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Cutting Edge: Lupus Susceptibility Interval Sle3/5 Confers Responsiveness to Prolactin in C57BL/6 Mice

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Prolactin is of interest in the pathogenesis of systemic lupus erythematosus (SLE) because almost 25% of SLE patients display hyperprolactinemia, and serum prolactin correlates with disease activity in some patients. Furthermore, hyperprolactinemia causes early mortality in lupus-prone mice and induces a lupus-like phenotype in nonspontaneously autoimmune mice. We show here that the immunomodulatory effects of prolactin are genetically determined; hyperprolactinemia breaks B cell tolerance and causes a lupus-like serology in BALB/c mice expressing a transgene encoding the H chain of an anti-DNA Ab but not in C57BL/6 transgenic mice. In C57BL/6 mice that express both the H chain transgene and the lupus susceptibility interval, prolactin induces increased serum titers of anti-DNA Ab and glomerular Ig depositions. The increase in costimulation due to prolactin-mediated up-regulation of both CD40 on B cells and CD40L on T cells would appear to play a central role in lupus induction in this model. The Journal of Immunology, 2006, 177: 1401–1405.

Systemic lupus erythematosus (SLE) is an autoimmune disease with an unclear etiology and a highly variable clinical presentation. It is characterized by a strong gender bias, with a female to male ratio of 9:1 during child-bearing age, suggesting that female sex hormones may play a crucial role in pathogenesis (1, 2). Genetic factors also affect the incidence of SLE, with African-Americans and Hispanics 2–4 times more likely to develop the disease than Caucasians (3).

Prolactin is a pituitary hormone best known for its lactogenic effect. Data accumulated over the past two decades have demonstrated that lymphocytes secrete prolactin and express receptors for it, pointing to a potential immunomodulatory role for the hormone (4, 5). Furthermore, both clinical studies in SLE patients and experimental studies in mice implicate prolactin in the development of autoimmunity (6) and in the pathogenesis of SLE (7, 8). Approximately 25% of SLE patients display some degree of hyperprolactinemia, usually mild to moderate (1), and some studies have found that the degree of hyperprolactinemia correlates with global disease activity (8). Hyperprolactinemia accelerates lupus activity and causes early mortality in lupus-prone mice (9). Doubling the serum prolactin level of BALB/c mice transgenic for a H chain of the pathogenic anti-DNA Ab R4A-y2b breaks tolerance and induces a lupus-like phenotype. The same prolactin treatment has no effect on C57BL/6 mice bearing this transgene, indicating that the responsiveness of the immune system to prolactin is genetically determined (10).

In the study reported here, we demonstrate that genes responsible for this effect of prolactin on B cell tolerance lie within the lupus susceptibility interval Sle3/5. The Sle3/5 interval, derived from New Zealand mixed (NZM) 2410 lupus-prone mice, mediates an increase of the CD4:CD8 ratio in C57BL/6 mice and an increase in the number of activated CD4+ T cells (11), but prolactin is required to produce a lupus-like syndrome in this mouse strain. Prolactin up-regulates both CD40 and CD40L, which may be sufficient to mediate B cell rescue for apoptosis.

Materials and Methods

Mice

B6.NZW-Sle3 (NZM 2410/Aeg) (Sle3/5 C57BL/6) mice were a gift from Dr. E. Wakeland (University of Texas Southwestern Medical Center, Dallas, TX). R4A-y2b C57BL/6 mice were bred at the animal facility of the Albert Einstein College of Medicine (Bronx, NY). The R4A-y2b C57BL/6 mice were generated by backcrossing the R4A-y2b transgene onto the C57BL/6 background for >15 generations.

Pellets of placebo or prolactin (Innovative Research of America) were implanted subcutaneously in place of standard pellets of placebo and yielded serum prolactin levels of 68.3 ± 20.75 ng/ml, representing a mild increase over the normal serum prolactin levels (30.5 ± 19.7 ng/ml) (10) similar to the degree of hyperprolactinemia observed in patients with SLE. Placebo or prolactin treatment of the mice was maintained over a 5-wk period.

ELISAs

Serum DNA-reactivity was evaluated by ELISA using Immunolon-2 plates (Dynex Technologies) coated with calf thymus DNA (Sigma-Aldrich). The assays were developed with an anti-IgG2b Ab (12).

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; NZM, New Zealand mixed.

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ELISPOT assay

Splenocytes from placebo- or prolactin-treated mice, added in serial dilutions to plates coated with dsDNA, were incubated at 37°C for 6 h. Biotin-conjugated goat anti-mouse IgG2b Ab (Southern Biotechnology Associates) at a 1/5000 dilution was added, followed by streptavidin-conjugated alkaline phosphatase (Southern Biotechnology Associates) diluted at 1/1000. The plates were developed with 5-bromo-4-chloro-3-indolyl phosphate as substrate (Sigma-Aldrich). DNA-reactive ELISPOT assays were counted under a dissecting microscope.

Immunohistochecistry

Formalin-fixed, paraffin-embedded kidney sections from placebo- and prolactin-treated mice were deparaffinized in alcohol and then stained with biotinylated anti-mouse IgG Ab (Jackson Immunoresearch Laboratories) and FITC-labeled anti-IgG2b Ab (BD Pharmingen), both at a 1/200 dilution.

Flow cytometry

After RBC lysis with NH4Cl2, we performed surface staining of splenocytes for CD19, γ2b, CD21, CD23, AA4.1, CD3, CD4, CD40, and CD40L with antibodies-conjugated to FITC, PE, allophycocyanin, or PE-Cy7 (BD Pharmingen) at 4°C for 30 min. The cells were then washed and fixed in 2% paraformaldehyde. Data was acquired by FACS Calibur flow cytometer (BD Biosciences) and analyzed with Flowjo software (Tree Star).

Statistical analysis

Data were analyzed with standard statistical tests (mean value, SD, two-tailed Student’s t test, and Kruskal-Wallis nonparametric test).

Results

DNA reactivity in prolactin- and placebo-treated mice

The lupus susceptibility genetic interval Sle3/5 is associated with T cell hyperactivity (11), and induction of lupus in the R4A-γ2b BALB/c mouse model is T cell-dependent (8, 10). To determine whether the presence of Sle3/5 might allow prolactin to induce a lupus-like serology in R4A-γ2b C57BL/6 mice, we generated Sle3/5-bearing C57BL/6 mice that also carried the R4A-γ2b transgene. After treatment with prolactin for 5 wk, these mice had higher serum levels of anti-DNA Abs and an increased number of activated DNA-reactive B cells in comparison with placebo-treated mice as determined by ELISPOT assay (Fig. 1).

Lupus nephritis

Sle3/5-bearing, R4A-γ2b transgenic C57BL/6 mice treated with prolactin developed features of lupus nephritis, including proteinuria (Fig. 2A) and IgG deposition in the glomeruli (Fig. 2B), indicating that hyperprolactinemia enabled the production of high affinity nephritogenic anti-DNA Abs.

Thus, the R4A-γ2b transgene and the lupus susceptibility locus Sle3/5 do not by themselves induce lupus in C57BL/6 mice, but together they provide a susceptible genetic basis upon which other immunostimulatory factors such as prolactin can act to break B cell tolerance.

Transgene-expressing B cells

Like R4A-γ2b BALB/c mice and in contrast to R4A-γ2b C57BL/6 mice (10), Sle3/5 R4A-γ2b C57BL/6 mice responded to prolactin treatment with an expansion of the transgene-expressing B cell population (Fig. 3A). The increased number of transgene-expressing B cells was confirmed by immunohistochemical studies of the spleen. The increased number of γ2b-expressing B cells was primarily localized to the follicles (Fig. 3B).

B cell maturation

To evaluate the effects of hyperprolactinemia on B cell maturation in Sle3/5 R4A-γ2b transgenic C57BL/6 mice, we compared splenic B cell subsets in prolactin- and placebo-treated mice. Consistent with our previous data, prolactin-treated mice displayed a significantly decreased number of transitional T1 B cells (CD19⁺AA4.1⁺CD21⁻CD23⁻), leading to a significantly lower T1:T2 ratio than that present in placebo-treated mice (Fig. 4).

Increased serum prolactin levels did not significantly increase the numbers of mature marginal zone (CD19⁺AA4.1⁺CD21highCD23⁻), and follicular (CD19⁺AA4.1⁺CD21intermedCD23high) B cells (Fig. 4B), but did modestly bias the maturation pattern of γ2b-expressing B cells toward a follicular phenotype (Fig. 4C). The latter was confirmed by histology demonstrating an accumulation of transgene-expressing B cells in the splenic follicles of prolactin-treated mice (Fig. 3B). These observations indicate that hyperprolactinemia specifically induces the maturation of autoantibody producing B cells to the follicular phenotype. This finding is similar to our previous observations of prolactin-treated transgenic mice with the BALB/c background, where we have found that the follicular subset harbors the DNA-reactive B cells that spontaneously secrete autoantibodies.

FIGURE 1. DNA reactivity. Mice were implanted subcutaneously with placebo or prolactin slow release pellets (n = 5, for each group) (100 μg/day). A, Peripheral blood samples were taken before the treatment was started and after 5 wk of treatment. Sera were diluted 1/500, and anti-dsDNA Ab levels were determined by ELISA. At the end of the treatment, prolactin-treated mice displayed higher anti-dsDNA levels than placebo-treated mice (p = 0.01). B, After 5 wk of treatment, mice were sacrificed. DNA-reactive B cells were enumerated. Prolactin-treated mice displayed a higher number of spontaneously secreting DNA-reactive B cells than placebo-treated mice (p = 0.01).
Because follicular B cells are T cell dependent and T cells are necessary for the development of prolactin-induced lupus in mice with a BALB/c background (10), we assessed the potential role of costimulation in lupus induction by prolactin. As demonstrated by flow cytometry, increased serum prolactin up-regulated CD40 on B cells and also increased the number of activated CD4⁺ T cells expressing CD40L (Fig. 5), indicating that heightened CD40-CD40L interactions could play a crucial role in the B cell hyperactivity in these mice.

Discussion

A doubling of serum prolactin breaches B cell tolerance in R4A-y2b BALB/c mice and induces a lupus-like syndrome. The increased serum prolactin alters B cell development, blocks negative selection of autoreactive specificities, and leads to an increased number of activated transgene-expressing B cells with a follicular phenotype. The effects of prolactin on B cells are accompanied by an up-regulation of Bcl-2 and CD40 expression (13). The same prolactin treatment has no effect on B cell development, survival, or activation in C57BL/6 mice bearing the same R4A-y2b transgene (10). Hyperprolactinemia in these mice also does not alter the threshold for negative selection as assessed by the degree of apoptosis of isolated B cells on BCR engagement (E. Peeva and B. Diamond, unpublished data).

The prolactin-induced increase in CD40 expression may contribute to the development of a lupus-like syndrome by at least two mechanisms. First, ligation of CD40 may induce the expression of the anti-apoptotic proteins Bcl-xL and Bcl-2 (14) and, thus, may cause an increased survival of autoreactive transitional B cells. In addition, increased CD40 on B cells, along with a prolactin-mediated increase in the number of CD40L-expressing CD4⁺ T cells, may enhance activation and autoantibody production by mature B cells.

Genetic predisposition is a crucial factor in the susceptibility to human and murine lupus (15). Over 50 chromosomal regions containing genes responsible for lupus susceptibility or resistance have been identified (reviewed in Ref. 16). By linkage analysis of susceptibility to antinuclear Ab production and glomerulonephritis in lupus-prone NZM 2410 mice, Wakeland...
and colleagues (11) identified genetic intervals on several chromosomes that can induce specific autoimmune manifestations when transferred to nonlupus-prone mouse strains. Lupus susceptibility intervals on chromosome 1 (Sle1), chromosome 4 (Sle2), chromosome 7 (Sle3/5), and chromosome 17 (Sle4) were transferred onto a C57BL/6 genetic background producing congenic strains, each of which displayed an individual pattern of an autoimmune diathesis but not an autoimmune disease unless they were transferred together (11).

We were specifically interested in the Sle3/5 C57BL/6 mouse strain, which is characterized by an expanded CD4+ T cell compartment and production of polyclonal and polyclonal IgM and IgG autoantibodies (11, 17). These mice display an increased CD4:CD8 ratio that is present in all age groups, whereas autoantibody production increases with age. Also, mice of all ages exhibit an increased number of activated CD4 cells expressing CD69, CD25, and CD44, whereas B cell activation is late in onset; only mice that are 9 mo and older display activated I-Aβ, CD44+, and B7.2-expressing B cells (17). The T cell phenotype appears to be secondary to hyperreactivity of dendritic cells.

Sle3/5 is a 40 cM genetic interval. The lupus susceptibility locus Ibu5, which carries susceptibility to nephritis, and locus Lmb3, which is associated with lymphoadenopathy and antibodies against dsDNA Ab production, have been mapped to the vicinity of this interval. The Sle3/5 interval also contains genes important for lymphocyte survival, signaling, and activation, including Bax, IL-4i1, CD22, CD37, TGF-β, and Bcl-3, all of which may play a role in the pathogenesis of lupus.

Prolactin down-regulates Bax in mammary glands (18) as well as in the Nb2 lymphoma cell line (19), and prolactin-mediated down-regulation of Bax may contribute to the prolactin-modulated survival of autoreactive B cells. IL-4i1 is a lysosomal l-amino acid oxidase (20) involved in peptide processing, and its altered expression may affect the MHC class II peptide repertoire (21). CD22 is a coreceptor that down-regulates BCR signaling and determines whether Ag-stimulated B cells undergo apoptosis or proliferation (22). CD22 knockout mice develop an autoimmune syndrome characterized by B cell hyperactivity with increased serum IgM and antinuclear Ab levels. In BALB/c mice, estrogen-up-regulated CD22 contributes to a breakdown of B cell tolerance and the development of a lupus-like syndrome (23), whereas tamoxifen-down-regulated CD22 appears to play a crucial role in the abrogation of estrogen-induced lupus (24). The importance of CD22 in the pathogenesis of human lupus has also been recognized, and a pilot clinical trial with anti-CD22 mAb demonstrated promising results (25). Although prolactin does not affect CD22 expression in BALB/c mice (10), the presence of other susceptibility genes and different background genes in Sle3/5 C57BL/6 mice may allow for certain epistatic relationships that can lead to a prolactin-mediated modulation of B cell activation via CD22. Finally, it is possible that the major contribution of Sle3 is to enhance the activation of dendritic cells.

Systemic lupus is a multifactorial disease, with genetic and hormonal factors implicated in its pathogenesis. This is reflected in the murine model used in our study, where neither the anti-DNA-encoding transgene nor the lupus susceptibility interval Sle3/5 was sufficient to induce lupus in a nonsusceptible mouse strain, but together they provided a genetic base upon which other immunostimulatory factors such as prolactin can act to break tolerance and produce disease.
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Disclosures
The authors have no financial conflict of interest.

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