PHENOTYPE AND PROLIFERATION OF EARLY B LYMPHOCYTE PRECURSOR CELLS IN MOUSE BONE MARROW

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The primary development of B lymphocytes in the bone marrow can be divided into two major phases based on the expression of the lineage-specific molecule, IgM. In the later of the two phases, the progressive expression of IgM molecules provides a precise identification of phenotypically distinct subsets of B lymphocyte precursors. This has permitted detailed studies of the population sizes, proliferation, and functional maturation of precursor cells in mouse bone marrow (1–11). Actively proliferative large pre-B cells containing free cytoplasmic μ heavy chains ($c\mu$)[1] give rise to small pre-B cells, which without further division mature into B lymphocytes bearing surface μ heavy chains ($s\mu$) in whole IgM molecules. The population of newly formed B lymphocytes turns over rapidly in the bone marrow as the cells acquire the other surface receptors characteristic of mature B cells and they migrate to peripheral lymphoid tissues as functional B cells (12–22). In contrast, cells of the earlier phase of B cell genesis have not been directly characterized. These populations of precursor cells lack μ heavy chains, but include cells undergoing Ig heavy chain gene rearrangements. The number and proliferative amplification of such cells would be influential factors in determining the variety and size of new B cell clones being generated per unit time and potentially becoming available to the immune system.

The B cell lineage in mice is associated with a large (220 kD) surface membrane glycoprotein (23, 24) detected by several monoclonal antibodies (mAb), including the 14.8 clone developed by Kincade et al. (25). The 14.8 mAb binds to all B lymphocytes, as well as to both small and large pre-B cells in mouse bone marrow, but the total number of 14.8-binding cells exceeds that of the μ chain-bearing cells (26). Removal of 14.8^+ cells leads to temporary deletion of B lymphocyte production in vitro (11, 25). These findings suggest that mouse bone marrow contains a population of early B cell precursor cells that bind 14.8 antibody before expressing μ chains. Their proliferative activity in vivo is unknown.

Terminal deoxynucleotidyl transferase (TdT) is an intranuclear enzyme restricted to immature lymphoid cells in the thymic cortex and bone marrow (27–32) that appears to be involved in generating immunological diversity. In the B

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[1] *Abbreviations used in this paper:* $c\mu$, cytoplasmic μ chains; Imet, metaphase incidence or index; NCS, newborn calf serum; $s\mu$, surface μ chains; TdT, terminal deoxynucleotidyl transferase; TRITC, rhodamine isothiocyanate.

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cell lineage, recent studies (33, 34) suggest that TdT inserts additional short nucleotide sequences, not in the original template sequence, at VJ and DJ junctions during Ig heavy chain gene rearrangements. The bone marrow of human subjects and rats contain TdT^+ cells that exhibit B cell lineage–associated markers (31, 35–37). TdT^+ cells found within mouse bone marrow could thus be early B lineage precursors during the stage of Ig heavy chain gene rearrangement, but the characteristics of such a TdT^+ cell population and its possible relationship to the B lineage–associated antigen detected by mAb 14.8 are unclear.

We wondered whether the degree of coexpression of the foregoing cell markers on individual cells in mouse bone marrow would permit a direct characterization of early B lymphocyte precursor cells and their properties. Double immunofluorescence labeling of individual cells for the presence of the 14.8 antigen, TdT, and μ chains in various combinations has quantitated three phenotypically distinct cell subsets with characteristic cell size distribution profiles and, in conjunction with a mitotic arrest technique, has established their normal proliferative dynamics in vivo. The three cell subsets are proposed to be successive differentiation steps in a working scheme of primary B lymphocyte production in the bone marrow.

Materials and Methods

Animals

C3H/HeJ male mice were purchased from The Jackson Laboratories, Bar Harbor, ME, maintained under conventional conditions, and used at 8–9 wk of age.

Antibodies

Rat mAb 14.8 (25, 26) was kindly provided by Dr. P. W. Kincade, Oklahoma Medical Research Foundation, Oklahoma City, OK. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Cooper Biomedical Inc., Malvern, PA) diluted 1:10 in PBS, pH 7.2 (Gibco Laboratories, Grand Island, NY) was used as a second layer after cell surface binding of the 14.8 antibody at 1:20 dilution. Rabbit anti-TdT (Supertechs Inc., Bethesda, MD) was used at 1:20 dilution with a rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG $F(ab')_2$ second layer (1:20 dilution; Jackson Immunoresearch Laboratories Inc., Mississauga, Ontario). TRITC- and FITC-conjugated affinity-purified goat antibodies to mouse μ chains (Kirkegaard & Perry Laboratories, Gaithersburg, MD) (anti-μ–TRITC and anti-μ–FITC) were diluted 1:10 in PBS. All antibodies were centrifuged for 30 min in an Airfuge (Beckman Instruments, Palo Alto, CA) to remove aggregates just before use.

Bone Marrow Cell Samples

Mice were killed by cervical dislocation. Both femoral shafts were flushed with 1 ml cold Eagles' MEM, pH 7.2, containing 10% (vol/vol) Millipore-filtered newborn calf serum (MEM/NCS, Gibco Laboratories). After thorough resuspension, large particles were removed by sedimentation into 1 ml NCS for 5 min. The marrow cells were then centrifuged through 1 ml NCS (7 min, 200 *g*, room temperature) and resuspended in 1 ml MEM/NCS. The nucleated cells recovered from two femurs were counted with an electronic particle counter (model B; Coulter Electronics, Hialeah, FL).

Metaphase Arrest

Mice were given vincristine sulfate (Eli Lilly, Toronto, Ontario, Canada; or Sigma Chemical Co., St. Louis, MO) i.p. in a dose of 1 mg/kg body weight to stop cells in

metaphase (8, 38). For stathmokinetic studies, marrow cells were sampled from uninjected mice, and at four precise intervals, from mice injected with vincristine at 7:00 a.m.

Immunofluorescence Labeling

Double labeling of 14.8 and μ chains. To detect surface membrane 14.8 antigen, 100 μl of marrow cells (4×10^7 nucleated cells/ml) were incubated for 30 min on ice with 100 μl of mAb 14.8. The cells were washed twice by centrifugation (4°C, 10 min, 220 *g*) through NCS, exposed to FITC-conjugated goat anti-rat IgG (30 min, 4°C), washed twice through NCS, and resuspended in 1 ml 0.15 M NaCl, with 2.7 mM disodium EDTA (Fisher Scientific Co., Fairlawn, NJ) and BSA (Gibco Laboratories), 5% (wt/vol). In a cytocentrifuge (Cytospin; Shandon Southern Instrument Inc., Sewickly, PA) samples of 4×10^5 cells were deposited (1,000 rpm, 5 min) onto glass slides previously coated by centrifugation of 100 μl PBS/BSA 3% (wt/vol) (10 min, 2,000 rpm). The samples were quickly air-dried, fixed in precooled 5% (vol/vol) glacial acetic acid in absolute ethanol for 12 min on ice, and then washed three times and overnight in fresh PBS at 4°C.

Double labeling of surface and cytoplasmic μ chains. Bone marrow cell suspensions were incubated with anti-μ–FITC (30 min, 4°C) to label sμ, then washed, cytocentrifuged, fixed, and exposed to anti-μ–TRITC, thus labeling total μ chains, including both cμ and sμ (8).

Double labeling of 14.8 and TdT. After surface 14.8 labeling, as described above, the cells were cytocentrifuged, fixed with absolute methanol, and then stained with rabbit anti-TdT (2 h, 4°C) followed by TRITC-conjugated goat anti-rabbit IgG $F(ab')_2$ (30 min, 4°C).

Double labeling of μ chains and TdT. Bone marrow cells were cytocentrifuged, fixed with 5% acetic acid in ethanol and then exposed to anti-μ–FITC followed by anti-TdT labeling, as described above.

Slide preparations were mounted in 90% (vol/vol) glycerol (Fisher Scientific Co.) in PBS, pH 8.0, containing 0.1% (wt/vol) *p*-phenylenediamine (Fisher Scientific Co.) to reduce fading of the fluorescein during microscopy (39). An epifluorescence microscope (Carl Zeiss of Canada Ltd., Don Mills, Ontario, Canada) equipped with a HBO 50 mercury lamp, phase-contrast optics, and a × 100 oil-immersion phase-contrast objective was used for analysis.

Immunofluorescence Analysis

Individual marrow cells were scored for (*a*) single labeling with either FITC alone (14.8, sμ) or TRITC alone (cμ, TdT), or (*b*) double labeling with FITC plus TRITC (14.8 + cμ, sμ + cμ, TdT + 14.8). Fluorescing cells were examined by phase-contrast to measure the cell diameter, using an eyepiece micrometer scale, and to detect cells in metaphase arrest. The incidence of $c\mu^+s\mu^-$ pre-B cells, $s\mu^+$ B cells, $14.8^+\mu^-$ cells, and $14.8^+\mu^+$ cells, as well as the proportion of $14.8^+\mu^-$ cells in metaphase (metaphase index, Imet) were derived by examining at least 1,000 nucleated cells. The counts included at least 100 cells of each of the four phenotypes and at least 20 $14.8^+\mu^-$ cells in metaphase. The absolute number of marrow B lineage cells was calculated from their incidence and the total bone marrow cellularity. The incidences of $TdT^+\mu^-$, $TdT^+14.8^+$, and $TdT^+14.8^-$ cells were calculated by examining more than 3,000 nucleated cells, including at least 100 cells of each of the three phenotypes. To determine their size distribution, 300 TdT^+ cells were measured by ocular micrometer.

Results

Populations of 14.8^+ Cells Lacking μ Chains in Bone Marrow

Incidence. Approximately one-quarter of all the cells that bound the B lymphocyte lineage–associated mAb 14.8 in normal bone marrow lacked detectable μ chains either in the cytoplasm or at the cell surface (Table I). Such $14.8^+\mu^-$ cells represented ~5% of all the nucleated bone marrow cells or 8×10^5 cells

TABLE I

Analysis of 14.8⁺ and μ Chain–bearing B-lineage Cells in Mouse Bone Marrow by Double Immunofluorescence Staining

Cell markers			Incidence (percent of total nucleated cells)*	Actual number ($\times 10^{-5}$) per femur*
14.8	cμ	sμ		
+	−	−	5.2 ± 1.2‡	8 ± 2
+	+	+	17.0 ± 2.2‡	27 ± 4
	+	−	10.9 ± 1.2§	17 ± 1.8
		+	7.8 ± 0.8§	12 ± 1.2

* Means ± SD; $n = 11$.

‡ Double immunofluorescence labeling for surface binding of mAb 14.8 and for the presence of μ chains (cμ and sμ).

§ Double immunofluorescence labeling for sμ and for cμ (±sμ).

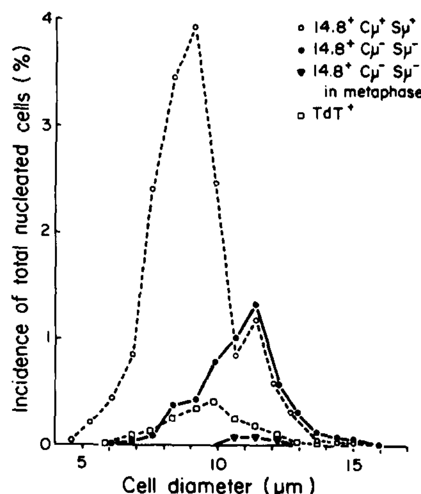

FIGURE 1. Size distribution of cell populations bearing 14.8 antigen, either with or without the simultaneous presence of μ chains (cμ + sμ), and of TdT⁺ cells in cytocentrifuge preparations of mouse bone marrow.

per femoral shaft. On the other hand, the numbers of 14.8^+ cells bearing μ chains corresponded with those of pre-B cells ($c\mu^+ s\mu^-$) plus B lymphocytes ($s\mu^+$), determined separately.

Size distribution. Measurements of cytocentrifuged $14.8^+\mu^-$ cells showed them to be mostly large cells, 7–16 μm in diameter with a single size distribution curve and a modal value of 11.5 μm (mean, 10.9 μm) (Fig. 1). This contrasted with the broad biphasic curve of μ chain–bearing 14.8^+ cells, 4.5–15 μm in diameter, peaking at 8–9 μm and 11.5 μm, representing small B lymphocytes and nondividing pre-B cells, as well as large replicating pre-B cells (6, 8, 10). The $14.8^+\mu^-$ cells thus resembled closely the large proliferating pre-B cells in size distribution.

Morphology. The largest $14.8^+\mu^-$ cells were undifferentiated blasts with a circular, indented, or irregularly-shaped nucleus and copious cytoplasm; others resembled large lymphoid cells with little cytoplasm and a circular or slightly indented nucleus having a fine chromatin pattern.

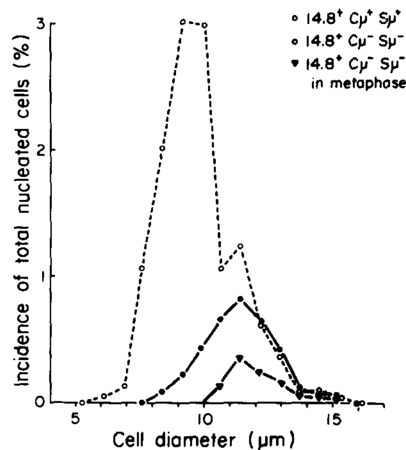

FIGURE 2. Size distribution of $14.8^+\mu^-$ cells arrested in metaphase, total $14.8^+\mu^-$ cells, and $14.8^+\mu^+$ cells in mouse bone marrow 4 h after administering vincristine sulfate.

Mitoses. A few (2–4%) $14.8^+\mu^-$ cells were normally observed in mitosis by phase-contrast microscopy. These cells were almost all larger than 10 μm diameter (Fig. 1).

Proliferative Dynamics of $14.8^+\mu^-$ Cells in Bone Marrow

$14.8^+\mu^-$ cells in vincristine-treated mice. To examine the kinetic properties of $14.8^+\mu^-$ cells, vincristine sulfate was given in vivo. After 4 h, a large proportion (40%) of the $14.8^+\mu^-$ cells had accumulated in metaphase arrest (Fig. 2), as distinguished under phase-contrast by a characteristically condensed, irregular chromosomal mass. The mitotic index increased with cell size, almost all the largest $14.8^+\mu^-$ cells (>13 μm) having entered mitosis within 4 h after vincristine administration. At the same time, the size distribution of the total $14.8^+\mu^-$ cell population shifted to the right, showing some decrease in smaller cells and increase in larger cells in vincristine-treated mice (Fig. 2) relative to untreated mice (Fig. 1).

After vincristine treatment, the cellularity of the bone marrow declined to 83% of normal values by 4 h, while the $14.8^+\mu^-$ and $14.8^+\mu^+$ cell populations both fell to about 65% of normal values (Table II), as previously noted for pre-B cells (8).

Rate of entry into mitosis and cell population turnover. Groups of mice were examined at four time intervals from 2 to 4 h after vincristine administration (Fig. 3). The accumulation of $14.8^+\mu^-$ bone marrow cells in metaphase followed a linear curve throughout this period of time. Regression analysis and extrapolation of the data indicated an initial lag time of 50 min before the vincristine began to arrest $14.8^+\mu^-$ cells in metaphase (Fig. 3). Thereafter, metaphases accumulated at a rapid rate, representing the rate of entry of $14.8^+\mu^-$ cells into mitosis, indicating a rapid turnover and a short apparent cell cycle time of cells in this phenotypic compartment (Table III). Combined with the population size (Table I), these data revealed a high production rate, totalling 0.4×10^8 $14.8^+\mu^-$ cells/d/mouse (Table III).

TABLE II

$14.8^+\mu^-$ and $14.8^+\mu^+$ Cells in Bone Marrow of Normal and Vincristine-treated Mice

Mice		Total bone marrow cellularity per femur (× 10^{-6})	Incidence (percent of total nucleated cells)		Actual number (× 10^{-5}) per femur	
Experimental group	n		$14.8^+\mu^-$	$14.8^+\mu^+$	$14.8^+\mu^-$	$14.8^+\mu^+$
Normal	3	15.7	5.2	15.6	8.1	24.5
After vincristine						
2 h	3	14.5	4.5	15.1	6.6	21.9
2 h, 40 min.	3	13.7	4.2	11.3	5.8	15.5
3 h, 20 min.	3	13.4	3.9	12.6	5.3	16.9
4 h	3	13.0	4.0	13.0	5.3	15.6
Total	15	14.1	4.4	13.5	6.2	18.8

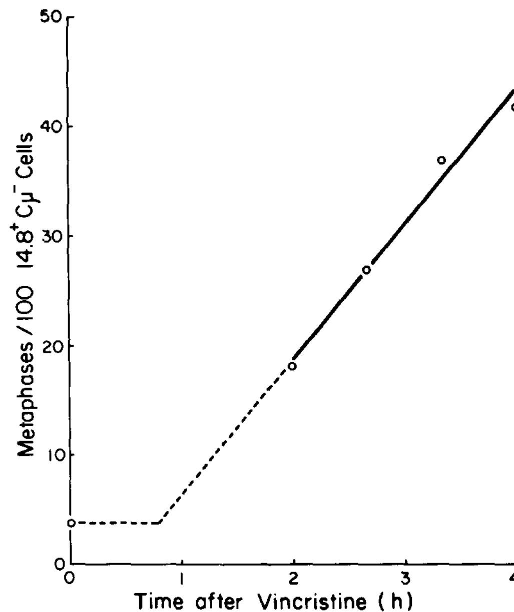

FIGURE 3. Imet of $14.8^+\mu^-$ bone marrow cells after vincristine. The curve was obtained by linear regression analysis of the values for Imet derived from four groups of three mice given vincristine.

Cell Populations Defined by TdT and 14.8 Antigen in Bone Marrow

Incidence. Cells showing a patchy intranuclear localization of TdT formed a small but well-defined population, totalling 1.8% of all nucleated cells in normal bone marrow. In double immunofluorescence labeling experiments, one-half of the TdT^+ cells simultaneously expressed the 14.8 surface antigen (Table IV), but of a sample of 200 TdT^+ cells, none had detectable μ chains ($c\mu$ or $s\mu$).

Size distribution. The TdT^+ cells as a whole were mainly medium-sized cells, corresponding in diameter with the smaller cells of the $14.8^+\mu^-$ population and peaking at 10 μm (range, 7–13 μm; mean, 9.7 μm) (Figs. 1 and 4). The TdT^+ cells binding mAb 14.8 tended to be somewhat larger than those lacking 14.8^- antigen, the size distribution curve of $TdT^+14.8^+$ cells (peak and mean, 10 μm)

TABLE III

Kinetics of 14.8⁺μ⁻ Bone Marrow Cells

Parameter	Value
Entry into mitosis*	13.5%/h
Turnover time‡ (average apparent cell cycle time)	7.4 h
Number of cells produced per:	
Femoral shaft§	1.1×10^5 cells/h
Total bone marrow‖	410×10^5 cells/d

C3H/HeJ ♂ mice, 8–9-wk-old.

* Slope of line obtained by linear regression analysis of the Imet for $14.8^+\mu^-$ cells after vincristine (Fig. 3).

‡ Turnover time = 100/rate of entry into mitosis.

§ Calculated as (number of cells per femur from Table I) × (0.01) × (rate of entry into mitosis).

‖ Calculated as (cells produced per femur) × 15.8 × 24 h. 15.8 is the conversion factor for one femur to whole-body bone marrow (8).

TABLE IV

Expression of TdT and 14.8 Antigen by Three Populations of Presumptive Early B Lymphocyte Precursor Cells in Bone Marrow

Group	Phenotype			Incidence (percent of total nucleated cells)	Actual number ($\times 10^5$) per femur
	TdT	14.8	cμ		
1	+	–	–	0.9 ± 0.2*	1.4 ± 0.3
2	+	+	–	0.9 ± 0.2*	1.4 ± 0.3
3	–	+	–	4.3 ± 0.3‡	6.6 ± 0.4

* Analyzed by double immunofluorescence labeling for nuclear TdT and for surface binding of mAb 14.8.

‡ Calculated by subtracting values for $TdT^+14.8^+$ cells from those of $14.8^+\mu^-$ cells (Table I).

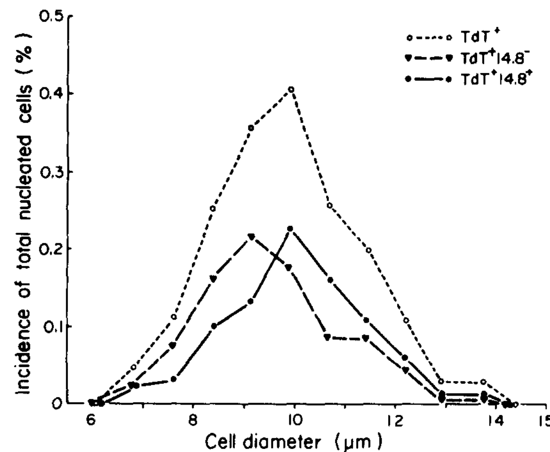

FIGURE 4. Size distribution of cells bearing TdT and 14.8 antigen in normal mouse bone marrow.

being consistently shifted slightly to the right of that of $TdT^+14.8^-$ cells (peak, 9 μm; mean, 9.5 μm) (Fig. 4).

Mitoses. Some mitotic cells were observed to have detectable TdT dispersed throughout the cytoplasm.

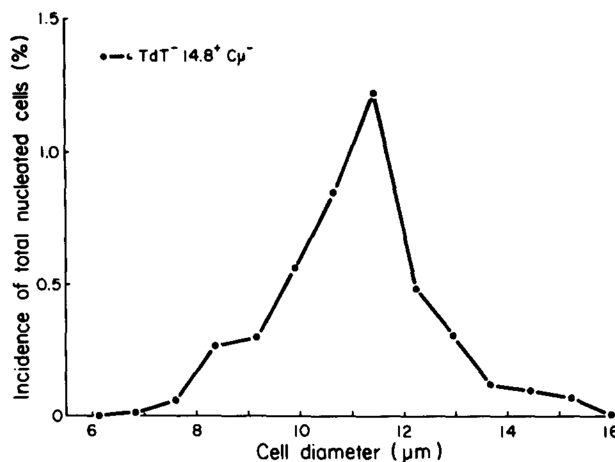

FIGURE 5. Size distribution of $TdT^-14.8^+\mu^-$ cells in normal mouse bone marrow (derived by subtracting data for $TdT^+14.8^+\mu^-$ cells [Fig. 4] from those for total $14.8^+\mu^-$ cells [Fig. 1]).

14.8^+ Cell Population Lacking TdT and μ Chains in Bone Marrow

The foregoing data demonstrate that the population of $14.8^+\mu^-$ cells in normal bone marrow (Fig. 1, Table I) includes a subset of TdT^+ cells (Fig. 4, Table IV). By subtracting values for $TdT^+14.8^+$ cells, all of which were demonstrated to be lacking μ chains, from those for $14.8^+\mu^-$ cells, a population of $TdT^-14.8^+\mu^-$ cells could be defined with respect to incidence (Table IV) and diameter (Fig. 5). These cells represented the larger $14.8^+\mu^-$ cells and showed a single, sharp size-distribution curve (peak, 11.5 μm; mean, 11.7 μm) (Fig. 5).

Three Populations of Presumptive Early B Lymphocyte Precursors in Bone Marrow

Three phenotypically distinct subsets of cells have thus been identified by double immunofluorescence labeling for the expression of TdT and 14.8 in the absence of detectable μ chains ($TdT^+14.8^-$, $TdT^+14.8^+$, and $TdT^-14.8^+$), as summarized in Table IV and Figs. 4 and 5.

Discussion

Using cytological markers, the present work defines three subsets of cells in mouse bone marrow, putatively representing steps in the development of early B lymphocyte precursors before the synthesis of μ heavy chains. These cells, which would include the phase of μ heavy chain gene rearrangement, have now been shown to be in active cell cycle, and their normal production rate has been quantitated in vivo. The work provides a working model of the phenotype and proliferation of early B cell progenitors, and raises questions concerning the potential effect of cellular events at this stage of development on the number and size of B cell clones ultimately delivered to the immune system.

Cells that bind the B lineage–associated mAb 14.8 but lack μ heavy chains form a substantial population in mouse bone marrow. Accounting for ~5% of all nucleated bone marrow cells, such $14.8^+\mu^-$ cells are 1.5 times as numerous as

the large proliferative $c\mu^+s\mu^-$ pre-B cells, previously quantitated in the same mouse strain (8). The population size of $14.8^+\mu^-$ cells determined in the present work by double immunolabeling accords with values that can be derived from the reported incidences of 14.8^+ cells and of μ^+ cells, measured separately (26). In the latter reports, using a different mouse strain (CBA), with lower frequencies of pre-B cells, the numbers of $14.8^+\mu^-$ cells, would be equivalent to approximately one to two times those of all pre-B cells (26). From its magnitude alone, the $14.8^+\mu^-$ cell compartment would appear likely to represent the major population of pre-μ chain precursor cells in mouse bone marrow.

The present findings now demonstrate an active proliferation of B precursor cells after they first express 14.8 antigen and before developing $c\mu$. Essentially the whole population of $14.8^+\mu^-$ cells appears to be in cell cycle, as suggested by the shape of its size distribution curve as well as the shift in size from small to large $14.8^+\mu^-$ cells during metaphase arrest, a finding that reflects the continuing growth of cells in interphase not being replaced by the usual production of small postmitotic cells. The rate of accumulation of metaphases during mitotic arrest indicates a turnover time and mean cell cycle time of 7.4 h for $14.8^+\mu^-$ cells. Both the size distribution profile and cell cycle time of $14.8^+\mu^-$ cells resemble closely those we have observed previously for the large proliferating subset of $c\mu^+s\mu^-$ pre-B cells (8).

The effects of mitotic arrest on the $14.8^+\mu^-$ cell population suggest that the developmental boundaries marking the beginning and end of this compartment do not coincide with the mitotic phase of the cell cycle. During mitotic arrest, as well as their shift in size distribution, the $14.8^+\mu^-$ cells in femoral marrow show a progressive drop in absolute number. This phenomenon could be due to three factors. First, it could be a toxic effect. The dose of vincristine used, however, was well within the nontoxic range established for proliferating large pre-B cells in mouse bone marrow (8), and the rate of accumulation of mitosis remained linear over the 4-h period of observation. Vincristine toxicity, on the other hand, causes a decline or plateau in the accumulation of mitoses after a few hours. Second, a decline in cell number after vincristine will occur if the population consists of a mixture of dividing and nondividing cells. As the proliferating cells accumulate in mitosis, inflow from proliferating cells of the preceding phenotypic compartment ceases, yet the nondividing cells continue progressively to develop the features of the next phenotypic compartment and are thus perceived as being lost from the compartment under study. Such is the case for the population of $c\mu^+s\mu^-$ pre-B cells (8). After vincristine, the small pre-B cells decline in number as they continue to develop $s\mu$ without cell division and pass from the pre-B cell to B lymphocyte compartment. In the present work, the 17% decline in total cellularity of the bone marrow after vincristine reflects the continuing emigration and loss of nondividing end cells during the arrest of their proliferating precursors. The $14.8^+\mu^-$ cells, however, as already noted, are probably all in cell cycle. The third possibility is that the phenotypic transition from one compartment to the next occurs some hours after the last mitosis within a completely cycling compartment. The postmitotic cells in early interphase at the onset of mitotic arrest continue to leave the compartment for some hours as they develop the

phenotypic markers of the next differentiation stage. Because the number of cells within a given compartment doubles in successive generations, the loss of postmitotic cells will exceed any input from the preceding compartment over the same period of time. The latter explanation fits all the observed data for the $14.8^+\mu^-$ cell population, suggesting that the cells first express μ heavy chains during interphase at least 3 h after the beginning of cell cycle. This would mark the point of transition from the $14.8^+\mu^-$ cell to the $c\mu^+s\mu^-$ pre-B cell stage.

Quantitation of the proliferation of $14.8^+\mu^-$ cells in vivo, calculated from their observed number and turnover time, indicates a total turnover of 0.4×10^8 cells/d throughout the entire bone marrow. This value represents the total turnover of cells within the $14.8^+\mu^-$ cell compartment itself. It is not clear, however, whether this value represents cells passing either concurrently through a single cell cycle or sequentially through two or more cell cycles. The number of successive cell generations of $14.8^+\mu^-$ cells will determine the degree of cellular expansion within the compartment and the relationship between the calculated turnover of $14.8^+\mu^-$ cells and the number of cells flowing from this developmental stage per unit time. If the calculated turnover represents a single cell cycle, the progeny of dividing cells will leave the compartment at twice this rate. In contrast, if each $14.8^+\mu^-$ cell undergoes multiple cell cycles, the cells in the final generation will number only approximately half of the total $14.8^+\mu^-$ cells, and the calculated turnover of the whole population will approximate the actual outflow from the compartment. The rate at which cells need to enter the compartment to maintain the steady state may be only a small fraction of this. Thus, the present study suggests that the production of cells leaving the $14.8^+\mu^-$ compartment is in the range of $0.4–0.8 \times 10^8$ cells/d, depending on the number of generations within the phenotypic compartment. This number is not known. The rate of fall in number of $14.8^+\mu^-$ cells after vincristine suggests that the ratio of outflowing cells to inflowing cells is greater than would be the case if only a single cell division had occurred. In vitro maturation assays of bone marrow depleted of $14.8^+\mu^-$ cells (11, 25, 26) as well as a precursor B cell cloning assay in vitro (40) also accord with the possibility of more than one cell cycle within the $14.8^+\mu^-$ phenotype. More direct indications are required of the number of generations of $14.8^+\mu^-$ cells and thus the total time spent and the overall expansion achieved by cells entering this phenotypic compartment in vivo.

A small but well-defined subset of μ^- lymphoid cells in mouse bone marrow exhibits intranuclear TdT. These TdT^+ cells appear to be mainly B-lineage cells, including the stage of Ig heavy chain gene rearrangement. The present work reveals that half of the TdT^+ cells in mouse bone marrow also express the B lineage–associated antigen, 14.8. Depletion studies suggest that this antigen is expressed by virtually all the immediate precursors of B lymphocytes (11, 25, 26). Furthermore, some cells transformed by infection with the Abelson murine leukemia virus are $14.8^+\mu^-$ cells showing Ig heavy chain gene rearrangement (11). Human and rat bone marrow also contain small subsets of TdT^+ cells (28, 32, 37, 41) which normally coexpress various B lineage–associated antigens (35, 37). In addition, TdT has been detected in human pre-B cell leukemias (42, 43), and in a few normal pre-B cells in human subjects (35) and C57BL/6 mice (44).

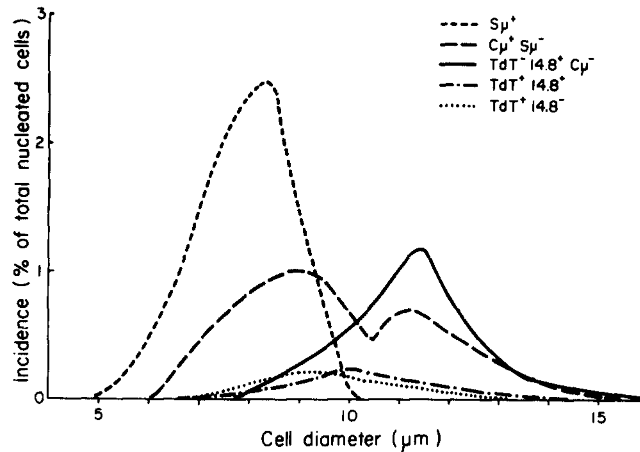

FIGURE 6. Incidence and size distribution of μ-bearing B-lineage cells (sμ+ B lymphocytes; cμ+sμ− pre-B cells) and μ− candidate B lineage cells (14.8+TdT− cells; TdT+14.8− and TdT+14.8+ cells) in mouse bone marrow.

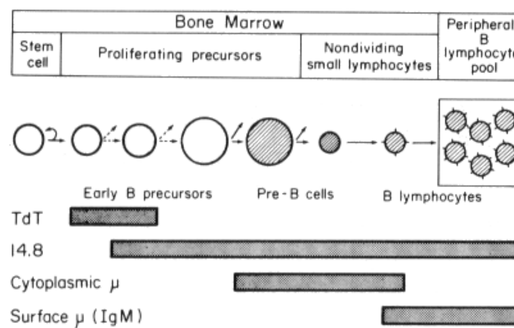

FIGURE 7. Scheme of differentiation of B lymphocytes in mouse bone marrow.

TdT itself has recently been implicated as the enzyme catalyzing the insertion of additional nucleotide sequences, the N regions, at the junctions of Ig heavy chain gene segments, one mechanism of immunological diversification (33, 34). The presence of TdT would therefore provide a cellular marker of the stage of Ig heavy chain gene rearrangement. It has yet to be shown, however, whether the presence of either TdT or 14.8 antigen is an obligatory step in B cell genesis.

Unlike Ig, neither the 14.8 antigen nor TdT in the bone marrow are necessarily entirely specific for the B cell lineage. The 14.8 antigen is expressed in small amounts on a subset of peripheral T lymphocytes (25, 26). TdT characterizes immature lymphocytes of both B and T lineages. TdT+ cells can be induced to appear in bone marrow cultures by thymic hormones (45), and bone marrow cultures can show high incidences of TdT+ cells lacking both B and T lineage markers (46). Thus, it cannot be excluded that some TdT+ cells in bone marrow may be prethymic T cell precursors or other undifferentiated lymphoid cells.

Based on the expression of TdT and 14.8 antigen, three phenotypically distinct subsets of μ− cells can be distinguished as presumptive early B lineage precursors. Fig. 6 compares both their incidence and size distribution with those of previously

defined B lymphocyte lineage populations. In Fig. 7, these subsets of cells are depicted in a scheme of B lymphocyte differentiation, proposing a sequential expression of TdT, 14.8 antigen, and cμ by individual cells. This scheme represents the simplest interpretation consistent with the present data and provides a model for testing the precursor-product relationships of the cell subsets now described.

The demonstration of a mitotic step and of the presence of TdT at the $14.8^+\mu^-$ stage of B lymphocyte development, in addition to the proliferative phase previously noted in large pre-B cells (6, 8), are important in considering the number of different clones of B cells and the number of cells per clone potentially available for delivery to the immune system. The present kinetic data also suggest that the total production of $14.8^+\mu^-$ cells is somewhat in excess of that of previously calculated for large pre-B cells (8), thus pointing to some cell loss, possibly reflecting ineffective gene rearrangements at this stage. The phenotypes and proliferation rates now established for candidate precursor cells in normal bone marrow form the basis for continuing work on perturbations and the factors which normally regulate primary B cell production in vivo.

Summary

Bone marrow cells were examined by double immunofluorescent labeling techniques to detect determinants for the B lineage monoclonal antibody, 14.8, the nuclear enzyme, terminal deoxynucleotidyl transferase (TdT), cytoplasmic μ chains (cμ), and surface μ (sμ). In 8–9-wk-old C3H/HeJ mice, 14.8^+ cells totalled 22.2% of all marrow cells (35×10^5 cells/femur). While many 14.8^+ cells were $c\mu^+s\mu^-$ pre-B cells and $s\mu^+$ B lymphocytes (17.0%), the remainder (5.2%) were large cells lacking μ chains. After injecting vincristine sulfate, these $14.8^+\mu^-$ cells accumulated in mitosis at a rate of 13.5%/h (turnover time, 7.4 h). Their calculated total production rate (41×10^6 cells/whole marrow/d) exceeded that previously determined for large pre-B cells, suggesting some cell loss from the B lineage. TdT^+ cells made up 1.8% of marrow cells and were mainly medium-sized cells. They all lacked μ chains, but half (0.9%) bound 14.8 antibody at low to medium intensity. Three discrete cell populations were thus defined, differing in mean cell diameter (*a*) $TdT^+14.8^-\mu^-$, 9.5 μm; (*b*) $TdT^+14.8^+\mu^-$, 10 μm; and (*c*) $TdT^-14.8^+\mu^-$, 11.5 μm, presumptively representing a sequence of cell stages preceding the expression of μ chains in large pre-B cells ($TdT^-14.8^+c\mu^+s\mu^-$, 11.5 μm). This work provides a tentative model of early progenitor cells and their proliferation in normal marrow as a basis for studies of perturbations and the control of B lymphocytopoiesis.

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