Suppression of B Lymphopoeisis during Normal Pregnancy

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Summary

We describe a dramatic reduction in numbers and activity of committed B lymphocyte precursors in the bone marrow of normal pregnant mice. Changes in cells responsive to IL-7 were evident as early as 6.5 d of pregnancy and values were <10% of normal at parturition. B lineage precursors, identified by display of CD45R and absence of surface IgM, were also substantially depressed, and subpopulations representing different stages in the B lineage were assessed by three-color flow cytometry. Early pro-B cells are medium to large in size and have been previously characterized by low expression of the heat-stable antigen (HSA). This category of cells was not reduced, and in fact may have been slightly elevated, during pregnancy. In contrast, all subsequent populations of B lineage precursors, defined by patterns of expression of heat-stable and CD43 antigens, were substantially depressed. The immediate precursors of B cells (small pre-B cells) were identified by small size, expression of CD45R, absence of CD43, and lack of surface IgM. These were the most reduced of any phenotypically defined population in bone marrow. Numbers of newly formed B cells, characterized by the presence of sIgM, but not sIgD, were also diminished. However, B cells with a mature phenotype ($sIgM^+$, $sIgD^+$) were present in normal to somewhat elevated numbers. Mitogen-responsive B cells clonable in a semisolid agar assay were not significantly affected. A bromodeoxyuridine (BrdU) labeling technique was used to evaluate mitotic activity, which revealed an increased proportion of long-lived lymphocytes in the bone marrow of pregnant mice. These observations indicate that B lymphopoiesis is markedly downregulated during pregnancy and that all precursor populations beyond the early pro-B cell stage are affected. The pregnancy-related changes in bone marrow were selective for B lineage precursors, as cells expressing myeloid and erythroid markers were not reduced. In spleen, evidence was obtained for partial depletion of one subset of B cells. These cells, which have been reported to be recent immigrants from marrow, are characterized as having high levels of sIgM and HSA. Changes in other major B lymphocyte subsets in the spleen were less remarkable. When considered with results from the BrdU labeling procedure, the findings indicate that both production and export of lymphocytes from marrow may be substantially decreased. Numbers of B cell precursors were higher in postpartum animals whose litters were removed at birth, suggesting that lactation may prolong regeneration of lymphocyte production. We also found that acute estrogen treatment selectively reduced numbers of B lymphocyte precursors in bone marrow. These observations raise the interesting possibility that endocrine mechanisms involving sex steroids may be important for normal regulation of B lymphopoiesis.

B lymphocyte formation involves the coordinated rearrangement of Ig genes in committed precursor cells, expression of many other lineage restricted genes, and cell division. Longterm marrow culture models have permitted some progress to be made in understanding local regulatory mechanisms that influence these events (1). For example, it is now clear that at least one proliferation factor, IL-7, as well as certain pairs of adhesion molecules–ligands, such as VLA-4 and VCAM-1, are critical (2). In contrast, relatively little is known about systemic mechanisms that might control the daily output of B cells. Depletion of mature B cells with antibodies does not influence the kinetics of B cell formation and this has been interpreted to mean that there is no feedback from the pool of peripheral cells (3). The process is certainly influenced by chronic antigen exposure, injections of polyclonal IgG, graft-vs.-host disease, malaria, age, and cyclic neutropenia (4–8). However, little information is available about mechanisms involved in those phenomena or what controls movement of the site of B lymphopoiesis from liver and spleen to bone marrow during late embryonic life (1).

It has long been appreciated that there is a relationship between the size and activity of the thymus and sex hormones.

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For example, it was reported in 1898 that castration resulted in increased thymus size, and transient involution of the thymus during pregnancy was first described in 1913 (9–11). Subsequent studies revealed that estrogen injections caused similar atrophy of the thymus cortex and indicated that the relevant receptors for this hormone were probably on cells of the microenvironment (12, 13). B lymphopoiesis has not previously been studied in this context in mammals, but testosterone is known to ablate the bursa of Fabricius of birds (14, 15). Many studies have been conducted to explore the influence of pregnancy and hormones on autoimmune disease (16–18). However, a connection between hormone effects on immune system function and the production of new lymphocytes remains obscure.

We now demonstrate marked reductions in B lymphopoiesis that occur during normal pregnancy and show that B lymphocyte precursors are selectively depressed after a single injection of estrogen. Although many aspects of these responses require further study, there are parallels to the effects of pregnancy and estrogen on the thymus. Sex hormones could be important negative regulators of B, as well as T, lymphopoiesis.

Materials and Methods

Animals. BALB/c mice were obtained from our Laboratory Animals Resource Center or The Jackson Laboratory (Bar Harbor, ME). All females were studied at 2–5 mo of age and controls were age matched. For staged pregnancies, females were housed well separated from the males until mating, at which time one male was placed with two females. The appearance of a vaginal plug after overnight mating was labeled as day 0.5 of gestation. Pregnancy was unambiguously determined by implantation sites from 6.5 d of gestation. Controls for most experiments had been successfully mated, but were subsequently found not to be pregnant. All mice analyzed postpartum were allowed to retain their litters unless otherwise noted.

Cell Preparation. Single cell suspensions were prepared from bone marrow by injecting RPMI 1640 with 5% heat-inactivated FCS, into both femurs and tibia using a syringe with a 26-gauge needle. The cells were then centrifuged and resuspended at 10^7/ml. Spleen and thymus suspensions were prepared by gentle mincing over a metal screen with a syringe plunger followed by a 5-min incubation on ice to remove large aggregates.

Colony Assays. Bone marrow (5×10^4) or spleen (2.5×10^4) cells were prepared and suspended in 1 ml of assay medium as previously described (19). The semisolid agar cloning assay for B lymphocyte precursors was done with 1 ng recombinant mouse IL-7 (Upstate Biotechnology Inc., Lake Placid, NY). The granulocyte–macrophage progenitor assay (CFU-c) was done with 25 μl of 10 times concentrated L cell–conditioned medium. Clonable B cells were enumerated in semisolid agar containing 25 μg of LPS and 2-mercaptoethanol (19). 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. Bone marrow, spleen, or thymus 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/per ml. For three-color flow cytometry, the bone marrow was depleted of sIgM-positive cells by two rounds of depletion on affinity-purified goat anti–mouse μ-coated petri dishes. 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 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). Antibodies used in this study were as follows: biotinylated-14.8 (CD45RA), biotinylated-1126C-1 (anti-IgD) (20), Mac 1 (American Type Culture Collection, Rockville, MD), Ter 119 (a generous gift from Dr. T. Kina, Kyoto University, previously published in reference 21), goat anti-mouse IgM (PE or FITC) from Southern BioTechnology Assoc., Birmingham, AL (heat-stable antigen [HSA][1]), M1/69 FITC (PharMingen, San Diego, CA), or as culture supernatant from hybridoma TIB125 (American Type Culture Collection), Thy1.2-FITC (Becton Dickinson & Co.), anti-CD3 (purified from the 145-2C11 hybridoma generously donated by Dr. Jeff Bluestone, University of Chicago), goat anti–hamster Ig (H + L chain) FITC (CALTAG Labs, S. San Francisco, CA), biotinylated-S7 (CD43) purified and biotinylated in our lab from the hybridoma obtained from the American Type Culture Collection, CD45R (6B2/B220) PE (PharMingen). 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. Conditions used for each analysis are specified in Results. Cytocentrifuged bone marrow cells were evaluated for nuclear terminal deoxynucleotidyl transferase (TdT) according to a published protocol (22). Briefly, cells were fixed for 20 min at 4°C, rehydrated with 50% methanol in PBS for 5 min, and washed with cold PBS. The slides were blocked by incubation with normal goat serum and stained overnight with rabbit anti-TdT serum, followed by FITC-labeled goat anti–rabbit Ig (Supertechs, Bethesda, MD).

Bromodeoxyuridine (BrdU) Incorporation. Mice were given drinking water containing 1 mg/ml BrdU and 5% glucose (to overcome taste aversion) for a consecutive 3 d. For simultaneous surface staining and BrdU labeling we used a slight modification of the method described by Forster et al. (23). Briefly, 10^6 cells suspended in 200 μl cold PBS were injected into 5 ml 70% methanol and incubated 15 min at room temperature. After fixation, the cell suspension was centrifuged for 5 min at 400 *g* and the methanol was removed. For denaturation of the DNA, the cell pellet was resuspended in 2 ml 1 N HCl containing 0.5% Tween 20 (added immediately before use) and incubated for 15 min at 37°C. After centrifugation, the HCl was removed and the pH neutralized by addition of 200 μl of 0.1 M borax, pH 9.0. The cell suspension was then diluted into 5 ml of cold PBS, containing 3% heat-inactivated FCS and 0.1% sodium azide (washing buffer), and centrifuged. The cells were then stained with FITC antibody to BrdU (Becton Dickinson & Co.) for 15 min on ice. The cells were diluted with washing buffer, centrifuged, then stained with PE-labeled antibody to CD45R for 15 min on ice. After two more washes, the cells were resuspended in washing buffer and kept on ice until flow cytometric analysis.

Estrogen Treatment. Female mice at least 8 wk of age were given a single 1-mg injection of water soluble estradiol (Sigma Chem. Corp., St. Louis, MO) intraperitoneally and examined 1–5 d later for clonable bone marrow cells.

[1] *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; HSA, heat-stable antigen; TdT, terminal deoxynucleotidyl transferase.

Results

B Lymphocyte Precursors Are Reduced in Pregnant Mice. An IL-7 dependent colony assay was first used to determine that B lineage precursors are markedly diminished during and after pregnancy. This analysis included >77 pregnant and postpartum animals (Fig. 1). We picked 6.5 d after mating as the earliest time when pregnancy could be unambiguously established. Even at this early stage, clonable precursors were substantially reduced and this declined further to less than 10% of age matched control values by the end of gestation. Over the course of pregnancy (day 6.5 to birth), this averaged 13 ± 11% of control values. For these experiments, litters were not removed and numbers of functional progenitors returned to normal after delivery in only a few instances.

Two-color flow cytometry was then used to determine if actual numbers, as well as IL-7 responsiveness, of B lymphocyte precursors were affected by pregnancy. Cells in the B lineage were identified by expression of CD45R, with or without surface IgM (24). A typical result is shown in Fig. 2, where numbers of CD45RA$^+$/sIgM$^-$ cells (*upper left quadrants*) were significantly ($p \leqslant 0.001$) reduced during pregnancy. When values for animals between 6.5 and 19.5 d of gestation were pooled, the pregnant group averaged only 35% of controls (Table 1). B lineage precursors (CD45R$^+$, sIgM$^-$) were further resolved into small and large populations by low angle light scatter (Fig. 2, *bottom*). While both were significantly diminished during pregnancy, there was preferential reduction in small precursors (Table 1). Previous studies have shown that half of the cells in these two populations are pre-B cells, defined by the presence of cytoplasmic, but not surface, IgM (25). Slides prepared and evaluated for cytoplasmic immunoglobulin also revealed a marked reduc-

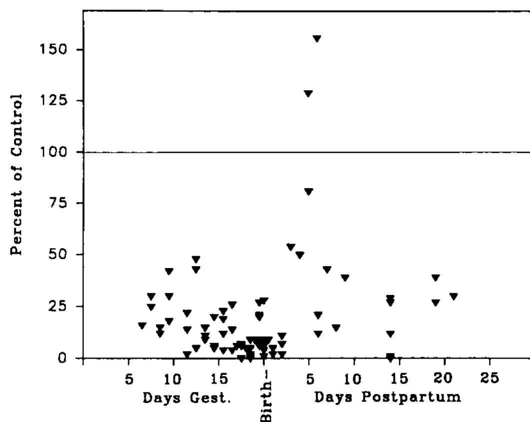

Figure 1. IL-7-responding cells in maternal bone marrow are reduced during pregnancy in mice. Points represent results obtained with a semisolid agar cloning procedure, where 77 pregnant and postpartum mice were compared with 51 age-matched controls. The control values averaged 85 ± 6 colonies per 10^5 cultured bone marrow cells and experimental values were normalized to this pooled average. A majority of the controls had been mated, but were not pregnant, and their results were indistinguishable from normal female mice that had not been mated.

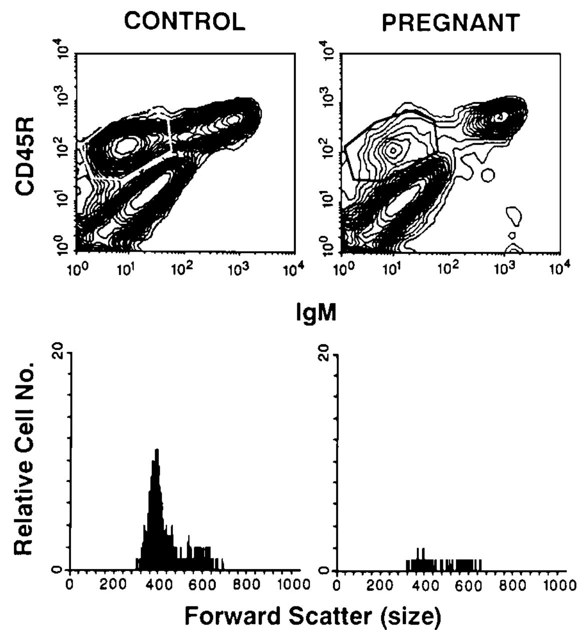

Figure 2. B lymphocyte precursors in bone marrow are greatly diminished during pregnancy in mice. Two-color flow cytometry was used to detect cells with expressed CD45R, but not surface IgM (top panels). This population was gated as indicated and examined for low angle forward light scatter as a size parameter (lower panels). The results of this pregnant mouse at 18.5 d of gestation are typical of more than 19 independent experiments and the average results are given in Table 1.

Table 1. *Selective Depletion of B Lymphocyte Precursors during Normal Pregnancy*

	Controls	Pregnant
Nucleated cells	142.9 ± 7.0	126.3 ± 7.0
Total CD45R$^+$,sIg$^-$	28.1 ± 3.0	9.8 ± 2.0
Large CD45R$^+$,sIg$^-$	7.0 ± 1.2	2.3 ± 0.3
Small CD45R$^+$,sIg$^-$	16.6 ± 2.0	5.1 ± 1.0
TdT$^+$	1.3 ± 0.4	0.5 ± 0.2
Fraction A	1.7 ± 0.3	2.1 ± 0.4
Fraction B + C	4.1 ± 0.8	1.7 ± 0.3
Fraction D	27.0 ± 0.5	5.1 ± 0.1
TER119$^+$ cells	30 ± 1.0	40 ± 4.0
Mac-1$^+$ cells	54 ± 3.0	49 ± 3.3
CFU-c	92 ± 9*	97 ± 10*

Except where otherwise indicated, values represent mean numbers of cells × 10^{-5} ± SE per bone. Results were pooled from a large number of experiments, where pregnant animals were examined between 6.5 and 19.5 d of gestation, and compared with age matched controls that were usually mated, but not pregnant. Significant differences and p values are noted in the text.

* Mean number of myeloid progenitor cells (CFU-c) ± SE per 10^5 cultured cells.

tion in numbers of pre-B cells in pregnant animals (data not shown).

Expression of the nuclear enzyme TdT spans several populations of early lymphocyte precursors in bone marrow (26). While the average number of these was reduced during pregnancy, the decrease compared to nonpregnant mice did not reach significance (Table 1). We then depleted B cells from bone marrow suspensions with antibody-coated plates and used three-color flow cytometry to resolve subpopulations of precursors in the B lineage. Hardy et al. (27) have defined an early population of progenitors ("Fraction A") that express CD45R and CD43, but have little or no HSA. Fraction A cells represent ~30% of the $CD45R^+/CD43^+$ population and 1% of total nucleated cells. They were not significantly ($p = 0.535$) changed during pregnancy (Fig. 3 and Table 1). The presumptive progeny of these cells (Fractions B and C) were identified on the basis of HSA expression, and these were significantly reduced ($p = 0.008$) during pregnancy. Small pre-B cells, defined as $CD45R^+/HSA^{hi}/CD43^-$ by flow cytometry (Fraction D), were even more depressed in pregnant mouse marrow (Fig. 3 and Table 1). In summary, the earliest pro-B cells (Fraction A) were not numerically affected during pregnancy. However, subsequent stages of differentiation, including IL-7-responding precursors, were progressively depleted and numbers of small pre-B cells were reduced by an average of fivefold.

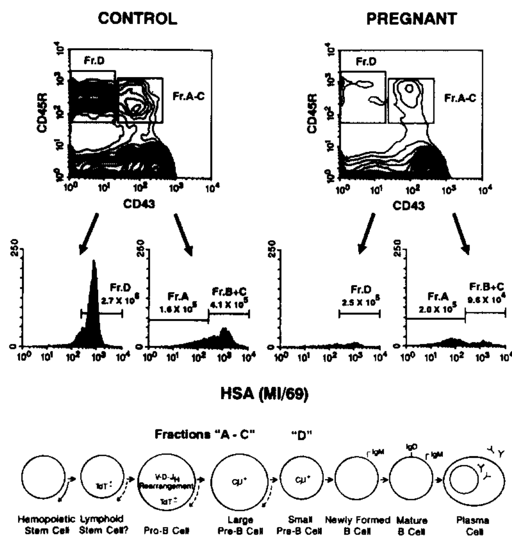

Figure 3. Pregnancy selectively affects subpopulations of B lineage cells in bone marrow. Cell suspensions from control and pregnant mice were first depleted of surface IgM^+ B cells with antibody coated plates. The remaining B lineage cells were then identified by staining with antibodies to CD45R and resolved further into subsets that were CD43 positive or negative (top panels). The boxed populations represent small pre-B cells (*Fr. D*) and a more complex category, which includes pro-B cells and large pre-B cells (*Fr. A-C*). The expression of a third marker, HSA (with monoclonal antibody M1/69) for each of the gated populations is depicted in histograms (lower panels). This allowed resolution of the earliest known population of pro-B cells (fraction A), which is $CD45R^+$, $CD43^+$, and HSA^{lo}. Total numbers of cells of each category in both femurs and tibias are indicated. The pregnant mouse was 18.5 d of gestation in the example shown. Illustrated are the major events in B lineage differentiation in relationship to fractions A–D.

Pregnancy Related Changes Are Restricted to B Lineage Cells. Numbers of total nucleated cells in bone marrow did not significantly change during pregnancy (Table 1). To evaluate myeloid cells, we assayed numbers of granulocyte–macrophage progenitors by colony formation in response to L cell-conditioned medium in semisolid agar, and found no significant differences in numbers of colonies between the marrow of control and pregnant animals ($p = 0.684$). Similarly numbers of myeloid cells expressing CD11b/CD18 (detected with monoclonal antibody Mac-1 or other myeloid markers (data not shown) were not reduced during pregnancy ($p = 0.324$). Cells in the erythroid lineage, defined by expression of the monoclonal antibody TER119 (21), were slightly elevated during pregnancy, but this change did not reach significance ($p = 0.169$). Thus, pregnancy related changes in marrow were restricted to B lineage cells.

Mitotic Activity of B Lineage Cells during Pregnancy. The population changes that we describe could result from decreased proliferation, and/or differentiation of early precursors, increases in apoptosis, and/or more rapid migration of cells from the marrow. We administered BrdU for 3 d to late-stage pregnant and control mice in order to identify cells that had divided during this interval. All myeloid cells were labeled in bone marrow from both groups of animals (data not shown). In three control animals, most of the small (88.8 ± 3.2%) and large B lineage lymphocytes (93.5% ± 3.0%) had undergone division during this time (Fig. 4, *upper quadrants*). This was more variable in three pregnant animals, but percentages of cells incorporating BrdU were reduced in small (33.7 ± 26.4%, $p = 0.023$) and large (70.5 ± 11.8%, $p = 0.031$) B lineage lymphocytes. These observations indicate that in addition to the depletions that occurred in numbers of B lineage precursors, there was also reduced mitotic ac-

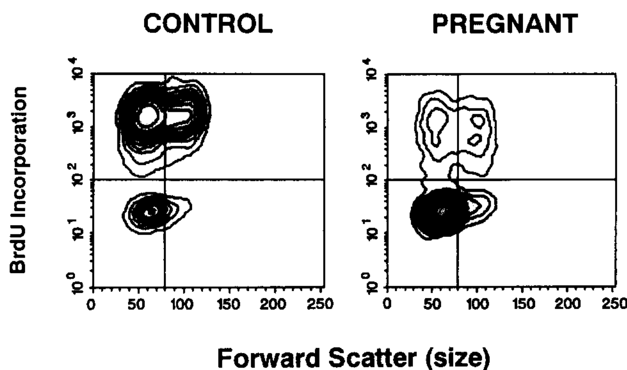

Figure 4. Mitotic activity of B lineage lymphocytes is reduced in bone marrow of pregnant mice. Mice were given BrdU in the drinking water for 3 d and flow cytometry was used to reveal lymphocytes that had incorporated the label. Cells were gated for expression of CD45R and further resolved into small and large populations with low angle light scatter. Upper left and right quadrants of each histogram correspond to labeled small and large lymphocytes, respectively. The pregnant mouse in the example shown was studied at 18.5 d of gestation.

Table 2. *B Lymphocyte Populations in Pregnant Mice*

	Cells	Control	Pregnant
Bone marrow	μ^+,δ^{-*}	7.7 ± 0.9	3.5 ± 0.7
	μ^+,δ^+	2.6 ± 0.6	3.4 ± 0.7
	CFU-B‡	158 ± 19	164 ± 32
Spleen	μ^+,HSA^{hi}	6.2 ± 0.7	2.7 ± 0.4
	μ^+,HLA^{lo}	42.4 ± 1.4	36.5 ± 1.3
	CFU-B	1,240 ± 103	1,362 ± 118

* B cells with "immature" and "mature" phenotypes were resolved by two-color flow cytometry and the results represent mean percentages of total nucleated cells with these characteristics ± SE.

‡ Mitogen-responsive B cells were enumerated with a semisolid agar (CFU-B) cloning procedure with results given as numbers of colonies per 10^5 cells cultured.

tivity in the remaining lymphocytes. We used the total numbers of small lymphocytes (Tables 1 and 2) and the fraction that incorporated BrdU to estimate the absolute number of interphase cells. Nonmitotic lymphocytes increased by approximately threefold in marrow of pregnant animals.

B Lymphocyte Populations in Marrow and Spleen of Pregnant Mice. A mitogen-dependent cloning assay (28) was used to assess the proliferative capability of B cells in pregnant animals (Table 2). There were slight increases in incidences of colony-forming cells in marrow and spleen, which were not statistically significant (p = 0.895 and p = 0.438, respectively). Flow cytometry was then used to evaluate B lymphocyte subsets in the two organs. Presumably immature B cells in bone marrow with the phenotype IgM$^+$, IgD$^-$, were decreased by ~60% (p = 0.003). However, B cells expressing IgM and IgD were present in normal or slightly elevated numbers. These findings indicate that while newly formed B cells are affected by pregnancy, the most mature B cells are exempt. This accords with the somewhat expanded population of long-lived, small lymphocytes identified by BrdU labeling (see previous section).

Recent B cell immigrants from the bone marrow to the spleen have been described as expressing high levels of HSA and sIgM (29). The number of these cells in pregnant mouse spleen was only 43% of that in control mice (Table 2, p = 0.008). The more abundant population of splenic B cells, which expressed low amounts of HSA, were less markedly reduced by pregnancy (p = 0.013). Therefore, the most notable change in splenic B cells was in a subset that had recently immigrated from the bone marrow. Less dramatic changes occurred in the most prevalent population of B cells in spleen. It should be noted that total numbers of nucleated cells per spleen did not change significantly during pregnancy (data not shown).

Influence of Lactation on B Lymphopoiesis in Postpartum Mice. In order to determine if lactation had an effect on the timing of recovery, three separate experiments were done where two females, delivering on the same day, with approximately the same size litters (always more than five in a litter), were compared. The litter of one female was removed, while the other was allowed to nurse. 14 d later, numbers of IL-7-responsive precursors were assessed. In each case, the nursing female had substantially lower numbers of IL-7 colonies compared with the nonlactating mother. The values were compared to results obtained from many independent experiments with a total of 51 nonpregnant control mice. Lactating and nonlactating postpartum mice averaged 20 ± 16% and 88 ± 61% of normal, respectively. A flow cytometry analysis was also performed on lactating versus nonlactating postpartum mice (Fig. 5). While B lineage precursors were 27.0 ± 20.0% of normal in lactating mice 2 wk after delivery, corresponding values were 63.5 ± 28.7% in animals that were not allowed to nurse. These findings indicate that lactation may be a factor in the speed with which B lineage precursors regenerate postpartum.

Estrogen Treatment Selectively Depresses B Lymphocyte Precursors. Many studies have shown that estrogen administration results in involution of the thymus (12, 13, 30–32). We conducted preliminary experiments to determine if lymphocyte precursors in bone marrow are influenced by this pregnancy-associated hormone. Female mice were given a single 1 mg injection of water-soluble 17-β estradiol intraperitoneally and compared with untreated mice at intervals over a 5 d period (Fig. 6).

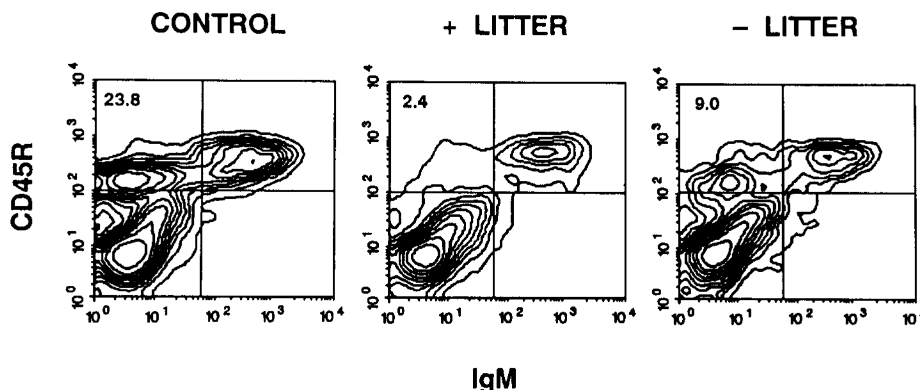

Figure 5. Lactation delays the regeneration of B lymphocyte precursors in bone marrow of postpartum mice. Two-color flow cytometry results are shown for two mice that delivered on the same day, with comparable litter sizes. One was removed at the time of birth and both animals, together with a normal control female, were evaluated 2 wk later.

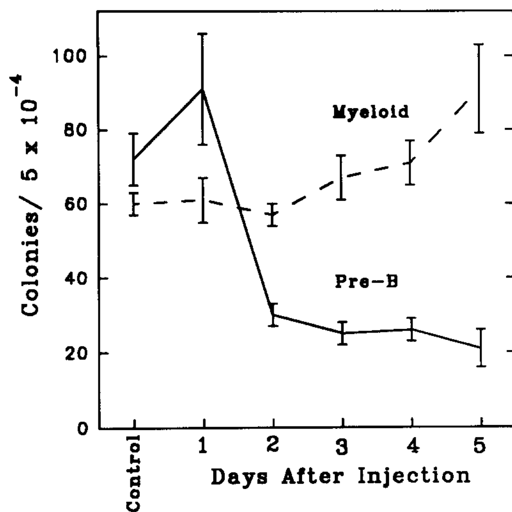

Figure 6. Selective depression of B lymphocyte precursors in bone marrow by estrogen. Adult female mice were given a single 1-mg injection of 17-β estradiol and evaluated at intervals with semisolid agar assays for myeloid progenitors (CFU-c) and IL-7-responding pre-B cells. The data are representative of three similar experiments and results are presented as mean numbers of colony forming cells ± SE.

This dose and time course of estrogen treatment did not cause significant changes in marrow nucleated cell numbers (data not shown). However, the incidence of IL-7-responsive precursors significantly declined ($p \leq 0.001$) within 48 h of treatment. In contrast, granulocyte-macrophage progenitors gradually increased in marrow, a change that was not statistically significant. Thus, absolute numbers of B lymphocyte precursors were selectively affected by systemic administration of this hormone.

Discussion

This is a first description of pregnancy-related effects on B lineage lymphocytes in bone marrow. We document a highly selective depression in numbers of IL-7-responsive precursors, beginning at a defined stage of differentiation, as well as consequences of this change on subsequent compartments in marrow and spleen. There are a number of similarities to changes previously documented in the thymus during pregnancy. Furthermore, we show that pre-B cells are selectively depleted in estrogen-treated animals. These findings demonstrate that B and T lymphopoiesis are subject to endocrine regulation and that pregnancy related hormones merit further study.

Lymphocyte precursors enumerated with a semisolid agar cloning procedure (19) dramatically declined within 6.5 d of successful mating and were almost totally absent by the time of delivery. Precursors might remain in marrow of pregnant mice, but lose IL-7 responsiveness. As other alternatives, cells detected by the assay might be produced from earlier precursors at a slower rate, or leave the marrow at an increased rate through apoptosis or migration. Our analysis does not completely rule out any of these possibilities. However, flow cytometry studies revealed substantial reductions in numbers of B lineage cells and made it possible to determine particular stages of differentiation that were affected.

Cells destined to become B lymphocytes can be identified in murine bone marrow by their expression of particular CD45 isoforms (1, 24, 25). Additional surface markers were used to evaluate subsets that differ in their positions in this differentiation lineage (27, 33–35) (Fig. 4). Cells at an early pro-B stage express CD45R, but little or no HSA, and are just beginning the rearrangement of D to J segments of μ heavy chain genes. This population, which represents only 1% of normal bone marrow, seemed unaffected by pregnancy.

An intermediate population of committed cells in bone marrow expresses both CD43 and moderate to high levels of HSA (27). These were significantly diminished (41% of control) in pregnant animals. This population of cells is thought to be dependent on IL-7, and probably includes the IL-7-dependent precursors detected with our cloning assay (19, 27). Rearrangement of immunoglobulin genes and expression of surrogate light chains are maximal at this stage (27, 35). As another milestone, dependence on growth signals provided by close contact with stromal cells gradually decreases as cells progress through these compartments (36, 37).

Even more diminished during pregnancy (19% of control) were small pre-B cells (Fraction D), that can be identified by their loss of the CD43 antigen (27). These immediate precursors of B cells are not themselves in cycle and are normally derived by differentiation of large dividing pre-B cells (22, 25). Defects in rearrangement and expression of immunoglobulins prevent cells from reaching this stage (22, 35). Reductions in small pre-B cell numbers during pregnancy could reflect diminished mitosis, survival, migration, or other alterations in earlier precursors.

Lymphocytes in marrow that express surface μ, but not δ, were reduced during pregnancy and these probably represent newly formed B cells (27). More mature cells that are μ^+, δ^+ were slightly elevated in the marrow of pregnant mice and this accords with numbers of mitogen-responsive B cells in that site. At least some of the mature B cells in marrow might be part of the recirculating pool.

Detailed studies of the mitotic activity and population dynamics in pregnant marrow have not been conducted. However, a BrdU labeling procedure revealed significant reductions in cells with evidence of division (Fig. 5). More than 89% of the small lymphocytes in control animals were labeled within a 3-d period, whereas only 34% did so in bone marrow of pregnant animals. Thus, both the total numbers of precursors and the proportion of recently formed lymphocytes were substantially decreased. These observations would be consistent with a greatly diminished production of new lymphocytes in marrow during pregnancy.

The normal contribution of newly formed B cells to the peripheral pool has been evaluated by a number of investigators and is the subject of some controversy (38, 39). A small but distinct population of B cells in the spleen have moderate to high levels of surface μ and high levels of HSA. It is thought that these cells represent recent immigrants from bone marrow (29). This population also declined during pregnancy, sug-

gesting that both productivity in the marrow and export to the spleen are downregulated.

Diminished production of B cells in marrow could somehow facilitate establishment of maternal–fetal tolerance during pregnancy. However, it is less clear how our findings relate to the well-known influence of sex steroids on autoimmunity (16–18). Diseases such as lupus, which are mainly dependent on polyclonal B cell activation and circulating immune complexes, are enhanced by estrogen and frequently worsen during pregnancy (16, 17). In contrast, autoimmune diseases in which inflammation is primarily dependent on T cell activation are suppressed by estrogen and usually improve during pregnancy (40). Although cell-mediated immunity may be altered during pregnancy, immune responsiveness in general has been reported to be unaffected (41–43). A decrease in production of new lymphocytes could ultimately result in simplification of the repertoire of antibody specificities. Although we found no significant changes in numbers of mature B lymphocytes in the marrow or the spleen, it remains to be determined if the process of somatic mutation in germinal centers (44) is affected during pregnancy. Pre-B cells in one autoimmune strain of mice appear earlier than normal during embryonic life, are elevated neonatally, and then decline more rapidly than in normal animals (45–47). We do not know if these characteristics relate to abnormal production or responsiveness to hormones. However, castration and hormone administration alter the disease course in such experimental models (18).

It has long been known that the thymus transiently involutes during pregnancy (10, 11) and there are several similarities to our findings with bone marrow lymphocytes. Numbers of mitotic cells and DNA synthesis decline in the thymus of pregnant animals (48). Likewise, our BrdU labeling is consistent with a large reduction in turnover of B lineage cells in bone marrow (Fig. 5). Lactation prolongs the suppression of lymphopoiesis in both tissues (Fig. 5 and reference 48). Furthermore, immature cortical cells in the thymus are selectively affected and cells with a mature phenotype are spared (48, 49). A similar pattern occurs after administration of estrogen, or, more rapidly, glucocorticoids (12, 13).

The thymus is described as one of the most estrogen responsive of nonuterogenic tissues and there have been many studies of estrogen-induced thymus atrophy (12, 13, 30–32). Interestingly, cells at an intermediate stage of differentiation ($CD4^+$, $CD8^+$, TCR^{lo}) were the most sensitive (13, 31, 32). Immature thymocytes ($CD4^-$, $CD8^-$, $IL\text{-}2R^+$) and mature cells ($CD3/TCR^{hi}$, $CD4^+$, or $CD8^+$) were both more estrogen resistant. While lymphocytes in the thymus may lack estrogen receptors, they are present on reticular-stromal cells in that organ (12, 30). Thus, the hormone may indirectly affect T lymphopoiesis via effects on the microenvironment. We obtained selective depletion of B lymphocyte precursors by injection of soluble estrogen (Fig. 6) and also determined that IL-7-responding lymphocyte precursors are unaffected by estrogen exposure in culture (Smithson, G., K. Medina, and P. W. Kincade, manuscript in preparation). This again suggests that the effect may be on the lymphopoietic microenvironment. As one possible target, estrogens have been shown to inhibit cytokine synthesis by marrow stromal cells (50).

There is another precedent for hormonal regulation of B lymphopoiesis. In birds, development of the bursa of Fabricius can be ablated by exposure to testosterone or, less effectively, to estrogen (15, 51, 52). Birds and mammals differ with respect to whether androgens or estrogens determine sex, and the suggestion was made that a similar difference might apply to which hormone effectively regulates immune system development (53). Experiments with birds demonstrated that testosterone selectively affects the bursal epithelium while cells with the potential to make B lymphocytes survive (54). Thus, diminished B lymphopoiesis in this model is also mediated by hormonal influence on the microenvironment.

Several recent reports indicate that peptide hormones such as insulin-like growth factor I may influence lymphohemopoietic progenitor cells (55–58). Another pregnancy-lactation related hormone, prolactin, has been extensively studied in terms of its production by, and effects on, immune system cells (59–63). A critical role in regulation of B lymphopoiesis has not been demonstrated but prolactin may stimulate development of T lineage cells. We are not aware of pregnancy-related peptide hormones that inhibit lymphopoiesis.

The depression of B lymphopoiesis during pregnancy calls attention to the possible importance of sex hormones in normal regulation of this process. Studies underway are aimed at learning if depletion of estrogen and/or other sex hormones increases lymphocyte formation. Success in this would have obvious implications for transplantation and treatment of immunodeficiencies. A better understanding of the relationship between hormones and the bone marrow microenvironment could improve the design of culture systems, which in turn might suggest new diagnostic and research strategies.

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