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17β-Estradiol, but not 5α-Dihydrotestosterone, Augments Antibodies to Double-Stranded Deoxyribonucleic Acid in Nonautoimmune C57BL/6J Mice*

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ABSTRACT

The influence of sex hormones on the immune response to foreign antigens as well as self-antigens is now recognized. In this study, we investigated the influence of gender and sex hormones on the development of antibodies to double-stranded DNA in nonautoimmune C57BL/6J mice. Immunoglobulin G (IgG) anti-dsDNA antibodies are commonly present in lupus patients and several autoimmune disease-prone murine strains. We found that C57BL/6J mice have detectable antibodies (IgM and IgG, but not IgA) to dsDNA. Interestingly, the incidence and level of IgG anti-dsDNA antibodies were lower in male than in female mice. Orchidectomy or administration of 5α-dihydrotestosterone to orchidectomized male mice had minimal effects on these antibodies. In contrast, administration of 17β-estradiol to orchidectomized or intact males significantly increased both the incidence and levels of anti-dsDNA antibodies. In female mice, ovariectomy decreased whereas administration of estrogen augmented the incidence and levels of anti-dsDNA antibodies in ovariectomized as well as intact female mice. Kinetic studies revealed that estrogen treatment of male and female mice induced earlier and sustained expression of IgG anti-dsDNA antibodies compared to controls. IgG subisotype analysis showed IgG2b to be predominant. In summary, our findings suggest that estrogen, but not dihydrotestosterone, promotes anti-dsDNA antibodies in normal mice. (*Endocrinology* **135:** 2615–2622, 1994)

ACCUMULATING evidence shows that there is a close interaction between the immune and endocrine systems (1–3). In humans, women in general have higher levels of immunoglobulins (Ig), and mount stronger responses to a variety of heteroantigens than men (reviewed in Refs. 1–3). This allows them to respond better to microbial and nonmicrobial antigens than their male counterparts (1–3). The greater immune responsiveness in women is also evident in their higher susceptibility to autoimmune diseases, especially during their reproductive years. For example, women to men susceptibility ratios for systemic lupus erythematosus (SLE), Sjogren's syndrome, and rheumatoid arthritis are 10:1, 9:1, and 2–7:1, respectively (1, 4). In autoimmune patients, the course of the disease is modulated by fluctuations in hormonal status, such as pregnancy, menses, or the use of oral contraceptives (1, 2, 4, 5). Further, female SLE patients have an abnormal metabolism of their sex hormones, resulting in an increased production of 16α-hydroxyestrone and estrol metabolites, which are thought to induce a chronic hyperestrogenic state. Male SLE patients have reduced levels of testosterone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate (4, 6). The above observations suggest that sex hormones may be involved in the pathogenesis of autoimmune diseases.

Analogous to the human situation, gender differences in immune capabilities have also been noted in a number of experimental laboratory animals (1–3, 7). For example, female mice have higher Ig levels and increased antibody responses to a variety of antigens compared to males. Further, female laboratory animals have a reduced incidence of tumors and reject allografts more rapidly than males (1, 7). This suggests a stronger humoral and cell-mediated immunity than males. The influence of sex steroids has been observed in several experimental animal models for autoimmune diseases (organ and nonorgan specific) (reviewed in Refs. 1 and 7). Female New Zealand Black × New Zealand White (NZBXNZWF$_1$) mice, a classical model for SLE, develop autoantibodies, lymphoadenopathy, arthritis, and immune complex glomerulonephritis and die earlier than their male counterparts (8). Administration of estrogen to B/W males resulted in accelerated development of severe nephritis accompanied by higher levels of anti-DNA antibodies and enhanced mortality (9). In contrast, 5α-dihydrotestosterone (DHT)-treated mice had reduced levels of anti-DNA antibodies, and their survival was prolonged. In other murine models of SLE, including NZB × SJL/J, NZB × CBA, NZB × C3H, and NZB × DBA/2, androgens have been shown to retard, whereas estrogens accelerate their expression of autoimmune disease and mortality (7). Sex hormone regulation of autoimmune reactivity has also been noticed in other models of

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autoimmune disease, including those prone to insulin-dependent diabetes mellitus (NOD mice) (10), polyarthritis (LEW/N rats), or autoimmune thyroiditis (PVG/c rats) (reviewed in Ref. 7). The effects of estrogen on the regulation of autoantibodies in autoimmune individuals is relatively well documented (1, 2, 4, 7). However, there is little information on the effects of this hormone on the induction or regulation of autoantibodies in healthy individuals. Therefore, in this study, we investigated for the presence of antibodies to double-stranded DNA (dsDNA) in C57BL/6J mice. This strain is currently considered nonautoimmune and employed as a control in numerous immunological studies. However, C57BL/6J mice have been shown to develop autoimmune-type lesions in old age (11, 12). Also, this strain constitutes part of the genetic background of mice that spontaneously develop autoimmune disease (BXSB/Mp ^{Yaa} mice) (12). Because autoimmune diseases are multifactorial (genetic, environmental, hormonal, infectious factors, *etc.*), C57BL/6J mice may represent a good model to study the influence of sex steroids on healthy individuals who may have some predisposing factors for autoimmune disease in their background.

In previous studies, we reported that estrogen treatment of normal mice markedly promoted autoantibodies to bromelin-treated erythrocytes (13), cardiolipin, and other phospholipids (14, 15). In the present study, we investigated the influence of sex hormones on the expression of IgG antibodies to dsDNA. Antibodies to DNA are present in normal healthy individuals as a part of their natural autoantibody repertoire. These antibodies mainly belong to the IgM class, have low affinity, react with several antigens, and have undergone little somatic mutation (16). Antibodies to dsDNA are commonly present in SLE and Sjogren's syndrome. These antibodies have undergone a high number of antigen-driven somatic mutations, react primarily with dsDNA, and are mainly of the IgG1 isotype in humans or the IgG2a, IgG2b, or IgG3 isotype in mice (16–18). Our observations suggest that estrogen can promote the induction of IgM and IgG anti-dsDNA antibodies in otherwise healthy mice.

Materials and Methods

Mice

Male and female C57BL/6J mice were initially purchased from Jackson Laboratories (Bar Harbor, ME) and subsequently bred in our laboratory animal facility. Mice were fed a commercial diet devoid of sex hormones and housed in standard cages (three to five mice/cage) with a 14-h light, 10-h dark schedule. The health of the mice was routinely monitored by the veterinarian in charge and laboratory animal resources technicians.

Sex hormone treatment

Four- to 5-week-old mice were given one of the following treatments under pentobarbital anesthesia, as described in Table 1. Mice were gonadectomized (orchidectomized or ovariectomized), as described previously (19). Silastic implants (Dow-Corning Co., Midland, MI) or commercial pellets (Innovative Research of America, Toledo, OH) containing 17β-estradiol (E_2) or DHT were surgically placed sc, as reported previously (13, 20). Mice with and without gonadectomies that received empty [placebo (P)] implants served as controls. The commercial pellets

TABLE 1. Sex hormone treatment groups

Male			Female		
Group	Hormone treatment	Orchidectomy	Group	Hormone treatment	Ovariectomy
P-O♂	Placebo	Yes	P-O♀	Placebo	Yes
P-S♂	Placebo	Sham	P-S♀	Placebo	Sham
DHT-O♂	DHT[a]	Yes	E_2-O♀	Estrogen	Yes
E_2-O♂	Estrogen	Yes	P-♀	Placebo	No
E_2-♂	Estrogen	No	E_2-♀	Estrogen	No

[a] 5α-Dihydrotestosterone.

are time-release capsules designed to slowly release sex hormones for 2–3 months. Silastic implants (7 mm long, containing 6–8 mg E_2; Sigma Chemical Co., St. Louis, MO) also release hormones slowly for several months (9, 20). In previous studies we found no significant difference between the two types of estrogen preparations in terms of induction of anticardiolipin antibodies (13) or T-cell proliferative response (Ansar Ahmed, S., R. M. Gogal, Jr., and J. E. Walsh, unpublished observations). In selected experiments, gonad-intact mice received estrogen or placebo implants (Table 1). In mice used for sequential studies, implants (estrogen or placebo) were surgically removed (under pentobarbital anesthesia) after 60 days to ensure that there was no residual release of E_2.

Serum collection

Serum from each mouse was collected retroorbitally and kept frozen at −70 C until tested for anti-dsDNA antibodies. To assess E_2 levels, because of the small volume of the serum obtained, it was necessary to pool sera from mice in each hormone treatment group (three to six mice per group). The samples were analyzed by RIA by Dr. T. J. Reimer (Diagnostic Laboratory, New York College of Veterinary Medicine, New York, NY). Frozen sera from (NZB × NZW)F_1 and BXSB/MP mice were obtained from the laboratory of Dr. V. Dannenberg, University of Connecticut (Storrs, CT).

Antibodies to DNA

Antibodies to dsDNA were detected according to previously reported standardized methods (21) (Rubin, R. L., Scripps Clinic and Research Foundation, La Jolla, CA, personal communication) with minor modifications. In our initial studies, poly-L-lysine (Sigma; 50 μl/ml) was used to coat 96-well flat bottom plates (High-Binding EIA/RIA Plate, Costar, Cambridge, MA). Plates were incubated for 1 h at room temperature and then coated with 70 μg/ml calf thymus dsDNA (Sigma) overnight at 4 C. To remove residual ssDNA, plates were incubated for 1 h at 30 C in a humid chamber with S1 nuclease [50 U/ml in S1 nuclease buffer (0.03 M sodium acetate, 0.1 M sodium chloride, 5 mM zinc chloride, and 0.1 mg BSA/ml)]. After washing with PBS, the wells were blocked with PBS containing 2% BSA (75 μl/well) for 90 min. Mice sera, at a 1:100 dilution (50 μl/well) in PBS containing 10% heat-inactivated adult bovine serum (JRH Bioscience, Lenexa, KS), were then added to the wells. Plates were incubated for 3 h at room temperature. After washing, 50 μl alkaline phosphatase-conjugated goat antimouse Ig (heavy chain specific; Caltag, South San Francisco, CA) in an optimal (1:1500 or 1:3000) dilution in PBS-10% adult bovine serum were added, and the plates were further incubated for 1 h at 37 C in a humid chamber. Plates were washed again, developed with *p*-nitrophenylphosphate (Sigma) in diethanolamine buffer (pH 9.8) and read after 60 min (405 nm; Molecular Devices, Menlo Park, CA). Negative controls included wells with all reagents except serum (no serum blanks), with known negative sera, or with no antigen (no antigen blanks). As known negative sera had similar optical densities (OD) as no serum blanks, no serum blanks were used to control for background in most instances. In addition, eight wells of each plate containing known positive sera from BXSB or MRL/lpr mice served as the positive control. Murine antibodies have recently been shown to bind nonspecifically to poly-L-lysine-coated wells without DNA (22). The coating method was changed to 2 μg/ml methylated BSA in PBS to decrease the possibility of nonspecific binding. The ODs obtained using poly-L-lysine were slightly higher than those obtained

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using methylated BSA (for the same sera); the differences between treatment groups, however, were consistent. To assess the specificity of the assay, inhibition studies were performed using monoclonal antibodies to dsDNA (s83) and to ssDNA (s10) that were kindly provided by Dr. T. Marion, University of Tennessee (Memphis, TN). Supernatants from these cell lines were preincubated on plates coated with increasing concentrations of calf thymus dsDNA (0–700 μg/ml). After incubation for 2 h at room temperature, the supernatants were aspirated and tested for the presence of antibodies to dsDNA. Preincubation with increasing concentrations of dsDNA absorbed out up to 90% of the anti-dsDNA antibodies binding to dsDNA-coated plates (Fig. 1).

Analysis of Ig isotypes and subisotypes

Serum Ig levels were estimated by enzyme-linked immunosorbent assay (ELISA). The procedure followed was similar to that described above with the following differences. 1) Plates were coated with 1 μg/ml heavy chain-specific goat antimouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b, or IgG3 antibodies (Caltag) in bicarbonate buffer. 2) Plates were blocked with PBS containing 1% BSA. 3) The conjugated antibodies used were alkaline phosphatase-conjugated goat antimouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3, respectively (Caltag).

Expression of ELISA data

Two methods of data analysis were chosen for this study: 1) incidence, and 2) specific OD. Incidence is a qualitative assessment of anti-dsDNA antibodies (*i.e.* presence or absence). The cut-off point was determined as 3 SD above the mean OD of eight background control wells included on each plate. The specific OD (OD of the sample minus mean OD of the background controls) provides a semiquantitative estimation of the antibody concentration.

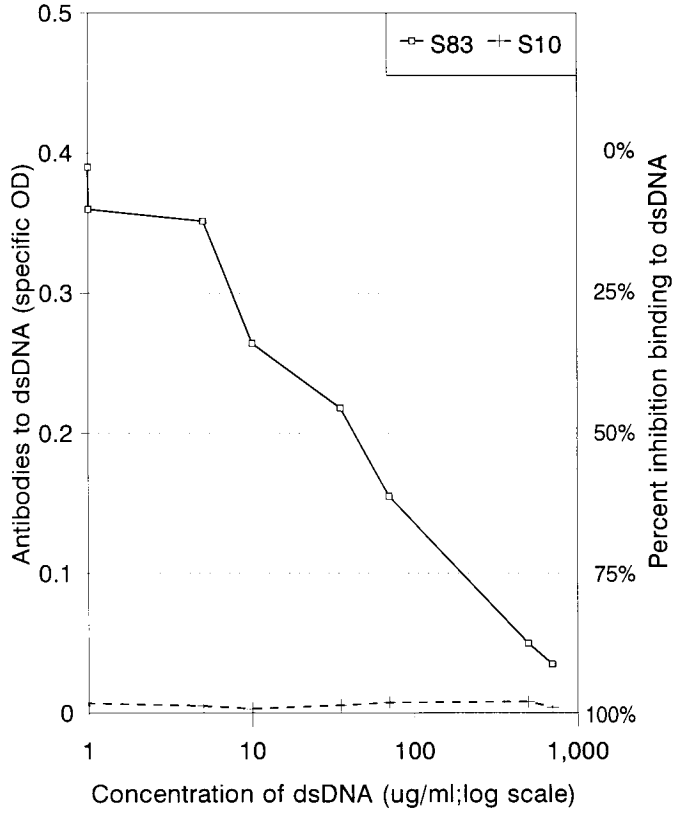

FIG. 1. Specificity of anti-dsDNA antibody ELISA. To validate the specificity of the anti-dsDNA ELISA, supernatants of cell lines secreting monoclonal antibodies to dsDNA (s83) or ssDNA (s10) were preincubated on plates coated with increasing concentrations of calf thymus dsDNA (0–700 μg/ml). This procedure absorbs out antibodies to dsDNA. After incubation at room temperature for 2 h, the supernatants were aspirated and tested for anti-dsDNA antibodies, as described in *Materials and Methods*. Note that the reduction in the binding of s83 (specific for dsDNA) is proportional to the concentration of dsDNA in which the supernatant was preincubated. As expected, the binding of s10 (specific for ssDNA) remains at background levels.

Statistical analysis

Nonparametric tests were employed given the lack of normality of the distribution of the data. Accordingly, the data are described as median values of the distribution (therefore, SEM are not indicated). The χ^2 test was used to determine the differences in incidence of antibodies between groups. The Mann-Whitney test was employed to test differences in the serum level of antibodies between groups.

Results

Normal C57BL/6J have low levels of IgG antibodies to DNA

To assess whether nonautoimmune C57BL/6J mice expressed these antibodies, sera from 16 female and 18 male mice were tested by ELISA for the presence of IgG antibodies to dsDNA. Two of 18 males (11.11%) compared to 10 of 16 females (62.5%) showed detectable levels of these antibodies ($P < 0.01$). As shown in Fig. 2a, the level of anti-dsDNA antibodies was also significantly higher in the female C57BL/6 mice than that in their male counterparts (0.008 and 0.006 median specific OD, respectively; $P < 0.05$). The levels of antibodies to dsDNA in autoimmune-prone mice have been included in Fig. 2b for reference. Previous work had shown that autoimmune disease-prone (NZB × NZW)F_1 and BXSB/MP mice [carrying the Yaa (Y-chromosome-linked accelerating) gene] manifest IgG antibodies to dsDNA (23).

Male hormones have minimal effects on the expression of IgG antibodies to dsDNA

The lower production of IgG antibodies to dsDNA in male C57BL/6J mice was not due to a protective effect of male hormones, because orchidectomized mice that received a placebo implant (P-O♂) did not differ from the placebo-treated sham-orchidectomized (P-S♂) group (0.008 and 0.007 specific OD, respectively; $P > 0.10$; Fig. 3). Further, treatment of orchidectomized mice with DHT did not alter the level (0.011 median specific OD; $P > 0.10$) of anti-dsDNA antibodies. There was no statistical difference between the level of anti-dsDNA antibodies in the surgical controls (P-S♂, 0.007 median specific OD; Fig. 3) and that in the untreated males (0.006 median specific OD; Fig. 2a; $P < 0.10$).

In male C57BL/6J mice, estrogen induces IgG antibodies to dsDNA

Administration of estrogen to male mice with or without testes had a significant enhancing effect on these autoantibodies (Fig. 3). The median level of antibodies to dsDNA for mice in the E_2-O♂-treated group (0.021 specific OD) was significantly higher than that in their controls (P-O♂, 0.008 specific OD; $P < 0.005$). Mice that received estrogen but were not orchidectomized (E_2-♂) had levels of antibodies to dsDNA (0.029 median specific OD) similar to those in the E_2-O♂-treated group ($P > 0.10$; data not shown). The mean

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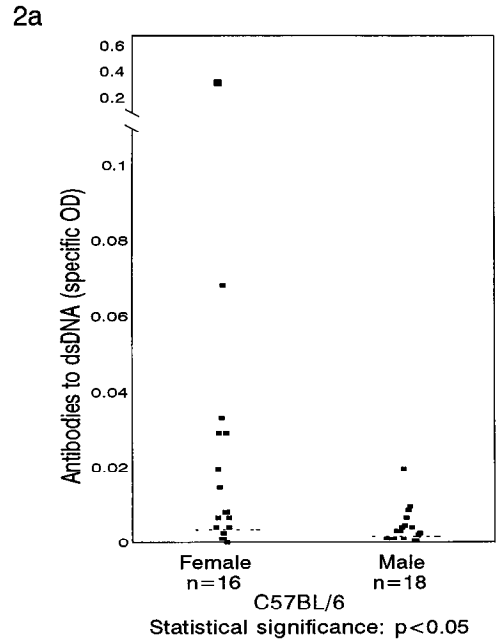

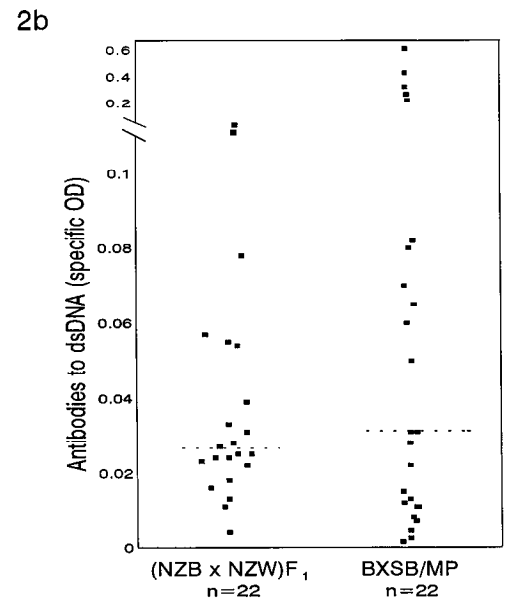

FIG. 2. Antibodies to dsDNA in C57BL/6J, (NZB × NZW)F1, and BXSB/MP mice. a, Male (n = 18) and female (n = 16) untreated C57BL/6J mice were tested for the expression of antibodies to dsDNA. The figure illustrates the level of anti-dsDNA antibodies in individual mice. The *dotted lines* represent the median for each group. Differences between male and female C57BL/6 mice were assessed by Mann-Whitney test ($P < 0.05$). Male mice (mean age, 10.4 months; range, 4–22 months). Female mice (mean age, 10.8 months; range, 3–21 months). b, Levels of antibodies to dsDNA in autoimmune-prone (NZB × NZW) F_1 and BXSB mice are shown as reference.

level of E_2 for untreated male mice was 59.3 pg/ml and 56.6 pg/ml for P-O♂, whereas that of E_2-O♂ was 211 pg/ml. This value was comparable to that of E_2-O♀ (193.7 pg/ml).

In female C57BL/6J mice, the expression of antibodies to dsDNA is markedly influenced by estrogen

As estrogen promoted the expression of IgG anti-dsDNA antibodies in male mice (E_2-O♂), we next determined whether

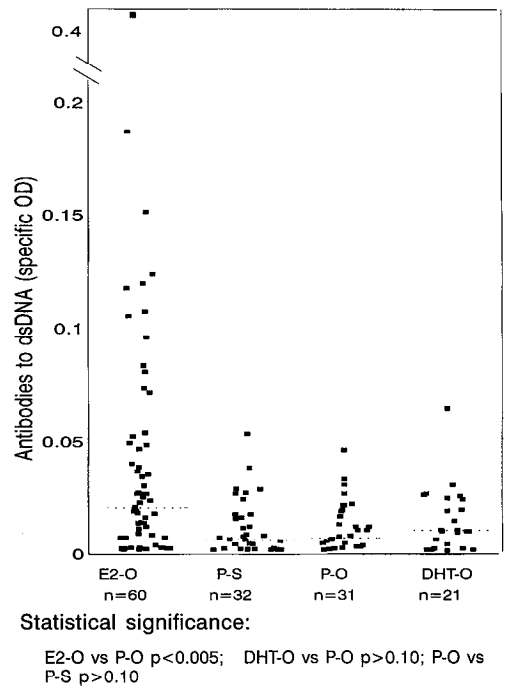

FIG. 3. Estrogen treatment of orchidectomized C57BL/6J mice induces anti-dsDNA antibodies. Four- to 5-week-old prepubertal male C57BL/6J mice underwent orchidectomy and were given DHT (DHT-O♂; mean age, 11.3 months; range, 3–13.3 months) or E_2 (E_2-O♂; mean age, 7.4 months; range, 3–11.4 months) implants. Control groups include orchidectomized mice with placebo implants (P-♂; mean age, 9.5 months; range, 3.3–13.3 months) and sham-orchidectomized mice with placebo implants (P-S♂; mean age, 8.3 months; range, 3.8–12 months). The figure illustrates the level of IgG anti-dsDNA antibodies of individual mice. The *lines* represent the median for each group. Data were analyzed by nonparametric Mann-Whitney test.

estrogen would further increase the production of these autoantibodies in female mice. Twenty-three female mice received estrogen implants. As observed in males, administration of exogenous estrogen to intact female mice (E_2-♀, 0.015 median specific OD) doubled the level of these antibodies compared to that in female mice that received placebo implants (P-♀, 0.007 specific OD; $P < 0.01$; Fig. 4a).

The effect of estrogen in the absence of ovaries was then determined. Female mice were ovariectomized and given either E_2 (E_2-O; n = 10) or a placebo implant (P-O♀; n = 10). An additional group of 10 female mice that were sham ovariectomized and received a placebo (P-S♀) served as surgical controls. The group of mice that was ovariectomized (P-O♀) and presumably had reduced levels of female hormones had lower levels of IgG antibodies to dsDNA (0.004 specific OD). There was no statistical difference in anti-dsDNA antibodies between P-O♀ and their surgical controls (P-S♀ = 0.005 median specific OD; $P > 0.10$; Fig. 4b). All ovariectomized female mice that received estrogen implants, however, had significantly higher levels of antibodies to dsDNA (0.026 specific OD; $P < 0.0001$). Together, these studies strongly suggest that estrogen promotes these autoantibodies.

Estrogen accelerates the expression of antibodies to dsDNA

Sequential studies were performed to study the kinetics of expression of these antibodies relative to age and estrogen

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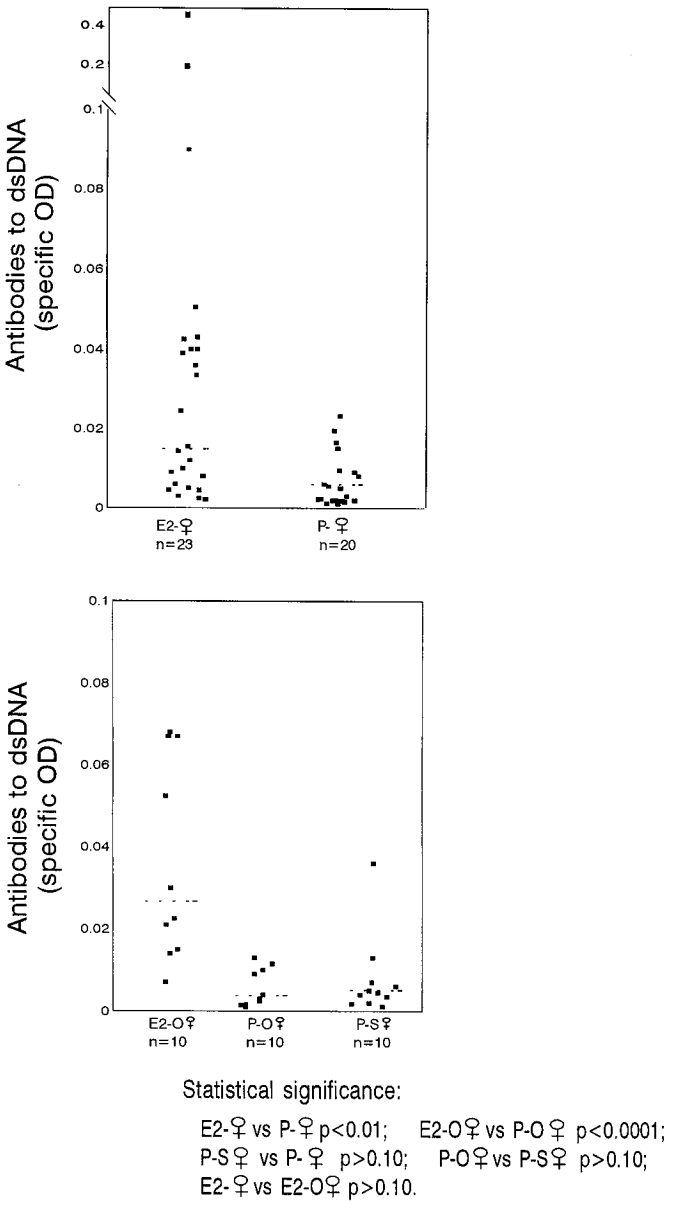

FIG. 4. Estrogen further increases anti-dsDNA antibodies in female C57BL/6J mice. Four- to 5-week-old female mice were given either E_2-♀ (mean age, 5.7 months; range, 3–7.5 months) or placebo implants (P-♀; mean age, 5.1 months; range, 3–7.8 months). a, IgG anti-dsDNA antibodies, assessed by ELISA. Note that there is a significant increase in the level of anti-dsDNA antibodies in mice treated with estrogen. b, Levels of anti-dsDNA antibodies in female mice that were ovariectomized and received E_2 implants (E_2-O♀; mean age, 3.5 months; range, 2.5–5.5 months) and their controls [P-O♀ (mean age, 4.2 months; range, 2.5–5 months old) and P-S♀ (mean age, 4 months; range, 2.5–5 months)]. The figure illustrates the levels of IgG anti-dsDNA antibodies in individual mice. The *dotted lines* represent the median for each group. Data were analyzed by nonparametric Mann-Whitney test.

treatment. Prepubertal (4–5 weeks) female (E_2-♀; 13 mice/group) and orchidectomized male (E_2-O♂; 9 mice/group) mice received E_2 implants. An equal number of mice received placebo implants (P-♀ and P-O♀). All implants were surgically removed 9 weeks postimplantation to ensure that mice would not be exposed to more exogenous estrogen. Antibodies to dsDNA (IgG, IgM, and IgA) were measured at the time of administration of the implant (4–5 weeks of age), upon removal of the implant (13–14 weeks of age), and at weeks 23 and 32. None of the mice had either IgM, IgG, or IgA anti-dsDNA antibodies at the time of administration of the implant. At the time of removal of the implant, over 80% of the estrogen-treated mice had detectable levels of IgM antibodies compared to less than 50% of the mice that received placebo implants. Estrogen treatment of mice also induced earlier and sustained expression of IgG anti-dsDNA antibodies in both male and female mice (Fig. 5). The incidence of anti-dsDNA antibodies in the female mice that received estrogen implants increased sharply with the administration of the hormone and remained elevated throughout the study. Interestingly, the incidence of antibodies to dsDNA in female mice that received a placebo increased over time, and by week 53 was similar to that in estrogen-treated mice (data not shown). In orchidectomized males that received a placebo, the incidence remained below 35% compared to over 55% in those that received estrogen implants. Overall, estrogen-treated mice attained earlier, higher, and more sustained levels of anti-dsDNA antibodies than the controls. IgA anti-dsDNA antibodies were not detectable in this strain, with the exception of one estrogen-treated female at week 23 (data not shown).

Analysis of isotype and subisotype of anti-dsDNA antibodies in estrogen-treated mice

C57BL/6J mice had IgM and IgG antibodies reactive to dsDNA. IgA antibodies were not detectable. It is not possible to directly compare the levels of the different isotypes and subisotypes of anti-dsDNA specific antibodies, because various reagents with differing degrees of sensitivity were employed. Nevertheless, a comparison between groups (estrogen-treated and placebo-treated mice) could be made. Upon estrogen treatment, female mice showed significantly increased levels of anti-dsDNA antibodies of the IgM and IgG isotypes ($P < 0.001$ and $P < 0.005$, respectively; Fig. 6). Although antibodies to dsDNA of all four subisotypes of IgG were augmented by treatment of female mice with estrogen, the largest increase corresponded to IgG2b ($P < 0.001$). In males, estrogen treatment also induced an increase in IgG anti-DNA antibodies ($P < 0.005$). IgG subisotype characterization indicated that estrogen increased anti-dsDNA antibodies of IgG2b and, to a lesser extent, IgG1 and IgG2a subisotypes (Fig. 6).

Discussion

Anti-DNA antibodies (mainly IgM) are present in normal individuals as part of their natural antibody repertoire. Antibodies to dsDNA (IgG) are commonly present in SLE patients. IgG anti-dsDNA antibodies have been found to correlate with the progression of the disease, and there is evidence of their association with tissue damage (16, 24). Their precise pathogenic role, however, has remained elusive (16, 24). DNA is a poor antigen and attempts to induce these antibodies by direct inoculation of native DNA into animals

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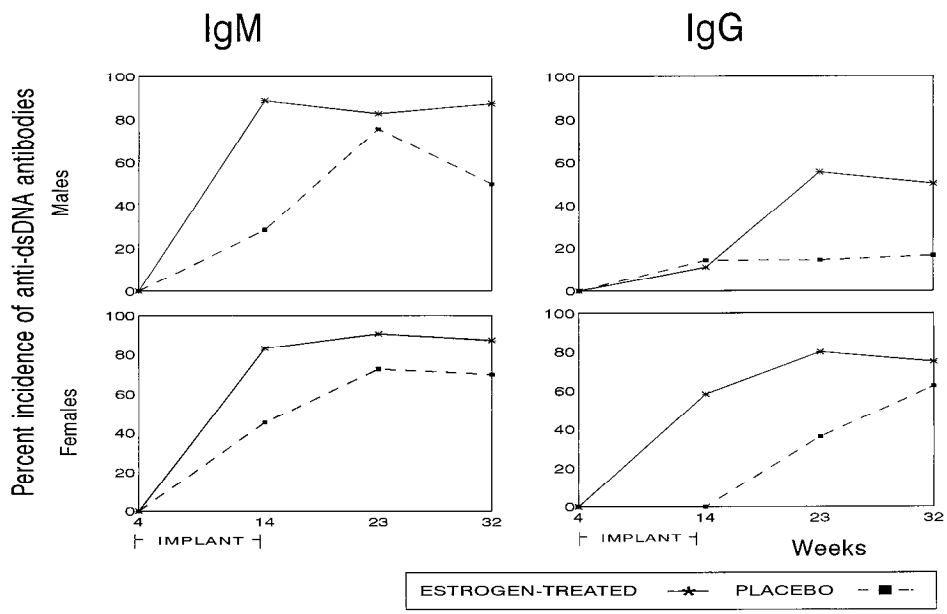

FIG. 5. Kinetics of estrogen-induced antibodies to dsDNA in (male and female) C57BL/6J mice. Four groups of mice [E_2-♀ (n = 13), P-♀ (n = 13), E_2-O♂ (n = 9), and P-O♂ (n = 9)] were tested, starting on the day of the implant (4–5 weeks), on the day of the removal of the implant (13–14 weeks), and later during weeks 23 and 32 for anti-dsDNA antibodies. Differences in incidence between E_2-O♂ and controls (P-O♂) and between E_2-♀ and controls (P-♀) were assessed by χ^2 test. (Note that two E_2-♀, one E_2-O♂, and one P-O♂ died before week 32. Upon postmortem examination, abnormal distension of the bladder was noted in the mice treated with estrogen.)

Statistical significance:

	IgM			IgG		
WEEK	14	23	32	14	23	32
E2-O♂ vs P-♂	<0.02	NS	NS	NS	<0.01	<0.01
E2-♀ vs P-♀	0.06	NS	NS	<0.005	<0.05	NS

have been largely unsuccessful. Recently, however, diverse approaches have been used to induce or enhance the production of anti-DNA antibodies in normal mice, including inoculation with heterologous (bacterial) DNA (25), DNA bound to immunogenic peptides (*e.g.* Fus-1) (26), or pathogenic antiidiotype (16/6) antibody (27). Despite the low immunogenicity of DNA, the characteristics of anti-DNA antibodies in SLE appear to be the result of antigen-driven, clonally selected, specific B-cell stimulation (26).

In this study, we found that unmanipulated nonautoimmune C57BL/6J mice, particularly females, have low levels of antibodies that react to dsDNA. Male hormones appeared to have minimal effects on the regulation of these autoantibodies, because neither prepubertal orchidectomy nor administration of DHT to orchidectomized mice significantly altered the levels of these antibodies. In contrast, administration of estrogen augmented the level of anti-dsDNA antibodies. Further, depletion of endogenous estrogen by ovariectomy in female mice resulted in lower anti-dsDNA antibody levels. Thus, the above studies suggest that estrogen promotes anti-dsDNA antibodies in nonautoimmune C57BL/6J mice, whereas male hormones do not. The autoantibody nature of the anti-dsDNA antibodies from estrogen-treated mice was assessed by comparing their binding to dsDNA from calf thymus (routinely used for testing anti-dsDNA antibodies) with that to dsDNA from liver of estrogen- and placebo-treated mice dsDNA (data not shown). The results show very similar binding patterns, indicating the autoantibody nature of these antibodies. Studies are underway to assess whether these antibodies have a pathological role.

Sequential studies showed that young animals exposed to estrogen produce IgM and IgG anti-dsDNA autoantibodies earlier and in higher amounts. Interestingly, the incidence of anti-dsDNA antibodies in E_2-O♂-treated mice increased after the implants were removed. This suggests that exposure to estrogen for a short period (9 weeks) has a long-lasting effect on the immune system. Further, the incidence of anti-dsDNA antibodies remained high after the removal of exogenous sources of estrogen, similar to what was observed for estrogen-induced anticardiolipin antibodies in C57BL/6J mice (Verthelyi, D., unpublished observations). Interestingly, the incidence of anti-dsDNA antibodies in the control groups increased steadily with age, especially in female mice. Estrogen, therefore, appears to have a long-lasting accelerating and enhancing effect on the expression of these autoantibodies. Studies are being performed on the effects of similar estrogen treatment on mature animals, which may correspond to women of postmenopausal age.

Under normal conditions, the synthesis of autoantibodies is down-regulated by several mechanisms, including clonal deletion and anergy, thus maintaining tolerance of self. There are several potential mechanisms by which estrogen may override tolerance and induce the expression of anti-dsDNA antibodies. First, estrogen may act directly on B-cell populations to override clonal anergy of autoreactive B-cells. However, to date, receptors for estrogen on B-cells have not been definitively identified. Second, estrogen may act through the hypothalamo-hypophyseal-thymic axis (28) to alter the neuro-immuno-endocrine regulatory circuits. Third, sex hormones may affect other cells of the immune system, such as macrophages or T-cells, to alter the production of cytokines that regulate B-cell activity and/or heavy chain isotype

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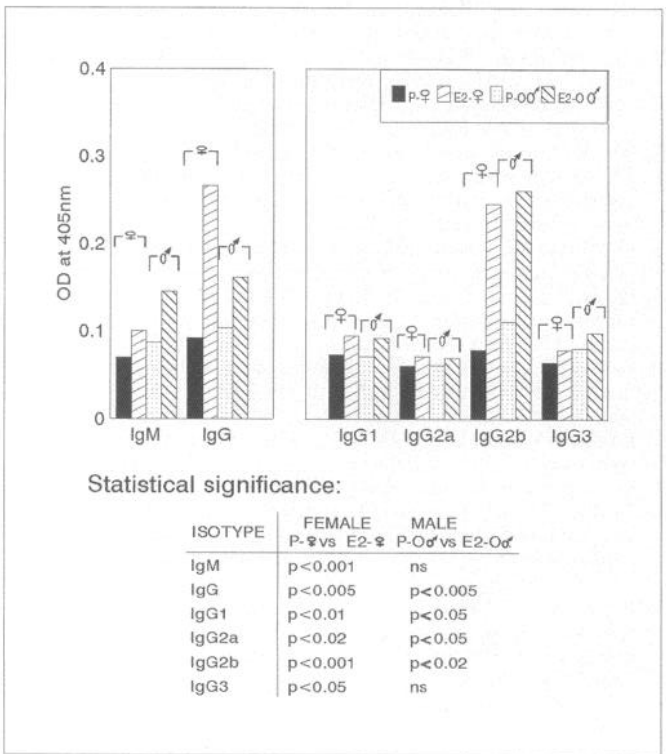

Statistical significance:

ISOTYPE	FEMALE P-♀ vs E2-♀	MALE P-O♂ vs E2-O♂
IgM	p<0.001	ns
IgG	p<0.005	p<0.005
IgG1	p<0.01	p<0.05
IgG2a	p<0.02	p<0.05
IgG2b	p<0.001	p<0.02
IgG3	p<0.05	ns

FIG. 6. Isotypes of anti-dsDNA antibodies in C57BL/6J mice. Shows the mean level of anti-dsDNA antibodies in the serum of mice in the following groups: P-♀ (n = 9), E_2-♀ (n = 8), P-O♂ (n = 15), and E_2-O♂ (n = 16). Due to the use of different subisotype-specific antibodies, it was not possible to directly compare among (sub)isotypes. However, it is possible to compare between hormone treatment groups. Note that almost all IgG anti-dsDNA subisotypes were increased in the sera of estrogen-treated mice.

switching (*e.g.* interleukin-4, -5, and -6 and transforming growth factor-β) (29). Fourth, estrogen may be toxic to cells, thereby increasing the exposure to intracellular molecules. Finally, the possibility that estrogen may activate an unknown latent virus or other infectious agents that could promote autoimmunity cannot be ruled out, especially as inoculation with bacterial DNA has been shown to be immunogenic in mice (25).

Estrogen treatment has been shown to induce antibodies reactive with cardiolipin and other anionic membrane phospholipids in C57BL/6J mice (13, 14). Preliminary data suggest that the cross-reactivity between estrogen-induced antibodies to dsDNA and cardiolipin is variable and, in general, low (data not shown). Polyreactive low affinity antibodies and monoreactive autoantibodies could both be induced by estrogen treatment.

There is a vast body of literature which documents that estrogen treatment of normal rodents augments their levels of antibodies to many exogenous antigens (1–4, 9, 30). Further, estrogen treatment of autoimmune mice has been shown to promote antibodies to a variety of autoantigens (7, 9, 31). In this study, we demonstrated that antibodies to dsDNA can be induced in normal C57BL/6J mice by manipulation of sex steroid levels. It must be emphasized that other than hormone treatment, these mice were not injected with DNA, cardiolipin, or any other antigen or preparation from infectious agents. These data together with our previous findings of estrogen-induced antibodies to cardiolipin and bromelain-treated erythrocytes support the concept that estrogen alters immunoregulatory pathways in otherwise normal subjects. This is important because prenatal exposure to diethylstilbestrol, a nonsteroidal estrogen, has been shown to enhance the susceptibility to neoplasms and the development of autoimmune disease (32, 33). Further, increasing numbers of women are taking estrogen-containing drugs as either contraceptives or replacement therapies. Therefore, it would be of relevance to ascertain whether the exposure to pharmacological levels of estrogen in nonautoimmune individuals (with susceptible genetic backgrounds) could potentially alter their immune competence.

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