Hormonal Approaches to Immunotherapy of Autoimmune Disease[a]

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INTRODUCTION

Autoimmune diseases are multifactorial, with genetic, environmental, hormonal, viral, and psychoneurological influences all playing a role in pathogenesis.[1] A striking feature of almost all autoimmune diseases is the marked female predominance. SLE is a good example. The female to male ratio in this disease is 10:1 when one considers patients in the childbearing age. This ratio falls to 3:1 when patients are premenopausal children or postmenopausal women. This simple clinical observation alerts one to a possible important sex hormone influence related to the menstruating reproductive years of a woman's lifetime.

Other clinical facts support this suggestion. For example, Klinefelter's syndrome (an XXY condition in men associated with feminizing features such as gynecomastia) is often accompanied by autoimmunity, including SLE and myasthenia gravis.[2-4] The medical literature includes a pair of monozygotic twins, only one of whom developed SLE some years after oophorectomy.[5] We are also following a patient with Noonan's syndrome (male Turner's syndrome) whose male hypogonadism is associated with SLE and lupus nephritis. SLE patients appear to be under a hyperestrogenic influence.[6,7] Furthermore, the use of estrogen-containing contraceptive pills can result in exacerbations of SLE and is generally contraindicated.[8,9]

In both experimental animals and in humans, normal immunologic reactivity is greater in females than in males.[10] The basis for this difference and the probable explanation for the influence of sex on autoimmune disease lie in the ability of sex hormones to modulate the immune response.[11] Early evidence suggested that sex hormones act on the thymus to influence lymphocyte development and on the reticuloendothelial system to regulate immune complex clearance. For example, suppressor T cells are one of the sensitive target sites for sex hormone action.[12,13] However, the picture now is more complicated with the recent findings in the field of neuroimmunomodulation and the known influence of sex hormones on the central nervous system (CNS).[14]

Our published studies on the ability of sex hormones to modulate the lupus-like

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disease of autoimmune mice are briefly reviewed in this report.[15-17] We present further evidence for the ability of estrogen to deplete suppressor T cells, along with recent findings on the ability of sex hormones to modulate the developing fetal immune system (a phenomenon that we call immunologic imprinting).

MATERIALS AND METHODS

Animals

Four- to seventeen-week-old normal C57BL/6 (B6) and eight- to ten-week-old NZW mice were purchased from Jackson Laboratories, Maine. Autoimmune mice are from our own colonies.

Sex Hormone Preparations

Estrogen (E_2-Ayerst Labs) and Depo-testosterone (Te-Upjohn) were used in these studies. Various doses of E_2 (100 ng and 10 μg/100 gram body weight) or Te (100 μg to 1000 μg/100 gram body weight) in sterile peanut oil were administered subcutaneously in a final volume of 0.2–0.25 ml. Te or E_2 were also prepared in the form of capsules as described earlier.[16]

Orchidectomy

A group of mice (C57BL/10) were prepubertally orchidectomized by procedures described earlier.[18]

Sex Hormone Administration

Orchidectomized C57BL/10 or intact C57BL/6 were given either Te or E_2 implants for 3–8 months. Adult female mice were given various doses of Te or E_2 in oil on an alternate day basis for a period of two to four weeks.

Fourteen-day-old pregnant NZW, B/W, or B6-lpr mice were given three separate subcutaneous injections of sex hormones on alternate days until term. The offsprings of these mice were sacrificed at different ages and selected immunological parameters known to be influenced by sex hormones were studied.

Collection of Tissue Materials

Animals were weighed and bled by orbital exsanguination. The serum was collected and stored at -70°C for the analysis of serum autoantibodies. Thymus, spleen, and lymph nodes were collected under aseptic conditions and single cell suspensions were prepared as described earlier.[12] The detailed methodologies for FACS analysis, IL-2 production, and enumeration of serum autoantibodies to DNA have been described earlier.[12,16]

Autoantibody Plaque-Forming Cells to Bromelin-Treated Mouse Red Blood Cells (Br-APFC)

Syngeneic mouse red blood cells (MRBC) obtained from young mice (less than three months old) were washed repeatedly in cold RPMI medium. Equal volumes of washed packed MRBC and 20 mg/ml Bromelin (Br) (Calbiochem-Behring) were mixed and incubated at 37°C in a CO_2 incubator for 45 minutes. The cells were washed three times and a final suspension (20%) was made in complete RPMI.

Spleen cells were treated with ammonium chloride to lyse red blood cells and were either incubated in complete RPMI for four days or used directly. The cell concentration was adjusted to 4×10^7 cells/ml in complete media. Lymphocytes (100 μl) were admixed with 50 μl of Br-MRBC, 20 ml of MRBC absorbed complement (Gibco), and 30 μl of RPMI-1640 complete media. The mixture (100 μl) was loaded into a Cunningham-type glass slide chamber with the aid of a micropipet and the edges were sealed with a paraffin-vaseline mixture. These slides were incubated for three hours at 37°C and the number of IgM plaques formed in each chamber were enumerated.

Ly1$^+$ B-Cell Enumeration

Splenic Ly1$^+$ B cells were visualized and quantitated by flow cytometry after staining spleen lymphocytes with dual antibodies, FITC-F(ab')$_2$ fragments of rabbit anti-mouse IgM (RAM; Zymed Labs), and biotinylated anti-Ly1 (Becton-Dickinson). The methodology for staining was essentially similar to single-color staining as reported earlier.[12] Briefly, 2×10^7 cells/ml were stained with FITC-F(ab')$_2$ RAM for 30–45 minutes in the cold. After appropriate washing procedures, cells were stained with biotinylated anti-Ly1 (30 minutes) followed by Texas red avidin (30 minutes). Controls were as follows: (1) biotinylated anti-Lyt-1 plus Texas red avidin, (2) FITC-F(ab')$_2$ RAM, (3) Texas red avidin alone, and (4) unstained cells. The data were visualized as contour plots and analyzed with a PDP/11 computer by procedures standardized in this laboratory.

RESULTS

Modulation of Lupus in B/W Mice by Sex Hormones

We have reported on the ability of sex steroid hormones to modulate the spontaneous SLE-like disease that occurs in NZB/NZW (B/W) mice.[15–17] In this lupus model, the disorder appears first in female mice and then several months later in males. Androgens suppress the disease in females, even when administered later in life when clinical features are already present. Depletion of androgen by orchidectomy results in an accelerated disease expression comparable to that seen in females (TABLE 1). Estrogen worsens the disease, as evidenced by early mortality when administered to B/W mice of either sex (TABLE 1).

Mechanism of Action of Sex Hormones

We next investigated the mechanism underlying sex hormone action. Studies from several laboratories suggest that there are multiple mechanisms by which sex hormones can modulate immune responses in general and autoimmune diseases in

TABLE 1. Effect of Sex Hormones on Survival of B/W Mice[a]

Experimental Procedure	% Mortality at 8 Months
Males	
Sham surgery	8
Orchidectomized	60[b]
Orchidectomized + E_2	95[b]
Orchidectomized + DHT	10
Females	
Sham surgery	78
Ovariectomized	87
Ovariectomized + E_2	95
Ovariectomized + DHT	15

[a]B/W mice were prepubertally gonadectomized and given either 17β-estradiol (E_2), Dihydrotestosterone (DHT), or empty implants.
[b]$p < 0.05$.

particular.[10] This is not surprising since receptors for sex hormones are present in many different cells in these lymphoid organs. These include lymphocytes, macrophages, epithelial cells, and reticular cells.[19,20] In addition, sex hormone receptors are present in the brain and pituitary. We have recently suggested that sex hormones may also act via the CNS to influence or modulate autoimmune disease.[19]

Available data suggest that T cells are the primary targets for sex hormone action. Evidence in support of this view includes the profound effects of sex hormones on the thymus and T cells.[10] We and others have recently reported that sex hormones affect a subset of T cells, namely, suppressor T cells. For example, E_2 depletes Lyt-2 positive cells, while Te or DHT maintains it.[12,21] As shown in TABLE 2, the administration of E_2 to either C57BL/10 orchidectomized mice or B6 intact mice reduced Lyt-2 positive cells in the spleen. By contrast, Te treatment had no such effect. Furthermore, E_2, but not Te treatment, reduced suppressor cell activity (TABLE 3). This was assessed by the ability of Con-A induced suppressor cells from sex hormone treated mice to inhibit the lymphoproliferative response to PHA. A decrease in suppressor function might contribute to autoimmunity through failure to control emergent B-cell clones capable of producing autoantibodies.

TABLE 2. Estrogen Depletes Lyt-2 Positive Cells

Strain	Experiment No.	Treatment	Tissue	% Positive	% Decrease
C57BL/10	1[a]	Intact	Spleen	22	—
		Orchidectomized	Spleen	14	—
		Orchidectomized + testosterone	Spleen	18	0
		Orchidectomized + estrogen	Spleen	2	86
C57BL/6	2[b]	Intact	Spleen	28	—
		Intact + estrogen	Spleen	15	46
		Intact	Lymph nodes	54	—
		Intact + estrogen	Lymph nodes	26	52

[a]Four-week-old mice were prepubertally orchidectomized and given testosterone or estrogen capsules for three months.
[b]Four-week-old mice were given estrogen capsules, which remained in place for three months.

TABLE 3. Effects of Sex Hormones on Suppressor Cell Activity

Strain	Treatment[a]	Percent Suppression (1:1)[b]
C57BL/6	Oil	17
	100 μg Te	36
	100 ng E_2	0
	10 μg E_2	0
C57BL/6-lpr	Oil	59
	100 ng E_2	55
	10 μg E_2	24

[a]Five-week-old mice were given sex hormones for two weeks.

[b]Splenic responder to splenic suppressor cell ratio of 1:1. Fifty microliters of splenic responder cells (plus PHA) were cocultured with 50 μg of Con-A induced splenic suppressor cells for 24 hours in the presence of PHA (3 μg/ml).

Prenatal Effects of Sex Hormones

We have recently investigated the influence of sex steroid hormones on the developing fetal immune system by administering Te or E_2 to pregnant murine mothers in the final week of gestation (day 14). We looked for long-term immunologic effects in the offspring of these mothers that were similar to those that occurred spontaneously in autoimmune mice. A variety of immunoregulatory abnormalities or immunopathologic effects was seen. Autoimmune mice, for example, spontaneously produce significantly greater numbers of plaque-forming cells (APFC) to bromelin-treated mouse red blood cells (Br-MRBC) (TABLE 4). These APFCs are thought to represent a subset of B cells bearing the Ly1 antigen.[22,23] Indeed, in addition to the augmentation in APFCs to Br-MRBC, we also observed marked increases in $Ly1^+$ B cells in autoimmune mice (TABLE 5). Both APFCs and $Ly1^+$ B cells are increased by the administration of estrogen to autoimmune mice starting at four weeks of age (Ansar Ahmed, Dauphinee, and Talal, to be published). Moreover, a similar increase in APFCs and $Ly1^+$ B cells could be induced in mice following *in utero* exposure to estrogen (TABLE 6).

DISCUSSION

Extensive investigations in mice over the past decade have unequivocally established the involvement of sex hormones in the pathogenesis of autoimmune disease.[10,24] Evidence is also accumulating to indicate that sex hormones profoundly modulate a wide range of experimental autoimmune diseases in animals.[10,24] Overall, the data strongly suggest that male sex hormones prevent or delay the expression of autoimmunity, whereas female sex hormones accelerate autoimmune diseases.

TABLE 4. Increased APFC to Br-MRBC in Spleens of Autoimmune-Prone Mice

Strain	APFC to Br-MRBC ($\times 10^7$)
C3H	650
C3H/lpr	1680
C57BL/6	220
C57BL/6-lpr	1130

TABLE 5. Increased Ly1$^+$ B Cells in Autoimmune-Prone C3H/lpr Mice

Strain	% Ly1$^+$ B	% Ly1$^+$ B of Total B Cells
C3H	6.1	19.1
C3H/lpr	11.6	31.4

The suppression of autoimmunity by male sex hormones is of clinical significance because these agents can be exploited therapeutically. We have shown that male sex hormones have significant therapeutic effect in mice with lupus.[17] Clinically, the attenuated male hormone Danazol has been successfully used in the treatment of autoimmune idiopathic thrombocytopenic purpura and may hold some promise in the management of SLE.[25,26] Thus, one can view sex hormonal modulation of autoimmune disease in two directions: (1) the suppressive properties of male hormones, their therapeutic value in murine lupus, and their potential therapeutic value in patients; (2) the enhancing properties of estrogen, the potential dangers of a hyperestrogenic state, and its significance for the initiation of the lupus diathesis.

This paper demonstrates the long-term effect of estrogen on the developing immune system when administered *in utero* by injection into pregnant murine mothers late in gestation. These mothers gave birth to offspring who showed features later in life that were characteristic of spontaneously autoimmune mice. These features included the presence of APFCs to autologous erythrocytes and an increase in the Lyt-1$^+$ subset of B cells. Both findings are usually associated with B/W and other autoimmune mice,[22,23] and they are considered characteristic features of immune dysregulation and autoimmune disease.

Our studies suggest that permanent alterations can be induced by estrogen at a critical stage in the development of the fetal immune system. This may have biologic importance for the subsequent emergence of SLE, as well as for abnormalities that occur in children born to mothers with symptomatic or asymptomatic SLE.

There are now several studies that demonstrate a deficiency of androgen[27] and/or a hyperestrogenic state[28] in SLE patients. Thus, it is reasonable to presume that a human fetus growing in the uterus of a pregnant SLE patient could be exposed to hormonal alterations similar to those that we have induced experimentally in pregnant mice. The offspring of these lupus mothers might be born with a permanently altered immune system that is predisposed to latent or overt autoimmunity analogous to our findings of immune abnormalities in mice born to hyperestrogenized mothers. Thus, the tendency for autoimmune diseases to occur in families might be explained not only by direct genetic inheritance (e.g., MHC genes), but also by hormonal influences acting upon the developing fetus resident *in utero*.

Indeed, children born to autoimmune mothers producing anti-Ro (SS-A) antibodies may develop the neonatal lupus syndrome characterized by skin rash, congenital heart block, and other congenital cardiac malformations. The skin rash generally disappears upon clearing of maternal immunoglobulins from the newborn's circulation, but the cardiac lesions persist.[29,30] Since the heart, like the central nervous system and immune system, contains receptors for sex hormones, it is possible that hormonal factors may contribute to the cardiac lesions in neonatal lupus.

TABLE 6. Prenatal Exposure of Male B/W Mice to Estrogen

Group	APFC/Spleen	Ly1$^+$ B cells (%)
Oil	300	6.7
Estrogen	3045	12.0

It is well recognized that early exposure to sex hormones induces permanent neurological and behavioral changes. For example, injection of androgen into female rats in the first 24 hours of life results in an aggressive malelike behavior. These permanent changes in the CNS induced by sex hormones are referred to as "imprinting." Accordingly, we suggest the term "immunologic imprinting" to refer to permanent changes induced in the immune system as a consequence of exposure to sex hormones *in utero*.[19]

Finally, autoimmune disorders, like malignant diseases, develop as a consequence of a sequential process that, by analogy with carcinogenesis, can be divided into three stages called initiation, promotion, and progression. Aside from a relatively weak genetic predisposition linked to the MHC, particularly to the class II genes and HLA B8 DR3, little is known about the initiation stage. Almost all therapeutic attempts to date have concentrated on suppressing the immune inflammatory features that characterize the last two stages of disease. A nonvirilizing, but immunologically restorative sex hormone might prove a valuable adjunct to conventional therapy and might also have possibilities as a prophylactic agent in high risk individuals, particularly young women born into families with a high incidence of SLE.

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DISCUSSION OF THE PAPER

R. S. SCHWARTZ (*Tufts University School of Medicine, Boston, MA*): What do you think about the difference in the results with Danazol in autoimmune diseases of the blood, such as immune thrombocytopenia, where beneficial effects have been obtained, whereas the drug seems to be only marginally effective in systemic lupus?

Another point I would like you to comment on is the evidence of estrogen receptors in human lymphocytes.

N. TALAL (*University of Texas Health Science Center, San Antonio, TX*): I would not say that the effect of Danazol in hematologic autoimmune diseases represents a discrepancy, but rather a reason to be optimistic. There is some evidence that ITP is a disease very closely related to lupus. You are correct about estrogen receptors in human lymphocytes; they occur particularly on the OKT8 suppressor cells, which is a finding that fits very nicely with the data we are getting in mice.

UNIDENTIFIED DISCUSSANT: Do you think that the immunological imprinting is strictly a peripheral phenomenon within the immune system itself or might it be mediated by neural changes?

TALAL: We do not know. I think that the dramatic effects we are seeing suggest some effect on the immune system; however, whether there are additional effects on the central nervous system is certainly a possibility.