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Estrogen Induces the Development of Autoantibodies and Promotes Salivary Gland Lymphoid Infiltrates in Normal Mice

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There are important bidirectional interactions between the immune and the endocrine system. Sex hormones influence the immune system throughout life including postnatal and prenatal stages. For example, we find that administration of estrogen to normal mice markedly augments the ability of $CD5^+$ B cells to express their autoimmune potential by producing increased numbers of plaque-forming cells (APFC) to bromelain-treated mouse erythrocytes (Br-ME) [1].

The effect of sex hormones on immune function at the most critical stage of development, the prenatal period, remains unstudied. We hypothesize that an imbalance of the *in utero* sex hormone microenvironment critically influences the fetal immune system. We have termed this influence immunological imprinting [2]. After birth this imprinting could contribute to immune-mediated disorders. To test this hypothesis, we developed a mouse model in which normal mice were prenatally exposed to estrogens. In preliminary experiments, these mice produced higher numbers of APFC to Br-ME, particularly in the peritoneal cavity cell exudates. Furthermore, mice prenatally exposed to estrogens had accelerated development of autoimmune salivary gland lesions indistinguishable from Sjögren's syndrome (SS) in humans. Further experiments are warranted to confirm these findings. The prenatal effects of estrogen may have relevance for familial and neonatal autoimmune syndromes.

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Introduction

There are marked physiological differences in immune capabilities between the two sexes [3, 4]. Females in general are immunologically more reactive than males [3, 4]. For example, women have higher levels of immunoglobulins, respond better to heteroantigens and resist a variety of infections better than men [3, 5]. Therefore, it is not surprising that females are increasingly susceptible to the development of autoimmune disorders [3–7].

Sex hormones have been implicated in the pathogenesis of autoimmune diseases based both on direct experimental evidence in animal models for these disorders, as well as on indirect evidence in humans [3, 4, 6, 7]. In general, autoimmune NZB/NZW mice given estrogen had accelarated disease characterized by increased mortality, more severe renal lesions and heightened production of autoantibodies [8]. In humans, alteration of sex hormonal states such as pregnancy, administration of estrogen-containing oral contraceptives or menopause profoundly altered the course of autoimmune disorders [9–11]. Systemic lupus erythematosus (SLE) patients manifest abnormal sex hormone metabolism (hyperestrogenic/hypoandrogenic states) which could play a role in their disease [7, 12, 13]. Furthermore, reduced serum concentrations of androgens in SLE and rheumatoid arthritis (RA) have been described [12, 14].

A minor subset of B cell bearing the CD5 marker ($CD5^+$ B cells) is increased in SS and in RA human patients as well as in SLE and SS-prone autoimmune mice [15–19]. These cells are important in the context of autoimmune disorders because they produce autoantibodies to DNA, Br-ME, rheumatoid factor and thymocytes [17, 20]. These cells seldom produce antibodies to heteroantigens. In this report, we describe the effects of estrogen (post-natal and prenatal) on autoantibodies to Br-ME which are produced primarily by $CD5^+$ B cells.

We suspected that sex hormones could influence immune development throughout life. We created an animal model in which developing fetuses were, presumably, exposed to abnormal levels of estrogen to study their effect on the fetal immune system. Pregnant mice were deliberately given exogenous estrogens and their offspring examined at various ages for immunological and immunopathological changes. The relevance of this model to the familial clustering of autoimmune diseases is discussed.

Materials and methods

Sex hormonal administration

Pre-pubertal (4-week-old) mice were orchidectomized or left intact and given estrogen implants by procedures described earlier [8, 21]. Briefly, estrogen implants (0.062 in internal diameter × 0.125 in outer diameter by 0.5 cm in length) were packed with 17-β-estradiol. Control mice received empty implants of equal length. These implants were left for 2–3 months during which period there is a sustained release of sex hormones.

In other studies, 14-day-old pregnant mice were given three separate injections of estrogen in sterile peanut oil until term. Controls received equal quantities of oil only. The offspring of these mice were sacrificed at different ages and selected immunological parameters studies. In some experiments mice were given only one

injection. Preliminary studies revealed that in these animals serum estrogen levels were similar to that seen in pregnant females which received three injections (unpublished observations).

Autoantibodies to bromelian-treated mouse erythrocytes

The autologous plaque-forming cell to bromelain-treated mouse erythrocytes was determined as described [1]. Peritoneal exudate, bone-marrow or spleen cells derived from mice pre-pubertally or prenatally exposed to estrogens were utilized. In some experiments lymphocytes were cultured for 3 to 4 d in the absence of exogenous mitogens or antigens. This procedure increases APFC to Br-ME [1]. Briefly, 100 µl of lymphocytes at 20 million cells/ml were admixed with Br-ME, (50 µl), Br-ME absorbed complement (20 µl) and complete media (30 µl). The resultant mixture is plated onto a slide chamber, sealed and incubated at 37°C in a 5% CO_2 incubator for 2 h. Appropriate controls were also plated. The number of APFC per million (IgM) were enumerated after 1–2 h of incubation.

Dual color analysis for CD5$^+$ B cells

CD5$^+$ B cells were visualized by staining lymphocytes with anti-CD5 and anti-IgM antibodies by FACS according to procedures described [1]. Briefly, 10 million lymphocytes were stained with rat anti-mouse CD5 antibody followed by FITC-goat anti rat IgG. Cells were subsequently stained with Texas red anti-IgM (Jackson Immunoresearch Avondale, PA, USA). Suitable controls were also set up. Cells were subjected to two color-flow cytometry and were visualized by contour plots and three dimensional plots. The data were analyzed by PDP/11 computer.

Histopathology

The salivary glands (submandibular and parotid) of mice prenatally exposed to estrogens at different ages were processed histologically and stained with H and E. The degree of severity of autoimmune lesion was based on a histopathological scoring method routinely employed by one of us (T.B.A.) in grading salivary gland biopsies from SS patients. Briefly, autoimmune lesions in salivary glands were graded or assigned a focus score which correlates with the severity of infiltration. Focal, nodular or diffuse infiltrates of lymphoid cells form aggregates. An aggregate with 50 or more round cells constitutes a focus. The focus score is then expressed as the number of aggregates of 50 or more cells per 4 mm^2 of the salivary gland tissue. A focus score of 1 or greater is consistent with the diagnosis of SS.

Statistical analysis

Student's *t*-test or Chi-square test was employed to analyze the statistical validity of the data.

Results

Females produce greater numbers of autoantibody plaque-forming cells (APFC) than males

Lymphocytes derived from the peritoneal cavity, spleen, or bone-marrow of several normal strains produce greater numbers of APFC to Br-ME if they are from female

Table 1. *Estrogen increases autoantibodies to bromelain-treated mouse erythrocytes in normal mice*

Hormone treatment	Number of mice	APFC to Br-ME/10^6 Cells	*P* value
Sham orchidectomized + Sham implants	6	77.5 ± 15.8	
Orchidectomized + Estrogen	7	249.5 ± 41.1	$P \leqslant 0.005$

Prepubertal (4–5 weeks old) CBA male mice were orchidectomized and subcutaneously implanted with empty (sham) or estrogen implants for 4 months. Mice were sacrificed at 5 months of age and the spleens of these mice were utilized to determine APFC to Br-ME.

rather than male age-matched mice [1]. Interestingly, there is no corresponding increase in percent $CD5^+$ B cells, although they are the primary producers of autoantibodies to Br-ME as compared to $CD5^-$ B cells [1, 20]. Purified $CD5^+$ B cells obtained by FACS sorting produce higher numbers of APFC compared to similar numbers of $CD5^+$ B cells from males [1].

Estrogen markedly augments the ability of these mice to produce autoantibodies ($P < 0.005$, Table 1). There is no comparable increase in $CD5^+$ B cells. Purified B or $CD5^+$ B cells sorted from estrogen-treated mice produce higher numbers of APFC to Br-ME than control mice which receive empty implants. The increased ability of $CD5^+$ B cells to produce autoantibodies can also be demonstrated in autoimmune-prone mice (unpublished observations).

Prenatal exposure to estrogen augments Br-ME APFC

Initially, to establish a model for examining the effect of sex hormones on the developing immune system, pregnant NZW mice were injected with sex hormones at different stages of gestation.

Synchronous estrus was achieved by placing 7–9-week-old females in cages already occupied by male mice for 24 h. Two female mice were mated with one male during the evening and the presence of vaginal plugs was noted 16–24 h later to determine pregnancy. Pregnant mice were divided into three groups. In the first group, sex hormones in sterile peanut oil or oil only were administered on day 1 of pregnancy and given on alternate days until day 20 of pregnancy. In the second group, hormones were administered from day 7 until term on alternate days. The third group received hormones on day 14 of pregnancy which was then continued on alternate days until term. None of the treated mice other than controls (untreated/oil-treated) conceived and delivered in the first group. Viable offspring were delivered in the third group with some degree of success. Marginal strain differences were noted in sensitivity to estrogen in terms of successful parturition. In some experiments the day of delivery is considered as day 1 of conception. In our preliminary studies mice

Table 2. *Prenatal effects of estrogen on APFC to Br-ME*

Prenatal hormone groups	Tissue	APFC to Br-ME/10^6 cells (at age 4 to 6 months)
Oil	Peritoneal cell exudates	$21{,}390 \pm 7{,}130$
Estrogen	Peritoneal cell exudates	$47{,}500 \pm 2{,}500$*
Oil	Bone-marrow	540 ± 180
Estrogen	Bone-marrow	$1{,}013 \pm 253$*

C57BL/6J male mice prenatally exposed to oil only or estrogen were utilized. Four to 10 mice per group were sacrificed between 4–14 months of age. The APFC to Br-ME determined as before.
*$P < 0.05$.

prenatally exposed to testosterone had noticeable physical changes. Because testosterone can be converted to other hormones by aromatization, particularly in the placenta, the use of this hormone was discontinued.

C57BL/6J mice prenatally exposed to estrogen had increased APFC to Br-ME particularly in their peritoneal cavity cell exudates and bone-marrow compared to those mice exposed prenatally to oil only (Table 2). In general, the estrogen effects appeared to be more pronounced in males and the magnitude of response varied with age. In females, their own endogenous estrogens may interfere with the response.

Prenatal estrogen exposure augments the autoimmune salivary gland lesions

The increased incidence of salivary gland lymphoid infiltrates induced by estrogen was apparent at 5 months of age ($P < 0.01$). By 9–14 months of age, the incidence reached 100% compared to fewer and milder (lower focus score) lesions in the controls. Histologically, there were striking focal mononuclear cellular infiltrates with acinar atrophy and destruction (Figure 1). The infiltrates were polymorphous and comprised of lymphocytes, a few plasma cells and macrophages. There was a striking histopathological resemblance of these salivary gland lesions to those observed in patients with SS and in other animal models of SS [22]. It is noteworthy that untreated aged female (C57Bl/6J mice developed SS-like lesions more frequently than untreated age-matched males.

Discussion

B-cell hyperactivity, as evidenced by increased production of autoantibodies, is characteristic of autoimmune disorders. Recently, a minor but important subset of B cells bearing the CD5 marker has been identified. Several findings suggest that it may have relevance in autoimmunity both for mice and humans. For example, the cells are increased or activated in certain genetically autoimmune-prone mice such as NZB or

Table 3. *Prenatal estrogen induces salivary gland lymphoid infiltrates as determined by incidence (%) and severity (focus score)*

Prenatal treatment	Age (months)	
	4–5	9–14
Incidence (%)		
Oil	0	33
Estrogen	40*	100*
Severity (focus score)		
Oil	0	0.55 ± 0.49
Estrogen	0.40 ± 0.24	2.05 ± 0.46*

C57BL/6 mice prenatally exposed to oil only or estrogen in oil were sacrificed at different ages to examine salivary gland autoimmune lesions. There were four to six mice per group.
*$P < 0.01$.

'viable moth-eaten' and they preferentially produce autoantibodies to erythrocytes, DNA, rheumatoid factor and thymocytes. Further, these cells rarely produce antibodies to heteroantigens. In humans, $CD5^+$ B cells are increased in RA [15, 17] and SS [16] but surprisingly, not in SLE [16].

There is clonal proliferation of $CD5^+$ B cells in aged autoimmune mice. Further transplantation of bone-marrow cells for AKR thymectomized mice to syngeneic or $(AKR \times DBA/_2)$ F1 intact recipients led to the development of B-cell lymphomas which express the CD5 marker [23]. There is also elevated myc expression and C-myc amplification in $CD5^+$ B-cell lines. These findings suggest the potential for oncogenesis which may have relevance to B-cell lymphomas seen in some SS patients [24]

Although $CD5^+$ B cells were not significantly increased in normal untreated females compared to males, their activity as determined by the production of autoantibodies to Br-ME (APFC) is markedly augmented in females. This effect was mediated by sex hormones. Estrogen profoundly increased the ability of $CD5^+$ B cells to produce these autoantibodies. It has been suggested that $CD5^+$ B cells in humans can be activated by growth factors, oncogenes or viruses [25]. Estrogens may be an additional direct or indirect stimulatory factor [1].

Estrogen given prenatally similarly augmented the ability of $CD5^+$ B cells to produce autoantibodies in the offspring, particularly in the bone-marrow and the peritoneal cavity cell exudates. However, the magnitude of APFC to Br-ME in prenatally estrogen-exposed mice was lower than postnatal estrogen-tested mice. In some experiments we did not see increased autoantibodies in the offspring of mice given estrogen which may be due to technical problems such as expulsion or leakage of oil-based estrogen due to increased physical abdominal pressure in pregnant animals. Further, since there are numerous target sites for estrogen, adequate amounts of estrogen may not be available *in utero*. The amount of estrogen available

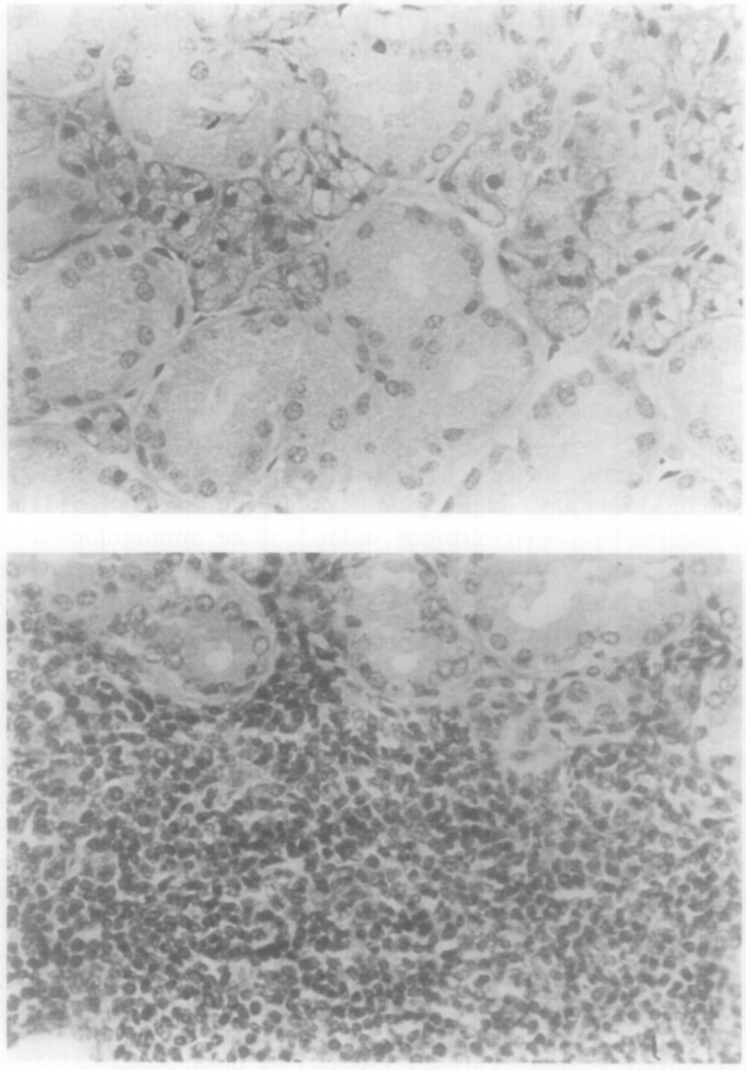

Figure 1. The top photomicrograph shows the salivary gland of a 9-month-old male C57BL/6J mouse prenatally exposed to oil. The acinar architecture is intact. The bottom photomicrograph shows the salivary gland of age and sex matched (C57BL/6J) mouse which was prenatally exposed to estrogen. Note the marked infiltration of mononuclear cells and macrophages and focal destruction of the glandular architecture.

in utero may vary with animals. We are currently determining the amount of estrogen available *in utero* (at placenta, uterus, fetus) by the use of radio-labeled estrogen.

The rationale for prenatal estrogen exposure studies stems from the following considerations. First, the immune system of mice develops during the late second week of pregnancy. The prenatal thymus of mouse embryos show rearrangements of T-cell receptor β-chain genes and transcripts of α and β chains and γ chains [26, 27]. Since there are significant concentrations of gonadal hormones *in utero*, it is highly plausible that these sex hormones could influence the differentiation and maturation

of lymphocytes. Secondly, SLE patients have been characterized as hyperestrogenic and hypoandrogenic [12, 13]. Human fetuses developing in autoimmune patients would be exposed to this abnormal hormonal microenvironment which might induce a predisposition to latent or over-autoimmunity. Early or prenatal sex hormone exposure of mice results in irreversible changes in central nervous system mediated reproductive behavior and physiology which is referred to as 'neurological imprinting' [28]. Similar findings have been noted in the metabolism of sex steroids in the liver and referred to as hepatic-steroid imprinting [29]. We have previously introduced the term 'immunologic imprinting' to mean long-term changes in the fetal immune system that can lead to the appearance of autoantibodies [2] and as reported here autoimmune tissue resembling SS.

Male fetuses situated *in utero* between two female fetuses had higher concentrations of estrogen in their amniotic fluid, and after birth had smaller seminal vesicular weights and were less aggressive than males located between two male fetuses [30]. Thus, it is conceivable that prenatal hormones could similarly exert major influences on the development and regulation of the immune system [31].

The mechanism by which estrogen induces these immunological changes is unclear. We are currently performing a systematic study to confirm and extend these studies. The possibility of activation of a virus or induction of autoantibodies or factors in the mothers which could cross transplacentally and influence the fetus cannot be discounted.

Male mice prenatally exposed to estrogens had accelerated and more severe development of salivary gland lesions compared to controls. These autoimmune lesions, characterized by mononuclear infiltration of lymphocytes, plasma cells and macrophages resulting in acinar architecture destruction, was indistinguishable from those seen in SS. These preliminary observations have implications for neonatal lupus. Although genetic inheritance may be the primary factor in the development of neonatal lupus, abnormal exposure to high levels of prenatal estrogen or its products could be a contributory factor.

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