PBS containing 10 or 20% ABS compared to PBS containing 1% ABS or 1% BSA. Sera incubated in PBS 1% ABS had binding of anticardiolipin antibodies comparable to PBS-1% BSA. Importantly, none of the sera that were negative for anticardiolipin antibodies when incubated in PBS-1% BSA or PBS-1% ABS became positive when the concentration of ABS in the buffer was increased.

Estrogen also induces antibodies to other phospholipids

We next investigated whether estrogen treatment also induces autoantibodies to other membrane phospholipids. Sera from individual mice (total of 75 mice) was tested for reactivity against a number of phospholipids. Estrogen treated mice also had antibodies reactive to phosphatidylinositol, phosphatidylserine, phosphatidylcholine and phophatidylethanolamine (Figure 3). Importantly, within a given animal

Figure 3. Sera from hormonally manipulated mice were simultaneously tested for the presence of antibodies to phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and cardiolipin. The figure shows representative data of the pattern of binding of antibodies to the above phospholipids. The horizontal line depicts the average mean of the 'no serum' blanks.

although estrogen increased antiphospholipid antibodies, the extent of increase differed among phospholipids. In mouse number 65 for example (Figure 3), the degree of binding of antibody was high to phosphatidylinositol, moderate to cardiolipin, phosphatidylcholine and phosphatidylserine, and low to phosphatidylethanolamine. In contrast, in another estrogen-treated mouse (e.g. No. 94) a high level of anticardiolipin antibodies but low to moderate level of antibodies to other phospholipid was noted (Figure 3). These findings have been reproduced at least twice.

Anticardiolipin antibody levels and platelet counts

There was no correlation between the levels of anticardiolipin antibodies and platelet numbers in mice ($r = 0.033$). This lack of correlation was observed regardless of the hormonal treatment (e.g. $r = 0.005$ for estrogen treated mice).

Discussion

In recent years there has been a surge of interest in antiphospholipid antibodies in general and anticardiolipin antibodies in particular [1, 4, 5]. Anticardiolipin antibodies have been detected in humans with diverse clinical conditions particularly autoimmune diseases (e.g. SLE, APS). Thus far, anticardiolipin antibodies have been detected only in certain autoimmune-prone, MRL/lpr and MRL+/+ [13], and (NZW X BXSB)F1 mice [14]. We have detected IgG anticardiolipin antibodies in the BXSB strain of mice, which develops lupus-like disease with lymphadenopathy (Ansar Ahmed, manuscript in preparation). Normal strains of mice such as Balb/c have been reported to be negative for anticardiolipin antibodies [13], even though they produce autoantibodies of other specificities [34, 35].

In this study, we find the presence of low levels of antibodies that react to cardiolipin in unmanipulated non-autoimmune C57BL/6J mice, particularly in females. Male hormones appear to have minimal effect in the regulation of these autoantibodies since neither prepubertal orchiectomy nor administration of dihydrotestosterone to orchiectomized mice significantly altered the incidence or levels of anticardiolipin antibodies (Table 3 and Figure 1). In contrast, administration of estrogen to orchiectomized mice profoundly increased the incidence and titers of this antibody. Further, estrogen markedly increases the incidence and levels of anticardiolipin antibodies in both gonadal intact female and male mice (Figures 1 and 2). These antibodies were found to be consistently increased in estrogen-treated mice when examined at different ages. It must be emphasized that other than hormonal treatment, these mice were not injected with cardiolipin or any autoantigens or preparations from infectious agents. Mice had increased anticardiolipin antibodies at a time when estrogen had returned to normal levels (data not shown). It thus appears that initial, rather than sustained, exposure to estrogen is sufficient to induce anticardiolipin antibodies. Long-term sequential studies involving short exposure of non-autoimmune mice to estrogen are underway to confirm this aspect.

Estrogen-treated non-autoimmune mice also developed antibodies which bound to other phospholipids. Overall, antibodies to phosphatidylinositol and phosphatidylserine tended to be moderate to high, while those to phosphatidylethanolamine

were barely detectable to negative. It is not known whether antibodies to other phospholipids in estrogen-treated mice are cross reactive antibodies or whether these are separate antibodies. However, we observed that some mice have high levels of antibodies to cardiolipin but not phosphatidylinositol and vice versa. There could well be two types of antibodies: (1) low titer antibodies which may be polyreactive (reactive to other phospholipids) and (2) high titer antibodies which may be monoreactive.

The contribution of the genetic background of C57BL/6J to the development of low levels of anticardiolipin antibodies is not known. However, under the influence of estrogen, the expression of these antibodies are markedly augmented. Several mechanisms may explain these observations. (1) Estrogen may act directly or indirectly on B cells to promote clonal expansion of autorective B cells producing antibodies to phospholipids. The indirect sites (e.g. hypothalamus-hypophysis, macrophages) through which sex hormones mediate their effects on the immune system have been reviewed in detail elsewhere [16, 36, 37]. (2) Estrogen may affect T cells to alter the production of cytokines which act on B cells (e.g. IL-2, IL-4, IL-5, IL-6) to promote autoantibody production. (3) Estrogen may be toxic to cells thereby exposing phospholipids such as cardiolipin to provoke an immune response. The above hypotheses are currently under investigation in our laboratory. Finally, the possibility that estrogen may activate an unknown latent virus or other infectious agents which could promote autoimmunity cannot be ruled out.

Beta$_2$ glycoprotein-I (β_2-GPI), a known inhibitor of the intrinsic coagulation pathway and prothrombinase activity of activated platelets, has been shown to be present in ABS [29]. A recent study reported that affinity purified anticardiolipin antibodies bind to cardiolipin on polystyrene plates only in the presence of β_2-GPI [26]. Subsequent studies show that the degree of enhancement of the binding of anticardiolipin to phospholipids in the presence of β_2-GPI is variable in APS sera [38]. Further, β_2-GPI was shown to block the binding of these antibodies in syphilis patients [39]. Our results show the following. (i) Increasing concentrations of ABS in the buffer do not increase the binding of anticardiolipin antibodies. Rather, it decreased the binding suggesting that ABS is acting as a blocking agent. (ii) The use of diluting buffer devoid of β_2-GPI does not diminish the incidence of these antibodies. Although it is possible that estrogen may also increase β_2-GPI in the sera of mice, the above data suggest that β_2-GPI does not increase the binding of estrogen-induced anticardiolipin antibodies. Moreover, throughout the study all sera were incubated with the same concentration of ABS in the diluting buffer. Further studies utilizing β_2-GPI are planned to confirm this aspect.

We found that, as observed in MRL/lpr mice [13], the levels of anticardiolipin antibodies did not correlate with platelet numbers in C57BL/6J mice despite the association of these antibodies with thrombocytopenia in humans.

In a brief report on human studies, it was observed that a small group of women with vascular complications who used estrogen-containing oral contraceptives did not develop anticardiolipin antibodies [30]. This apparent discrepancy with the present findings may be due to obvious differences in the species, disease/health status of the subjects, or dose, purity and route of administration of estrogen between the two studies. Further large studies are needed to definitively determine whether or not estrogen promotes antiphospholipid antibodies in humans. It would also be

interesting to determine whether post-menopausal patients on estrogen therapy develop anticardiolipin antibodies. Our present studies are in agreement with previous observations that estrogen induces B cells that produce anti-erythrocyte (bromelin pretreated autoantibodies in normal mice [23]. Further, this study also supports a vast body of literature which documents that estrogen treatment of normal rodents augments levels of immunoglobulins and antibodies to many exogenously administered foreign antigens [31–33, 36, 37]. Moreover, estrogen-treatment of autoimmune mice has been shown to promote antibodies to a variety of autoantigens [16–18].

The pathogenic significance (e.g. thrombosis or fetal loss) of anticardiolipin antibodies induced by estrogen needs to be determined. Nevertheless, it is important to note that estrogen can potentiate normal B cells to spontaneously produce autoantibodies. Further, this data supports the concept that estrogen may alter normal immunoregulatory pathways to induce B cell hyperactivity.

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