Enhanced Production of B Lymphocytes After Castration

By Cheryl A. Wilson, Susan A. Mrose, and David W. Thomas

Castration has long been recognized to stimulate thymic growth and augment cellular immunity. We sought to determine whether castration affects B lymphopoiesis by analyzing the phenotype of bone marrow and spleen cells from animals postcastration. In this report, we show that the bone marrow cells from castrated male mice show a sustained, twofold to threefold increase in numbers of B220+/IgM- cells and of newly formed B220+/IgM- B cells. Most of the expanded B220+/IgM- cell population consisted of small, HSA-CD43+ cells characteristic of pre-B cells. The castrated animals also showed increased numbers of splenic B cells, primarily consisting of small IgM-, IgD+, B220+, HSA- cells. Taken together, these results show that castration causes dramatic, long-lived enhancement of B lymphopoiesis in bone marrow and increased numbers of mature B cells in the periphery.

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MATERIALS AND METHODS

Animals. Castrated or sham-castrated C57BL/6 male mice were obtained from Taconic (Germantown, NY). Surgery was performed at 10 to 12 weeks of age.

Cell preparation. Mice were killed by CO2 inhalation. Single-cell suspensions of BM were prepared in RPMI 1640 plus 5% heat-inactivated fetal calf serum by cutting across the epiphyses of both femur and tibia and flushing medium through the ends of bone using a syringe and 25-gauge needle. BM extracts were passed through a 25-gauge needle and filtered through Nitex (Tetko, Inc). Cells were centrifuged, checked for viability by trypan blue exclusion, and resuspended at 10^7/mL. Single cell suspensions of spleen cells were obtained by pressing the spleens between the frosted ends of two glass slides, rinsing once in saline, lysing the red blood cells with Gey’s solution, and resuspending to 10^7/mL.

Cell phenotyping. Cells were washed twice with staining buffer (D-phosphate-buffered saline without Ca2+ and Mg2+, 1% bovine serum albumin, and 0.1% NaN3) and then stained for 30 minutes, on ice in 96-well round-bottom plates with a panel of fluorochrome-conjugated antibodies. Antibodies used in this study were as follows: goat antimouse IgM phycoerythrin (PE; Southern Biotechnology Associates, Inc, Birmingham, AL), antimouse CD45R (RA3-6Bu PE) (Pharminigen, San Diego, CA), antimouse granulocytes fluorescein isothiocyanate (FITC; Pharmigen), rat antimouse macrophage FITC (Caltag Laboratories, South San Francisco, CA), antimouse HSA (M1/69) PE (Pharminigen), antimouse CD43 (Leukosia- lin, S7) PE (Pharminigen), rat antimouse CD4 PE (Pharminigen), rat antimouse CD8 FITC (Pharmigen), antimouse IgD FITC (Pharminigen). Stained cells were washed twice with staining buffer and resuspended in 1% paraformaldehyde (BM) or staining buffer (spleen). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson & Co, San Jose, CA) using Lysis II software by collecting 10,000 events within a gate that eliminated red blood cells, platelets, and debris. Statistical significance was analyzed by Student’s unpaired two-tailed t-test using Statview 4.01 (Abacus Concepts, Berkeley, CA).

RESULTS

Phenotypic analysis of BM cells from sham-castrated versus castrated mice. The purpose of these studies was to determine whether there were specific effects of castration on lymphopoiesis in the BM of male mice. Accordingly, phenotype analysis was performed on BM cells from animals that were castrated or sham-castrated 13 days before harvest. As shown in Fig 1, the castrated mice had a threefold increase in the percentage of B220+ B-cell precursors lacking surface IgM expression (B220+/slgM-), a 1.5-fold increase in the percentage of B cells expressing slgM (B220+/slgM+),

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Fig 1. The effects of castration on the distribution of BM cell subpopulations. BM cells from 3 individual animals per group were harvested on day 13 postcastration as described. Dual-color analysis for B220 and slgM and single-color analysis for granulocytes and macrophages were performed by fluorescence-activated cell sorter (FACS) analysis, as described. Sham-castrated, (m); castrated, (■). The SEM is shown for each bar, and statistical significance is indicated by asterisks (*, P ≤ .01).

and a decrease in the percentage of cells expressing the GR-1 granulocyte or Mac-1 macrophage markers (62% and 67% of the sham-operated animals, respectively). To determine whether the castration-induced alterations in the BM subpopulations were caused by a temporary metabolic/regulatory imbalance after castration or by permanent changes in the cellular composition, animals were studied 3 to 86 days postsurgery (Fig 2). There was a threefold increase in the percentage of B220+/slgM- B cells at 2 weeks postcastration that plateaued to around a twofold increase, as compared with control animals, which was sustained through 86 days. This increase appeared to represent an overall increase in these cells because the overall number of BM cells was similar (5.32 × 10^7 ± 0.78 v 5.65 × 10^7 ± 1.25) for the sham-castrated and castrated animals. There was also an increase in the percentage of slgM+ BM cells (1.5-fold) that followed the increase in slgM- cells by several days. The B220+ BM cells from castrated animals showed a selective increase in the relative number of HSA^b+ cells and a selective increase in the number of CD43+ cells (Fig 3C). Two characteristics of cells that have matured to the pre-B-cell stages. The changes in these three parameters are consistent with a selective increase in small, pre-B cells and a smaller increase in large pre-B cells after castration. The slgM+ subpopulation of the BM cells is composed of two distinct B220 populations (Fig 3D). Cells from the castrated animals showed a selective increase in the population expressing lower levels of B220, whereas the population expressing higher levels of B220 remains fairly constant after castration. The B220^bh/IgM+ population most likely repre-

performed, as shown in Fig 3. Two weeks postsurgery, the B220^bh/lsgM- cells of the castrated animals showed a dramatic increase in the relative number of small cells (low forward scatter) as well as a smaller increase in the number of large cells. This shows that there was a preferential increase in the small B220^bh/lsgM- cells in the castrated animal compared with those of the control, because there was an increased percentage of these small cells relative to the large (79% and 21%, respectively, in the castrated animals and 72% and 28%, respectively, in the sham-operated animals). The B220+ BM cells from castrated animals showed a selective increase in the relative number of HSA^b+ cells and a selective increase in the number of CD43+ cells (Fig 3C), two characteristics of cells that have matured to the pre-B-cell stages. The changes in these three parameters are consistent with a selective increase in small, pre-B cells and a smaller increase in large pre-B cells after castration. The slgM+ subpopulation of the BM cells is composed of two distinct B220 populations (Fig 3D). Cells from the castrated animals showed a selective increase in the population expressing lower levels of B220, whereas the population expressing higher levels of B220 remains fairly constant after castration. The B220^bh/IgM^+ population most likely repre-
ENHANCED B LYMPHOPOIESIS AFTER CASTRATION

SHAM-CASTRATED

CASTRATED

GATE: B220+/slgM+

Fig 3. Phenotypic analysis of B220+ BM cells postsurgery. Dual-color FACS analysis was performed as described. (A) B220+/slgM- cells were gated, and forward scatter was plotted. A small cell equals less than 150 on the X-axis; large cells equal greater than 150 on the X-axis. (B) B220+ cells were gated, and HSA fluorescence was plotted. HSA- cells equal less than 6 × 10^2; HSAhi cells equal greater than 6 × 10^2 fluorescence intensity. (C) B220+ cells were gated, and CD43 fluorescence was plotted. CD43- cells equal less than 2 × 10^2; CD43hi cells equal greater than 10^2 fluorescence intensity. (D) sIgM+ cells were gated, and B220 fluorescence was plotted. B220hi cells equal less than 10^2 fluorescence intensity.

Fig 4. The effect of castration on the numbers of splenocytes. Splenocytes were harvested on day 13 from 3 sham-castrated and 3 castrated animals and analyzed individually by dual-color FACS analysis for B220/sIgM and CD4/CD8. The SEM is shown for each bar, and statistical significance is indicated by the asterisks (*, P < 0.01).

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The observations reported here show that castration has dramatic effects on the generation of B lymphocytes. We show that after castration, there is a threefold increase in the percentage of B220+/sIgM- cells (predominantly large and small pre-B cells), an increase in sIgM+ cells, and a...
decrease in granulocytes and macrophages in the BM. The spleens of castrated animals also contain increased numbers of lymphocytes comprised predominantly of B cells that appear to be recent immigrants from the BM. These results suggest that castration regulates the overall numbers of lymphocyte in animals, primarily through augmentation of the generation of new cells and not by the expansion of mature lymphocytes.

Our results and those of others suggest that gonadal hormones have a profound effect on the generation of pre-B cells. The inhibitory effect of sex hormones on B lymphopoiesis appears to require a functional hormone receptor, because we have observed that there is an increase in pre-B lymphocytes in Tfm mice (testicular feminized mice), which lack a functional androgen receptor (Wilson, Mrose, Thomas, unpublished results) similar to that in castration. How the excess of a sex hormone signal or the lack of it might regulate lymphopoiesis is unknown. It is unclear whether testosterone or estrogen could potentially exert their activity via direct binding to lymphocytes, blasts, stromal cells, or by some other mechanism. It has been shown, for example, that estrogen treatment of BM-derived stromal cells decreases their IL-6 production, demonstrating that sex hormones can affect cytokine production by stromal cells thereby altering proliferative or differentiation signals to other cells. Therefore, increased lymphopoiesis because of an absence of sex hormones may occur via derepression of proliferative signals.

The simplest explanation for our observations is that castration results in increased production of IL-7. Indeed, others have shown that injection of mice with IL-7 results in a threefold to eightfold increase in B220⁺/sIgM⁺ precursor B cells in the BM. In contrast, injection of mice with an anti-IL-7 monoclonal antibody results in a dramatic reduction in B220⁺/sIgM⁺/HSA⁺ cells (pre-B cells) in the BM without changing the CD43⁺/HSA⁺ pro-B–cell population. This phenotypic change is similar to that identified in pregnancy and after estrogen injection, suggesting that an elevation in gonadal hormones can initiate an effect similar to IL-7 interference by antibody. Our castration experiments have shown the opposite to be true as well. Reduced levels of gonadal hormones after castration results in a phenotypic change in the BM grossly similar to that obtained with increased IL-7 exposure (increased B220⁺/sIgM⁺ cells). We have characterized the expanded subpopulation in castration to be predominantly small pre-B cells, the same set of cells that is diminished in pregnancy and after estrogen injections.

Although the phenotype of the cells in castrated animals partly correlates with that of cells resulting from increased levels of IL-7 production, there is a difference between our results postcastration and those obtained with increased IL-7 exposure. IL-7 transgenic mice, mice transfected with an IL-7 expression vector, and mice injected with IL-7 all showed large numbers of immature sIgM⁺ pre-B cells and CD4⁺/CD8⁺ double-positive T cells in their peripheral organs. In contrast, castration did not result in sIgM⁺ pre-B cells or immature T cells in the spleens of animals at any time postcastration. Although the lack of immature cells in the periphery of castrated animals might simply be because of differences in the levels of IL-7 or other differentiation factors, we have found no evidence of increased IL-7 mRNA in animals 2 weeks postcastration (Wilson, Mrose, Thomas, unpublished observations). IL-7 receptor mRNA is increased in the BM of castrated mice (Wilson and Thomas, unpublished data), but this may simply reflect the increase in pre-B cells.

There are several other mechanisms by which pre-B cells might be expanded postcastration other than by upregulation of IL-7 or IL-7 receptor production. First, there could be an increase in stem cells committed to becoming B cells. Because these cells are so limited in BM preparations, it would be very difficult to detect a slight increase in this population. Second, there could be an increase in proliferation of cells committed to becoming B cells. This process may be driven by another known (such as SCF) or unknown factor. One such candidate factor, pre-B–cell growth-stimulating factor (PBSF), has been described recently by Nagasawa and coworkers. However, we have found that, similar to IL-7, PBSF and SCF do not appear to be increased in castrated mice BM (Wilson, Mrose, Thomas, unpublished data). Third, there could be a decrease in apoptosis of cells committed to becoming B cells. Experiments are in progress to examine these possibilities and to define the mechanism by which castration results in increased B lymphopoiesis.

In this report, we have defined the dramatic and novel phenotypic changes found in the BM and spleen of castrated animals. To define the mechanism by which this occurs, further studies are underway to determine whether there are alterations in the levels of known cytokines or cytokine re-
ceptors that would explain the postcastration phenotype, or whether the lymphopoietic changes might be caused by, as yet, unidentified differentiation factors.

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Enhanced production of B lymphocytes after castration

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