Increased B Lymphopoiesis in Genetically Sex Steroid-deficient Hypogonadal (hpg) Mice

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Summary

Interleukin 7 (IL-7) responsive B lineage precursors were greatly expanded in genetically hypogonadal female (HPG/Bm-hpg/hpg) mice that have a secondary deficiency in gonadal steroidogenesis. Estrogen replacement in these mice resulted in a dose-dependent reduction in B cell precursors. More modest increases were documented in genetically normal mice that were surgically castrated. These findings complement other recent observations that B lymphopoiesis selectively declines in pregnant or estrogen-treated animals. Sex steroids have long been known to influence such disparate processes as bone physiology and tumor growth, in addition to their importance for reproductive function. We now show that these hormones are important negative regulators of B lymphopoiesis.

Blood cell formation within bone marrow is thought to be controlled by close cellular interactions and the availability of cytokines that induce proliferation and differentiation of committed precursor cells. However, our understanding of this process is incomplete and especially so with respect to mechanisms that limit the production of particular blood cell types. We recently found that B lineage precursors, identified by their responsiveness to IL-7 and surface markers, were selectively depleted during normal pregnancy or after treatment with estrogens (1, 2). Since natural or artificial elevation of sex steroids suppresses B lymphopoiesis, it seemed possible that diminished endogenous levels of these hormones might result in expanded production of B lymphocytes. HPG/Bm-hpg/hpg (hpg) mice have a partial deletion of the hypothalamic gonadotropin releasing hormone (GNRH) gene and this results in a profound depression in synthesis of gonadotropins (follicle stimulating and luteinizing hormones) (3-5). We now show that B lymphopoiesis is abnormally increased in these sex hormone-deficient animals and is normalized by estrogen replacement therapy.

Materials and Methods

Animals. Hypogonadal HPG/Bm-hpg/hpg (hpg) mice have a deletion in the GNRH gene, resulting in nonexistent gonadal sex steroid secretion and infantile reproductive tracts (3-5). The hpg mutation was maintained segregating within the HPG/Bm inbred strain and phenotypically normal (+/+ or hpg/+; hereafter termed +/?) animals were used as controls. Mutant hpg mice were identified by Southern blot analysis as described (6) and confirmed by measurement of uterine weights. Doubly homozygous hypogonadal severe combined immunodeficient (hpg/hpg scid/scid) mice were produced as previously described (6). Castrated mice were obtained from Charles River Laboratories (Wilmington, MA).

Colony Assays. Bone marrow cells were prepared and suspended in 1 ml of assay medium as previously described (7). The semisolid agar cloning assay for B lymphocyte precursors (CFU IL-7) was done with 10 ng recombinant mouse IL7 (a gift from Immunex, Seattle WA). Mitogen responsive B cells were detected with 25 η/ml of endotoxin (Difco, Detroit, MI) and the granulocyte/macrophage progenitor assay (CFU-G/M) was done with 25 μl/ml of 10 times concentrated L cell conditioned medium. All cloning assays were performed in 35-mm dishes (Corning Glass Inc., Corning, NY) and incubated at 37°C, 5% CO2. Colonies were scored on day 6.

Immunofluorescent Staining and Analysis. Cells were suspended in staining buffer (PBS without CA2+ and Mg2+ with 3% heat inactivated FCS and 0.1% sodium azide) at a concentration of 107 cells/ml. Staining was performed by incubating cells with antibodies on ice for 15 min followed by washing with 10 vol of staining buffer. Unconjugated antibodies were revealed by a subsequent incubation with the appropriate fluorochrome-conjugated second antibody, or in the case of biotinylated primary antibodies, with streptavidin PE (Biomeda, Foster City, CA) or streptavidin Peridinin Chlorophyll (Becton Dickinson & Co., Mountain View, CA). B cells were identified by staining with FITC-labeled goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL). Subpopulations of B lineage precursors were then resolved using a second aliquot of the same cell suspensions with a modification of the procedures described by Hardy et al. (6). At a first step, B cells were depleted by adherence on anti-IgM-coated plastic dishes. The remaining cells were then stained with FITC-labeled M1/69 (Becton Dickinson & Co., Mountain View, CA). Colony assays were performed in 35-mm dishes (coming Glass Inc., Coming, NY) and incubated at 37°C, 5% CO2. Colonies were scored on day 6.

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and very early precursors (referred to by Hardy as Fraction "A"), were discriminated by their low expression of HSA. More mature cells (Fractions "B+C") display high levels of HSA. Bone marrow lymphocytes which are CD45R+ and CD43- were small pre-B cells ('Fraction "D""). All samples were analyzed with a FACScan® flow cytometer (Becton Dickinson & Co.). Parameters were established for discriminating total nucleated cells and lymphocytes by staining with appropriate antibodies, backgating on the positive cells, and setting forward and orthogonal scatter gates. Further details about these analyses are specified in our recent publications (1, 2).

Results and Discussion

IL-7 appears to be critical for B lymphocyte formation and IL-7 responding precursors (CFU IL-7) can be readily identified with a clonal assay (7, 9). Numbers of these precursors were dramatically elevated in bone marrow of female hpg mice (Fig. 1). Highly significant increases were also detected in total B lymphocyte lineage precursors (CD45R+, slgM-) enumerated by flow cytometry (10). Multiparameter flow cytometry was then used to resolve subpopulations of these cells at various stages of differentiation (8). The frequency of cells at an early stage (characterized as CD45R+, HSAhi, CD43- and termed Fraction A) were normal in hpg mice and were similarly unaffected by pregnancy or hormone treatment (1, 2). Subsequent compartments (CD45R-, HSAhi, CD43+ termed Fractions B + C), including the clonable IL-7 responding cells, were significantly elevated in hpg mice. However, small pre-B cells (CD45R+, HSAhi, CD43-; Fraction D) represented the most substantially increased subpopulation. This is reciprocal to the situation in pregnant or estrogen treated mice, where small pre-B cells were the most depressed of all B lineage cells (1, 2).

A variety of evidence indicates that large IL-7 responding cells normally give rise to small pre-B cells (Fraction D), which subsequently become newly formed B cells with an "immature" phenotype (7, 11, 12). We find significant elevations in B cells in bone marrow of hpg mice (1.7-fold increase, p <0.003; Fig. 1). This included not only cells with immature characteristics (slgM- and slgD-), but also slgM+ slgD- cells, that might be part of a recirculating pool of mature cells (data not shown) (12). B cells in the spleen were significantly increased (p <0.001) by approximately twofold and again, this applied to immature and mature populations (data not shown). The total number of nucleated splenocytes as a whole were increased by the same amount, but there was no significant change in the number of Mac-1+ cells (data not shown). Thus, changes in B lineage precursors within bone marrow were accompanied by some expansion of peripheral B lymphocytes.

The bone marrow simultaneously produces cells in eight lineages and it is remarkable that pregnancy or estrogen treatment preferentially affects precursors of B cells (1, 2). Similary, the elevations in cell number that we found in bone marrow of hpg mice were again highly selective (Fig. 1). Numbers of total nucleated cells were modestly, but significantly increased in hpg bone marrow, a change totally accounted for by increases in B lineage lymphocytes. Myeloid progenitors detected with a clonal assay (CFU-G/M), or myeloid and erythroid cells enumerated by flow cytometry (with Mac-1 and TER119 antibodies) were all within the normal range.

The hypogonadal mutation abolates synthesis of GNRH and gonadotropins (follicle stimulating and luteinizing hormones) (3-5). Either these, or the sex hormones they regulate, could be responsible for the changes we found. Hormone replacement experiments suggest that it is the deficiency in sex steroids that allows expanded lymphocyte production in the mutant mice (Fig. 2). Sustained elevations in serum estradiol were achieved with Silastic® tubing implants and this resulted in a dose-dependent decrease in B lineage cells. This procedure is known to cause an osteosclerotic reaction in the peripheral bones of normal mice (13), and we recovered fewer nucleated cells from estrogen treated hypogonadal mice. However, it was clear from subset analysis (data not shown) that this sex hormone preferentially depressed B lineage precursors and the highest dose brought their numbers even below the normal range. Thus, B lymphopoiesis in the mutant animals is sensitive to preferential negative regulation by this hormone, demonstrating again that systemic levels of sex steroids correspond reciprocally to the production of new cells within bone marrow.

Thymus and spleen cells were evaluated in a previous study of hpg mice (14). Small increases in thymus size and cell number were recorded in male, but not female hpg animals. There were also no significant changes in the thymuses of mice we examined (data not shown). This is in striking contrast to

![Figure 1](image-url)
parameters were variable in ovariectomized BALB/c mice and hormone deficiency that is less in magnitude and/or duration. Regardless, the deviation or B lymphopoiesis in uterine weight, an indicator of estrogen levels, was extremely low in hpg mice (12.2 ± 7.2 mg vs. 94.0 ± 35.3 mg in normal littermates), in agreement with previous studies (3). Castration did not decrease uterine weights as severely as in hpg mice (data not shown), which could be consistent with a hormone deficiency that is less in magnitude and/or duration. However, there must be other limitations because the magnitude of the latter changes was less than the elevations in IL-7 responding cells. Furthermore, the hpg mutation did not overcome the B cell deficiency in doubly mutant hypogonadal, severe combined immunodeficiency (hpgh/pgh scid/scid) mice (6), although there were no significant elevations in IgM+ , CD45R+ B lineage precursors (scid/scid = 1.43 x 10^6, and hpg/hpg scid/scid = 2.51 x 10^5 cells; p <0.001). A limiting amount of physical space or cytokines in the marrow, the need for successful rearrangement and expression of immunoglobulins, and/or the action of other negative regulators may determine the size of subsequent compartments. We found no evidence of B lineage precursors in the spleen of hpg mice, indicating that the normal site of B lymphopoiesis was unchanged (data not shown). Future studies should reveal the actual mechanisms through which sex steroid(s) control B lymphopoiesis. However, there is already reason to believe that they act via the microenvironment, rather than directly on B lineage precursors (18). The normal rate of B cell production can be diminished, or greatly elevated by changes in systemic hormone levels. However, the possibility also exists that steroid hormones can be produced locally by cells within the bone marrow microenvironment (19).

While there has been rapid progress in identifying cytokines and other molecules that may be potential regulators of lymphopoiesis, physiological relevance has been demonstrated for very few of them (20). When considered together with the findings from pregnant and hormone-treated mice, the results with hpg mice make a strong case that normal B lymphopoiesis is actively regulated by sex steroids. There is some reason to believe that this paradigm may extend to the production of other types of lymphocytes. The thymus has been reported to increase in size with castration or in some circumstances where there is an inability to produce and respond to sex hormones (21–24). In addition, the thymus decreases in size during pregnancy or after estrogen treatment (25, 26). However, as noted above, the thymus was not enlarged in female hypogonadal mice and this indicates that additional regulators may be operative in that organ. There are reports of mitogenic receptors for GNRH on thymocytes (27) and it is possible that the absence of a positive stimulus (GNRH) is compensated by loss of a negative regulator (estrogen) in hpg mice. Further studies of this kind offer promise for successful intervention in some immune deficiencies and highlight the need to better understand the consequences of hormone therapy on development and regulation of the immune system.
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