

## **SECOND AUTOIMMUNITY MEETING — TALAL'S DAY**

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This is the second meeting of autoimmunity in Israel, and our honored guest is Norman Talal, Professor of Medicine at the University of Texas Health Science Center at San Antonio, USA. Dr. Talal is one of the pioneers of our modern knowledge of autoimmunity. His paper, "Sex hormones, CD5<sup>+</sup> (Ly1<sup>+</sup>) B-cells, and autoimmune diseases," represents part of our recent advances in understanding the etiology of autoimmune conditions. The re-

search done on autoimmunity and the data subsequently reported led this year to the appearance of two additional journals on this subject: *Autoimmunity*, and the *Journal of Autoimmunity*. However, despite all these investigations, no curative treatment for these conditions has been found. Let us hope that at the next meeting we will hear reports on new avenues of therapy for these fatal conditions.

### **SEX HORMONES, CD5<sup>+</sup> (LY1<sup>+</sup>) B-CELLS, AND AUTOIMMUNE DISEASES**

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Clinical and experimental studies demonstrate that autoimmune diseases are multifactorial in origin. Genetic, viral, hormonal, environmental and psychoneurological factors all play a role in pathogenesis, and an interplay of these factors may be necessary to initiate autoimmune diseases. A striking feature of almost all autoimmune diseases is the marked female predominance (1-4). In systemic lupus erythematosus (SLE) for example, the female to male ratio is 10:1 among patients in the childbearing age and 3:1 among premenstrual children or postmenopausal women. This simple clinical observation suggests that a sex hormonal influence related to the reproductive years may contribute to a predisposition to the illness. Other autoimmune diseases (rheumatoid arthritis, Hashimoto's thyroiditis,

Sjögren's syndrome) also occur predominantly in women. This female predominance may reflect the greater immune responsiveness of women compared with men (1,2). Our laboratory has been working over the past decade to discover the scientific basis for this clinical factor.

Our early studies used NZB/NZW F<sub>1</sub> mice, a classic model for lupus in patients. In humans, as in NZB/NZW F<sub>1</sub> mice over 3 to 4 months of age, the disease is more often seen in females than in age-matched males. Female mice die by 8 to 9 months of age, while males live more than 1 year. Orchiectomy and estrogen replacement therapy enhance the expression of lupus in these mice, while the male sex hormone, dihydrotestosterone (DHT), delays it (5). Male sex hormones also have a protective influence

in MRL/lpr mice, another model for SLE (6). In this more aggressive form of murine lupus, autoantibodies, proteinuria and survival are all considerably improved by DHT treatment. Other forms of male sex hormones are also beneficial in murine lupus (2,7). Even when DHT therapy is delayed until the disease is active, it still has a therapeutic effect and can prolong survival (8).

SLE patients, as well as NZB/NZW F<sub>1</sub> mice, demonstrate a delayed clearance of particulate immune complexes. Female NZB/NZW F<sub>1</sub> mice exhibit this abnormality earlier than males. Androgens retard this defect in female mice, whereas estrogens accelerate it in male NZB/NZW F<sub>1</sub> mice. These results suggest an important influence of sex steroid hormones on the clearance of immune complexes (1).

Murine lupus is not the only disease in which sex hormones play an influential role. Chronic autoimmune thyroiditis in rats induced by thymectomy and irradiation occurs more frequently and with greater severity in females than in males (9,10). A similar sex susceptibility pattern is evident in chronic autoimmune Hashimoto's thyroiditis in humans (11). Testosterone administration is therapeutic in rats with established autoimmune thyroiditis (12). Androgens also suppress autoantibodies to thyroglobulin in acute thyroiditis induced in mice by thyroglobulin-adjuvant injections (13).

Women are approximately three times more susceptible to the development of myasthenia gravis than men. Experimental myasthenia gravis, induced in susceptible C56BL/6 mice by the administration of acetylcholine receptor in Freund's adjuvant, can be modulated by manipulating sex hormone levels (14). Ovariectomy suppresses, while orchectomy enhances, specific lymphocyte proliferative responses to acetylcholine receptor. Administration of testosterone reduces the lymphocyte proliferative response and antibodies to acetylcholine receptor.

Injections of cell-wall peptidoglycan-polysaccharide fragments derived from Group A streptococcal bacteria into LEW/N rats induce polyarthritis more readily in female rats than in males (15). The disease induced in rats resembles rheumatoid arthritis clinically, histologically and in the sex susceptibility pattern. Depletion of male hormones in rats by orchectomy, or by increasing estrogen levels in male rats by implanting estrogen capsules, renders these rats as susceptible as females.

We have recently studied the effect of estrogen on CD5<sup>+</sup> (Lyl<sup>+</sup>) B-cells, which are increased in autoimmune patients and in lupus mice (2,16). These Lyl<sup>+</sup> B-cells are increased in very young mice but then decline with maturity (17). They are the source of much autoantibody production, including anti-

bromelain-treated mouse erythrocytes (Br-ME) and antisingle-stranded DNA in NZB/NZW F<sub>1</sub> mice (17).

## MATERIALS AND METHODS

### Autoantibody plaque-forming cells to Br-ME

Syngeneic mouse red blood cells obtained from young mice (<3 months old) were washed repeatedly in cold RPMI medium. Equal volumes of washed, packed mouse red blood cells and 20 mg/ml Bromelain (Calbiochem-Behring, FRG) were mixed and incubated at 37°C in a CO<sub>2</sub> incubator for 45 min. 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 4 days or used directly. The cell concentration was adjusted to 4 × 10<sup>7</sup> cells/ml in complete media. Lymphocytes (100 µl) were admixed with 50 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 3 h at 37°C, and the number of Ig plaques formed in each chamber were enumerated.

### Lyl<sup>+</sup> B-cell enumeration

Splenic Lyl<sup>+</sup> B-cells were visualized and quantitated by flow cytometry after staining spleen lymphocytes with dual antibodies, Texas red-F(ab')<sub>2</sub> fragments of goat anti-mouse IgM (TR-GAM; Jackson Research Laboratories, USA), and rat anti-Lyl followed by fluorescein isothiocyanate-goat antirat (FL-GAR). The methodology for staining was essentially similar to single-color staining as reported earlier (14). Briefly, 1 to 2 × 10<sup>7</sup> cells/ml were stained with anti-Lyt1 followed by FL-GAR for 30 to 45 min at -4°C. After appropriate washing procedures, cells were stained with TR-GAM (30 min). Controls were as follows: a) anti-Lyt1 plus FL-GAR; b) TR-GAM; c) Texas red avidin alone; d) unstained cells; and e) FL-GAR. The data were visualized as contour plots and analyzed with a PDP/11 computer (Digital, USA) by procedures standardized in this laboratory.

## RESULTS

We investigated the influence of sex steroid hormones on the developing fetal immune system by administering DHT or estrogen to healthy or autoimmune pregnant rats in the final week of gestation. We found evidence for an acceleration of autoimmunity and autoimmune disease in the offspring of estrogen-treated mothers, in which a variety of immunoregulatory abnormalities and immunopathologic effects were observed.

Autoimmune mice spontaneously produced significantly greater numbers of autoantibody plaque-forming cells (APFC) to Br-ME than did normal mice (Tables 1 and 2). These APFC represent a

subset of B-cells bearing the Lyl antigen (17). In fact, APFC to Br-ME were increased by the administration of estrogen to autoimmune mice either at 4 weeks of age or following *in utero* exposure to estrogen (Table 3). We are currently investigating the mechanisms whereby estrogen induces these effects.

Table 1. Increased APFC to Br-ME in spleens of autoimmune-prone mice

Strain	APFC to Br-ME ( $\times 10^6$ )
C3H	65
C3H/1pr	168
C57BL/6	22
C57BL/6-1pr	113

Table 2. Autoantibodies to Br-ME and Lyl<sup>+</sup> B-cells<sup>a</sup>

Strain	APFC to Br-ME ( $\times 10^6$ )	Lyl <sup>+</sup> B-cells ( $\times 10^6$ )
C57BL/6J	50	3.0
NZB	425	6.4

<sup>a</sup>Spleen lymphocytes were used for the determination of APFC to Br-ME, and for dual-color flow cytometric analysis of Lyl<sup>+</sup> B-cells.

Table 3. Prenatal exposure of male NZB/NZWF<sub>1</sub> mice to estrogen

Treatment	APFC to Br-ME/spleen
Oil vehicle	300
Estrogen	3,045

## DISCUSSION

Sex hormones can influence the immune system through multiple pathways. It is generally believed that sex hormones induce their effects on target cells by first interacting with specific steroid receptors. Sex hormone receptors have been found not only in classical reproductive tissues but also in many other body systems, including the immune system. We suspect that the presence of sex hormone receptors in these nonclassical sites may indirectly affect the immune system.

Overall, it appears that T-cells are the primary targets of sex hormone action (1,2). Regulatory T-cells appear to be very sensitive to sex hormones, although it has been difficult to demonstrate sex hormone receptors on lymphocytes. This may be due to the low capacity of these receptors and the use of whole lymphocytes rather than sex hormone-sensitive lymphocytes. In humans, however, estrogen receptors have been demonstrated in peripheral blood on OKT-8-positive cells that have suppressor/cytotoxic activity. One mode of action of sex hormones may be a direct effect on lymphocytes, in which the function or expression of cell-surface

molecules necessary for immune reaction is altered.

Cells closely associated with lymphocytes, such as thymic epithelial cells (1), also contain sex hormone receptors. It has long been argued that the thymus is the primary lymphoid gland whereby sex hormones influence immune responses. Sex hormones may interact with thymic epithelial cells to alter the thymic microenvironment and the release of thymic hormones, which in turn profoundly influence the immune system.

Sex hormone receptors are found in several parts of the brain, including the brain stem, allocortical and mesocortical regions of the pallium, the ventral medial nucleus of the hypothalamus and the pituitary (18,19). Thus, it is conceivable that sex hormones induce neurological activity through the release of neuropeptides that influence the immune system.

We have recently developed a model in which a hyperestrogenic state was deliberately created through injections of estrogen in pregnant healthy and autoimmune-prone mice. During a follow-up of several months, offspring of hyperestrogenic mothers developed autoantibodies such as APFC to Br-ME largely produced by Lyl<sup>+</sup> B-cells. We coined the term "immunologic imprinting" to refer to the prolonged changes in the immune system due to the influence of prenatal sex hormones on the fetal immune system. These findings may be related to neurological imprinting in which neonatal sex hormone administration permanently alters subsequent neurologic behavior. Our studies suggest that prenatal estrogen administration at a critical stage in the development of the fetal immune system induces permanent alterations resulting in the subsequent emergence of autoimmunity.

## CONCLUSIONS

Sex hormones play a very important role in the prevention, induction and regulation of autoimmune diseases, and in manipulating immune responses to infectious agents. The mechanisms of sex hormone actions on the immune system are extremely complex, mainly due to the diversity of target tissue sites upon which sex hormones can act. With the rapid advancement of biotechnology (e.g., the availability of cDNA to estrogen receptors), these mechanisms may become clearer and thus lead to new preventive and therapeutic strategies.

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## LYMPHOKINES IN AUTOIMMUNITY—ROLES OF INTERFERONS IN SYSTEMIC LUPUS ERYTHEMATOSUS AND OTHER AUTOIMMUNE DISORDERS

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The pathogenesis of autoimmune diseases is thought to be multifactorial, with environmental, genetic and endocrine factors acting together. As one investigator very aptly put it—"the carriage that leads to overt SLE is drawn by 3 horses: a Troika"(1). However, the actual pathogenesis of autoimmunity remains enigmatic. We find it difficult to distinguish between primary dominant etiologic factors and secondary contributing events. In fact, additional factors may still be unrecognized. Interferon (IFN) was only recently identified as such a factor; it was discovered in 1957 as an antiviral substance, but its other effects on normal cells went largely unrecognized until about a decade ago. It was then that Gresser (2) established that, in addition to antiviral activity, IFNs affect cell growth, maturation, and dif-

ferentiation; even more importantly, they have a profound effect on many components of the immune system.

Is IFN involved in the pathogenesis of autoimmune disease? We addressed this question by looking into the origin of autoantibodies (autoAB), which are the hallmark of autoimmunity. By fusing lymphocytes from lupus mice and patients, hybridomas producing monoclonal autoAB were obtained and their idiotypes analyzed. These studies led to an important conclusion: the capacity to produce autoAB is not a specialized phenomenon occurring only in patients with autoimmunity but is a basic characteristic of normal B-cells; thus, autoAB are a natural phenomenon (3). In the laboratory of Schwartz in Boston, we were able to confirm this in humans by