Brief Definitive Report

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

By G. Smithson,* W. G. Beamer,‡ K. L. Shultz,‡ S. W. Christianson,‡ L. D. Shultz,‡ and P. W. Kincade*

*From the *Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; and ‡The Jackson Laboratory, Bar Harbor, Maine 04609*

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 IL-7 (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% CO_2. Colonies were scored on day 6.

Immunofluorescent Staining and Analysis. Cells were suspended in staining buffer (PBS without CA^{2+} and Mg^{2+} with 3% heat inactivated FCS and 0.1% sodium azide) at a concentration of 10^7 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 CP (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. (8). As 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 (HSA; heat stable antigen) either purchased from PharMingen (San Diego, CA) or produced in our laboratory from hybridoma TIB125 (ATCC; American Type Culture Collection, Rockville, MD), biotinylated-S7 (CD43) purified and biotinylated in our laboratory from the hybridoma obtained from the ATCC, and PE-labeled 6B2 (CD45R) (PharMingen). This three-color analysis was then performed and interpreted as follows. Pro-B and large pre-B cells are all $CD43^+$,

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/08/0717/04 $2.00
Volume 180 August 1994 717–720

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).

Hormone Replacement. 5–6-wk-old female *hpg* mice were implanted with Silastic® tubing (Dow-Corning, Midland, MI) containing 10, 20, or 40 μg/ml 17 β-estradiol in 8 mg cholesterol or containing cholesterol alone.

Statistical Analysis. The significance of differences were evaluated by paired *t* testing.

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$^+$, sIgM$^-$) 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$^+$, HSA^{lo}, CD43$^+$ and termed Fraction A) were normal in *hpg* mice and were similarly unaffected by pregnancy or hormone treatment (1, 2). Subsequent compartments (CD45R$^+$, HSA^{hi}, CD43$^+$; termed Fractions B + C), including the clonable IL-7 responding cells, were significantly elevated in *hpg* mice. However, small pre-B cells (CD45R$^+$, HSA^{hi}, 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 found 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 (sIgM$^+$, sIgD$^-$, and sIgM$^+$, HSA^{hi}), but also sIgM$^+$, sIgD$^+$ 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 in that site. 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). Similarly, 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.

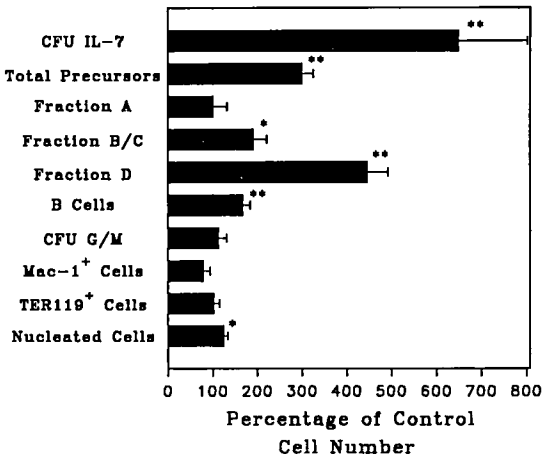

Figure 1. A comparison of lymphoid and myeloid cells in bone marrow of *hpg* and phenotypically normal +/? mice. IL-7 responding cells (CFU IL-7) and myeloid progenitor cells (CFU-G/M) were enumerated with colony assays (Materials and Methods). Flow cytometry was used to discriminate total B lineage precursors (CD45R$^+$, sIgM$^-$) and subpopulations (Fractions A–D) using a modification of the procedure originally described by Hardy and colleagues (1, 2, 8). All results were normalized to the normal (+/?) littermate controls (means ± SE) and statistical significance is indicated by asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$).

The hypogonadal mutation ablates 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 estrogen 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

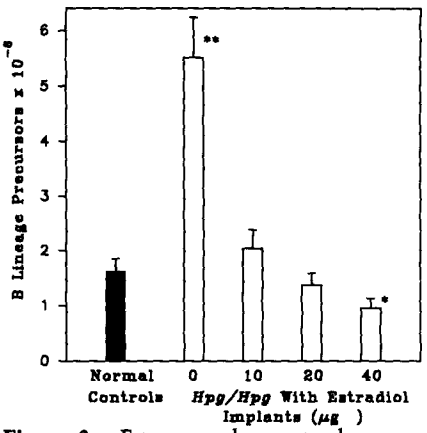

Figure 2. Estrogen replacement reduces numbers of B cell precursors in hypogonadal mice. B lineage lymphocyte populations in *hpg* and +/? littermate mice were evaluated 5 wk after implantation of 17 β-estradiol or cholesterol control tubing. Cells recovered from one femur and one tibia were analyzed by two-color flow cytometry for total B lineage precursors ($CD45R^+$, $sIgM^-$ cells). Highly significant (**, $p \leq 0.001$) and significant (*, $p = 0.036$) differences from control values are indicated.

the elevations we documented in bone marrow lymphocytes. While we found that the mutation significantly increased numbers of splenic B cells, this was not found in the earlier study, where the mutation was carried on a different genetic background.

We also evaluated B lineage lymphocyte precursors in castrated BALB/c mice (nine of each sex). In male mice, the numbers of IL-7 responsive cells and small pre-B cells in bone marrow increased approximately twofold as compared with sham operated controls ($p < 0.001$ for both). The same parameters were variable in ovariectomized BALB/c mice and no significant changes were found ($p = 0.863$ and 0.310, respectively). However, significant elevations (1.6-fold) in B lineage cells were found when phenotypically normal females of the same strain as the hypogonadal mice (HPG/Bm-+/?) were ovariectomized ($p = 0.022$).

It is not clear why the effects of surgical castration had less influence on B lymphopoiesis than the *hpg* mutation, but it could relate to the timing of sex hormone withdrawal. For example, it has been shown that GNRH is required for the normal development of the enzymes required for steroid production and that these enzymes are almost undetectable in the *hpg* mouse ovary (15). On the other hand, estrogen can be produced in the ovaries of normal mice as early as seven days after birth, thus possibly exposing B cell precursors to this steroid until the animals are castrated at 3–5 wk of age (15). Another possibility is that gonadotropins in castrated mice direct synthesis of sex steroids outside the gonads. Although, estrogen levels were below the reliable RIA detection range in both hypogonadal and castrated animals, 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. Regardless, the elevation of B lymphopoiesis in *hpg* mice was brought within normal values by estrogen replacement.

Detailed studies of the thymus have revealed that there may be multiple "control points" where critical events must occur in order for differentiation to proceed (16). Similarly, gene disruptions and natural mutations have been informative about essential steps in B lymphopoiesis (17). The results of the present study are consistent with our previous analyses of pregnant or hormone-treated mice which indicate that the negative regulatory effects of sex steroids must be at, or close to, the IL-7–responsive stage (1, 2). Elevations in such early precursors should produce an even greater number of differentiating cells and indeed we measured significant increases in small pre-B cell and B cell numbers. However, there must be other limitations because the magnitude of the latter changes were 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 (*hpg/hpg scid/scid*) mice (6), although there were significant elevations in IgM^-, $CD45R^+$ B lineage precursors (*scid/scid* = 1.43×10^6, and *hpg/hpg scid/scid* = 2.51×10^6 cells; $p \leq 0.001$). 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.

We thank Kay L. Medina for helpful advice on the experiments, as well as the manuscript.

This work was supported by grants AI-20069, AI-30389, CA-20408, and CA-54889 from the National Institutes of Health and The Ladies Auxiliary of the Veterans of Foreign Wars.

Address correspondence to Dr. Paul W. Kincade, Department of Immunobiology and Cancer, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104.

Received for publication 25 February 1994 and in revised form 2 May 1994.

References

1. Medina, K.L., G. Smithson, and P. W. Kincade. 1993. Suppression of B lymphopoiesis during normal pregnancy. *J. Exp. Med.* 178:1507.
2. Medina, K.L., and P.W. Kincade. 1994. Pregnancy related steroids are potential negative regulators of B lymphopoiesis. *Proc. Natl. Acad. Sci USA.* 91:5382.
3. Cattanach, B.M., C.A. Iddon, H.M. Charlton, S.A. Chiappa, and G. Fink. 1977. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature (Lond.).* 269:338.
4. Mason, A.J., S.L. Pitts, K. Nikolics, E. Szonyi, J.N. Wilcox, P.H. Seeburg, and T.A. Stewart. 1986. The hypogonadal mouse: reproductive functions restored by gene therapy. *Science (Wash. DC).* 234:1372.
5. Mason, A.J., J.S. Hayflick, R.T. Zoeller, W.S. Young, H.S. Phillips, K. Nikolics, and P.H. Seeburg. 1986. A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science (Wash. DC).* 234:1366.
6. Beamer, W.G., K.L. Shultz, B.J. Tennent, and L.D. Shultz. 1993. Granulosa cell tumorigenesis in genetically hypogonadal-immunodeficient mice grafted with ovaries from tumor-susceptible donors. *Cancer Res.* 53:3741.
7. Lee, G., A.E. Namen, S. Gillis, L.R. Ellingsworth, and P.W. Kincade. 1989. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β. *J. Immunol.* 142:3875.
8. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre–pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213.
9. Grabstein, K.H., T.J. Waldschmidt, F.D. Finkelman, B.W. Hess, A.R. Alpert, N.E. Boiani, A.E. Namen, and P.J. Morrissey. 1993. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J. Exp. Med.* 178:257.
10. Kincade, P.W., G. Lee, T. Watanabe, L. Sun, and M.P. Scheid. 1981. Antigens displayed on murine B lymphocyte precursors. *J. Immunol.* 127:2262.
11. Osmond, D.G. 1990. B cell development in the bone marrow. *Semin. Immunol.* 2:173.
12. Allman, D.M., S.E. Ferguson, V.M. Lentz, and M.P. Cancro. 1993. Peripheral B cell maturation: II. Heat-stable antigen^{hi} splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.* 151:4431.
13. Urist, M.R., A.M. Budy, and F.C. McLean. 1950. Endosteal bone formation in estrogen-treated mice. *J. Bone JT Surg. Am. Vol.* 32A:143.
14. Moscovitz, H.C., S. Schmitt, G.J. Kokoris, I.Z. Leiderman, and M.J. Gibson. 1988. Thymocyte maturity in male and female hypogonadal mice and the effect of preoptic area brain grafts. *J. Reprod. Immunol.* 13:263.
15. Mannan, M.A., and P.J. O'Shaughnessy. 1988. Ovarian steroid metabolism during postnatal development in the normal mouse and in the adult hypogonadal (*hpg*) mouse. *J. Reprod. Fert.* 82:727.
16. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today.* 14:547.
17. Rolink, A., and F. Melchers. 1993. Generation and regeneration of cells of the B-lymphocyte lineage. *Curr. Opin. Immunol.* 5:207.
18. Smithson, G.M., K.L. Medina, and P.W. Kincade. 1993. In vitro analysis of the effects of pregnancy-associated and other hormones on B lymphopoiesis. *J. Immunol.* 150:20a. (Abstr.)
19. Frisch, R.E., J.A. Canick, and D. Tulchinsky. 1980. Human fatty marrow aromatizes androgen to estrogen. *J. Clin. Endocrinol. Metab.* 51:394.
20. Kincade, P.W., K.L. Medina, and G. Smithson. 1994. Sex hormones as negative regulators of lymphopoiesis. *Immunol. Rev.* 137:119.
21. Calzolari, A. 1898. Recherches experimentales sur un rapport probable entre la fonction du thymus et celle des testicules. *Arch. Ital. de Biol.* 30:71.
22. Olsen, N.J., M.B. Watson, G.S. Henderson, and W.J. Kovacs. 1991. Androgen deprivation induces phenotypic and functional changes in the thymus of adult male mice. *Endocrinology.* 129:2471.
23. Olsen, N.J., M.B. Watson, and W.J. Kovacs. 1991. Studies of immunological function in mice with defective androgen action. Distinction between alterations in immune function due to hormonal insensitivity and alterations due to other genetic factors. *Immunology.* 73:52.
24. Greenstein, B.D., E.F. De Bridges, and F.T.A. Fitzpatrick. 1992. Aromatase inhibitors regenerate the thymus in aging male rats. *Int. J. Immunopharmacol.* 14:541.
25. Ito, T., and T. Hoshino. 1962. Studies of the influences of pregnancy and lactation on the thymus in the mouse. *Zeitschrift fur Zellforschung.* 57:667.
26. Screpanti, I., S. Morrone, D. Meco, A. Santoni, A. Gulino, R. Paolini, A. Crisanti, B.J. Mathieson, and L. Frati. 1989. Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation. Effects of 17 beta-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2 receptor. *J. Immunol.* 142:3378.
27. Marchetti, B., V. Guarcello, M.C. Morale, G. Bartoloni, Z. Farinella, S. Cardaro, and U. Scapagnini. 1989. Luteinizing hormone-releasing hormone-binding sites in the rat thymus: characteristics and biological function. *Endocrinology.* 125:1025.