Immunology 1984 **51** 333

Pre-B cells in bone marrow: size distribution profile, proliferative capacity and peanut agglutinin binding of cytoplasmic μ chain-bearing cell populations in normal and regenerating bone marrow

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Accepted for publication 22 August 1983

Summary. Pre-B cell populations in mouse bone marrow, identified by double immunofluorescence labelling of cytoplasmic and surface μ chains ($c\mu$, $s\mu$), have been characterized by cell size, proliferative capacity and the binding of peanut agglutinin (PNA). In the normal steady state of lymphocyte production the size distribution profile of cytocentrifuged $c\mu^+s\mu^-$ cells was bimodal. A population of large cells in rapid cell cycle was revealed by arresting cells in mitosis with vincristine. Many $c\mu^+s\mu^-$ cells, however, formed a nondividing population of small lymphocytes, resembling $s\mu^+$ cells in size distribution. During regeneration from sublethal whole body X-irradiation (150 rads) a marked enrichment of large $c\mu^+s\mu^-$ cells preceded small $c\mu^+s\mu^-$ and $s\mu^+$ cells; progressive changes in cell size distribution reflected a wave of B lymphocyte genesis. The $c\mu^+s\mu^-$ cells in foetal liver resembled those in regenerating marrow. Surface binding of PNA characterised all $c\mu^+s\mu^-$ cell populations in normal and regenerating bone marrow and in foetal liver, whereas only a minority of $s\mu^+$ cells and μ-negative marrow cells bound PNA strongly. The present size distribution analyses allow a correlation with other cytological and functional studies of marrow lymphocyte precursors in defining the place of pre-B cells in B lymphocyte genesis.

Abbreviations: $c\mu$, cytoplasmic μ chains; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PNA, peanut agglutinin; $s\mu$, surface μ chains.

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INTRODUCTION

The production of B lymphocytes in murine bone marrow maintains a pool of short-lived primary B cells in the peripheral lymphoid tissues (Osmond, 1980). Primary humoral immune responsiveness thus depends upon the continuous proliferation and differentiation of B lymphocyte precursors, pre-B cells, in the marrow. These precursor cell populations remain ill-defined, however, despite the use of various criteria to assay them.

The first evidence for the existence of precursors producing large numbers of lymphocytes in the marrow was provided by radioautographic DNA labelling of individual cells *in vivo* and in cultures (Osmond & Everett, 1964; Brahim & Osmond, 1970; Yoshida & Osmond, 1971). In cultured marrow cell fractions, lymphocyte precursors were identified within a population of large lymphoid cells visualized in radioautographic stained smears (Yoshida & Osmond, 1971). Analyses of the size distribution of DNA synthesizing cells revealed populations of dividing large lymphoid cells and non-dividing small lymphocytes, demonstrating a precursor–product relationship between them. The development of surface IgM molecules on B

cells did not coincide with the transition from dividing precursors to non-dividing small lymphocytes, however, but developed after a distinct post-mitotic period (Osmond & Nossal, 1974) together with other B lymphocyte surface markers (Yang, Miller & Osmond, 1975). Further analysis of lymphoid precursors based on morphological criteria alone was limited by their heterogeneity in proliferative and hemopoietic potential (Miller & Osmond, 1973; Rosse, 1976), and by the lack of specific markers for B lineage precursors.

Double immunofluorescence labelling of cells from bone marrow and sites of foetal B cell genesis has identified individual cells containing cytoplasmic μ chains ($c\mu$) without detectable surface membrane μ chains ($s\mu$), as presumptive late B lineage cells following the rearrangement and expression of immunoglobulin μ heavy chain genes (Raff *et al.*, 1976; Owen *et al.*, 1977b; Cooper & Lawton, 1979). Such $c\mu^{+}s\mu^{-}$ pre-B cells are heterogeneous in size and in proliferative capacity, as shown by [^{3}H]-thymidine uptake (Owen *et al.*, 1977b; Burrows *et al.*, 1978; Pearl *et al.*, 1978; Landreth, Rosse & Clagett, 1981), but the constituent populations have not been fully characterized. A proposed large to small cell sequence of development of $c\mu^{+}s\mu^{-}$ cells and their role in the genesis of $s\mu^{+}$ small B lymphocytes remain based on compelling but circumstantial evidence.

Pre-B cells have also been assayed indirectly by their capacity to give rise to functionally responsive B lymphocytes after an appropriate maturation period either *in vivo* or *in vitro* (Lafleur, Miller, & Phillips, 1972; Melchers, 1977; Paige *et al.*, 1981). These assays have analysed pre-B cell populations by their sedimentation velocity at unit gravity (Lau *et al.*, 1979) and by hydroxyurea deletion to reveal dividing cells (Rusthoven & Phillips, 1980; Freitas *et al.*, 1982). While they define some properties of pre-B cell populations, notably their relative cell size distribution as reflected by sedimentation velocity profiles, these assays do not distinguish individual pre-B cells from other cell types in the same sedimentation fractions nor the proliferative sequence of cell generations in B lymphocyte production.

Cytoplasmic μ chains provide a direct cytological marker for B lineage cells, yet such cell populations remain less well characterised than, and difficult to equate with, those defined by the other precursor cell criteria. The present work analyses the size distribution profiles of $c\mu^{+}s\mu^{-}$ and $s\mu^{+}$ cells in mouse bone marrow, as detected by double immunofluorescence labelling and measurement of individual cells under standardised cytocentrifuge conditions, as well as the proliferative subset of $c\mu^{+}s\mu^{-}$ cells identified after metaphase arrest by vincristine. $C\mu^{+}s\mu^{-}$ cell populations in the steady state of B cell genesis in normal mouse marrow are compared with those propagating waves of activity in regenerating marrow and in foetal liver. In each case, the binding of the lectin, peanut agglutinin (PNA), to individual cells is correlated with μ chain expression, as a potential means of distinguishing immature lymphoid cell populations (Reisner, Linker-Israeli & Sharon, 1976; Rose *et al.*, 1980; Newman & Boss, 1980). The results characterize further the $c\mu^{+}s\mu^{-}$ cell populations in bone marrow, indicating the place of these cells in a scheme of B lymphocyte differentiation and providing a basis for comparison with other pre-B cell assays.

MATERIALS AND METHODS

Animals

Male and female CBA mice were used at 7–9 weeks after birth and during foetal life at 14–19 days of gestation.

Cells

Bone marrow cells were flushed from the femoral shafts of pairs of mice, suspended in HEPES-buffered RPMI-1640 with 20% foetal calf serum (RPMI-FCS), pooled, washed by centrifugation through FCS at 4° and resuspended in RPMI-FCS. Foetal liver cells were treated similarly after teasing the organ in RPMI-FCS.

μ-Chain labelling

To label cell surface μ chains, cell aliquots ($2{\cdot}5 \times 10^6$) were centrifuged to a pellet, resuspended in 20 μl anti-μ antibody conjugated with fluorescein isothiocyanate (FITC) (Meloy Laboratories, Springfield, VA; 1:15 dilution), incubated on ice for 30 min and centrifuged twice through FCS. To label intracytoplasmic μ chains in addition to $s\mu$, as described (Raff *et al.*, 1976), aliquots of cells ($1–5 \times 10^5$) were next cytocentrifuged onto microscope slides, fixed in pre-cooled 5% acetic acid in ethanol (4°, 10 min) and washed by three immersions in phosphate-buffered saline (PBS; room temperature, 15 min). After removing excess PBS, each cytospot was covered with 10 μl anti-μ antibody conjugated with rhodamine isothiocyanate (affinity column purified anti-μ antibody supplied and rhoda-

mine conjugated by Dr M. D. Cooper, Birmingham, AL) incubated at room temperature for 30 min in a humidified chamber and washed in three changes of PBS. Cytospot preparations were mounted in 30% glycerol in PBS, sealed and examined by epifluorescence with 100X oil immersion objective for the presence of individual cells with rhodamine fluorescence alone ($c\mu$ only) and with double labelling by fluorescein plus rhodamine ($s\mu$).

Mitotic arrest

To examine the entry of μ-bearing cells into mitosis, mice were given vincristine sulphate ('Oncovin', Eli Lilly, Basingstoke; 0·1 mg/ml sodium chloride injection) intraperitoneally in a dose of 1 mg per kg body weight at 08.30 to 10.00 hours. After precisely timed intervals of 2 hr and 4 hr, femoral marrow cells from pairs of mice were sampled and analysed for $c\mu$ and $s\mu$, as above.

Irradiation

Mice were given a single whole body X-irradiation of 150 rads (50±4 rads/min, 300 kV, 5 mA; filters, copper 1·5 mm, aluminium, 0·5 mm) and maintained under conventional conditions with addition of Neomycin to the drinking water. Femoral marrow cells from pairs of mice were pooled at 3, 4, 5, 6, 7 and 10 days after irradiation and labelled for $s\mu$ and $c\mu$.

Peanut agglutinin binding

To examine the binding of PNA to $c\mu^+s\mu^-$ cells and $s\mu^+$ cells, aliquots of $2{\cdot}5\times10^6$ washed cells from normal marrow, regenerating marrow and foetal liver were exposed to 20 μl PNA-FITC (L'Industrie Biologique Française, Paris, Lot H536; 2 mg/ml in PBS) (1:10) for 30 min on ice, washed twice through FCS and then either exposed to anti-μ rhodamine in cell suspensions (30 min on ice) to label $s\mu$, or cytocentrifuged and exposed to anti-μ rhodamine (30 min, room temperature) after fixation with cold 5% acetic acid in ethanol to label all μ chain-bearing cells ($c\mu+s\mu$), as above. Cells were examined by epifluorescence for fluorescein labelling, indicating PNA binding, with or without simultaneous rhodamine labelling for either $s\mu$ or $c\mu+s\mu$, respectively.

Cell analysis

In every cell preparation, at least 1000 nucleated cells were examined in each of three ways: by phase contrast microscopy and epifluorescence excitation for fluorescein and rhodamine labelling, respectively. Metaphase figures were scored and the mean diameter of each cell was measured with an ocular micrometer scale. The size distribution profile and incidence of total μ^+ cells, $c\mu^+s\mu^-$ cells, $s\mu^+$ cells and $c\mu^+s\mu^-$ cells in metaphase were calculated.

RESULTS

Cells containing cytoplasmic μ chains in the absence of detectable surface membrane μ chains were readily identified and distinguished from $s\mu^+$ cells by double immunofluorescence labelling. In the marrow of 7- to 9-week-old CBA mice the incidence of $c\mu^+s\mu^-$ pre-B cells, detected under the prevailing technical conditions, was 5·5±0·5% and that of $s\mu^+$ B lymphocytes, 8·2±0·6% (Table 1).

Table 1. Incidence of cells bearing cytoplasmic and surface μ chains in postnatal bone marrow and foetal liver

Tissue	Age	Incidence of μ^+ cells (%)		
		Cytoplasmic μ	Surface μ	Total μ
Bone marrow	7 weeks	4·3	9·0	13·3
	8 weeks	5·7	7·1	12·8
	9 weeks	6·4	8·4	14·8
Foetal liver	19 days	10·2	4·5	14·7

Size distribution profile of pre-B cells in normal marrow

Direct measurements of individual pre-B cells and B lymphocytes in cytocentrifuged cell preparations revealed characteristic population size profiles (Fig. 1). Cells bearing $s\mu$ were all small lymphocytes, ranging from 5 to 10 μm in diameter, with a single sharp distribution curve, peaking at 7·5 μm. In contrast, $c\mu^+s\mu^-$ cells showed a broad size distribution, ranging widely in size from 7 to 15 μm in diameter, and tending to give a biphasic curve.

Apart from the presence of μ chains, indicating their B lymphocyte lineage, the largest $c\mu^+s\mu^-$ cells appeared only as large undifferentiated blasts with a nucleus of circular, indented or irregular outline and copious cytoplasm. Intermediate size $c\mu^+s\mu^-$ cells showed the morphology of large lymphoid or transitional cells, having a relatively small cytoplasmic volume and a circular or slightly indented nucleus with fine chromatin pattern. The small $c\mu^+s\mu^-$ cells (<10 μm), however, were typical small lymphocytes with a coarse pachychromatic nucleus and high ratio of

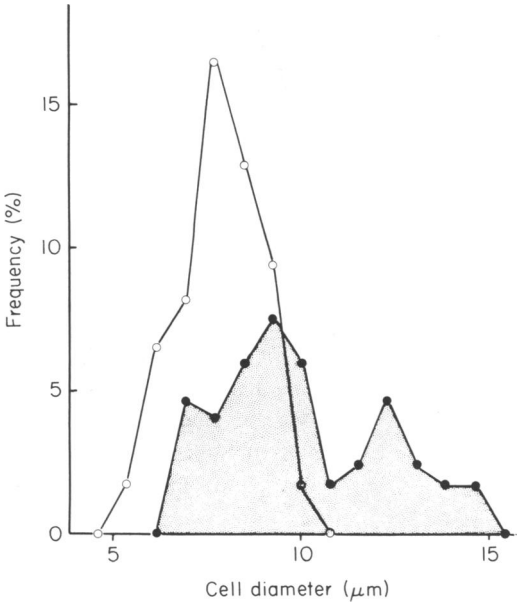

Figure 1. Size distribution of cells bearing (●) cytoplasmic and (○) surface μ chains in the bone marrow of normal mice.

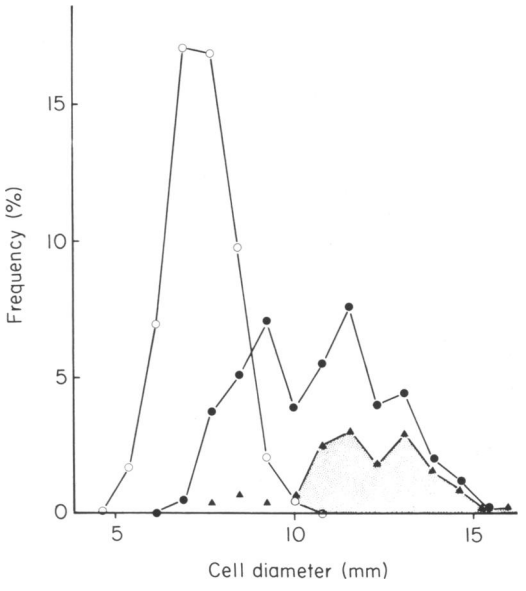

Figure 2. Size distribution of cells bearing (●) cytoplasmic and (○) surface μ chains and of (▲) cytoplasmic μ chain-bearing cells in metaphase in mouse bone marrow, 4 hr after administration of vincristine sulphate.

nucleus to cytoplasm. On morphological grounds alone, these cells would be indistinguishable from $s\mu^+$ cells.

Size distribution profile of proliferating pre-B cells

In normal marrow large $c\mu^+s\mu^-$ cells were occasionally observed by nuclear morphology to be in mitosis. To examine more fully the size characteristics of this cycling population, vincristine sulphate was administered to block cells in metaphase (Wright & Appleton, 1980). Four hours later, arrested metaphases in $c\mu^+s\mu^-$ cells were readily distinguishable under phase contrast by a characteristic dense and irregular chromosomal mass. Virtually all the mitoses were among the large $c\mu^+s\mu^-$ cells, forming a well-defined and broad cell size distribution profile ranging from 10 to 15 μm in diameter in cytospots (Fig. 2). Within the large $c\mu^+s\mu^-$ cell population (>10 μm) the proportion of total $c\mu^+s\mu^-$ cells of each size category arrested in metaphase increased progressively throughout the size range, almost all the largest cells (>13 μm) having entered mitosis within 4 hr after vincristine administration. At the same time, the size distribution profile of the total $c\mu^+s\mu^-$ cell population showed a shift to the right, with a relative decrease in small $c\mu^+s\mu^-$ cells and increase in large $c\mu^+s\mu^-$ cells relative to normal mice not given vincristine (compare Figs 1 and 2).

The rate of entry of $c\mu^+s\mu^-$ cells into mitosis was remarkably high. In one pair of mice, 2 hr after vincristine, 12·9% of all $c\mu^+s\mu^-$ cells were in metaphase. Two hours later in two further pairs of mice the values had risen to 27·2% and 39·7%, respectively, indicating a rapid turnover of the cycling $c\mu^+s\mu^-$ cells.

Thus, the $c\mu^+s\mu^-$ pre-B cells comprise two subsets, dividing and resting, distinguishable on the basis of their size distribution profile. The large $c\mu^+s\mu^-$ pre-B cells (>10) are entirely proliferating. The largest cells of all are late in the cell cycle, within 4 hr of entering mitoses. While other large $c\mu^+s\mu^-$ cells do not all reach metaphase within 4 hr, their proportions relative to those arrested in metaphase (Fig. 2), coupled with the evidence for a short total cell cycle time, are consistent with all these cells being at earlier stages of the cell cycle. In contrast, small $c\mu^+s\mu^-$ cells (<10 μm), coinciding in size distribution and morphology with $s\mu^+$ small lymphocytes, rarely if ever enter mitosis. The possiblity is not excluded that a few small $c\mu^+s\mu^-$ cells, somewhat less than 10 μm in diameter, may be G_1 cells which might subsequently enlarge during cell cycle to exceed 10 μm in diameter by the

time they enter mitosis. At least the large majority of the numerous small $c\mu^+s\mu^-$ cells encompassed by the size distribution curve below 10 μm in diameter, however, appear to be non-dividing cells.

Pre-B cell subsets in regenerating bone marrow

The size distribution profile of $c\mu^+s\mu^-$ cells in normal mice (Fig. 1) represents the balance achieved between the cycling and non-dividing pre-B cell subsets and B lymphocytes in the steady state *in vivo*, cell production balancing cell loss. To examine the relationships between these populations more fully their size distribution profiles were examined sequentially in a wave of B lymphocyte genesis during post-irradiation regeneration.

Following their initial depeletion after 150 rads whole body X-irradiation, $c\mu^+s\mu^-$ cells were detected in small numbers at 3–4 days, increased rapidly by 6 days and thereafter continued markedly to exceed normal incidences at 7 and 10 days (Table 2). At 6 days $s\mu^+$ cells were infrequent, but thereafter their incidence increased progressively to high normal values by 10 days.

Table 2. Incidence of μ-bearing cells in bone marrow after 150 rads whole body X-irradiation

Days after irradiation	Incidence of μ^+ cells (%)		
	Cytoplasmic μ	Surface μ	Total μ
4	1·0	2·7	3·7
5	3·4	7·4	10·8
6	14·0	1·4	15·4
7	12·3	3·6	15·9
10	11·7	7·7	19·4

The first $c\mu^+s\mu^-$ cells to reappear at 3 days (data not shown) and 4 days (Fig. 3) were exclusively large, putatively cycling cells, ranging in diameter from 10 to 15 μm. Some small $c\mu^+s\mu^-$ cells appeared at 5 days. Subsequently, as the number of $c\mu^+s\mu^-$ cells increased, their size distribution profile shifted progressively to the left, so that by 7–10 days a major portion of the curve corresponded to the subset of small non-dividing $c\mu^+s\mu^-$ cells (Fig. 3). In contrast, the size distribution profile of $s\mu^+$ cells remained essentially constant in shape.

Size distribution profile of $c\mu^+s\mu^-$ and $s\mu^+$ cells in foetal liver

In 19 day foetal liver, the $c\mu^+s\mu^-$ and $s\mu^+$ cells showed the same size ranges as in adult bone marrow (Fig. 4). The shape of the size distribution profile of $s\mu^+$ B lymphocytes was essentially identical to that in the marrow. $C\mu^+s\mu^-$ cells overlapped considerably the size distribution of $s\mu^+$ small lymphocytes and tended to show a bimodal distribution including many large blasts, generally resembling the marrow (Figs 1, 4). In incidence, relative to $s\mu^+$ cells (Table 1), and size distribution profile, however, the $c\mu^+s\mu^-$ cells in 19 day foetal liver most closely resembled those of regenerating marrow, 6–7 days after 150 rads X-irradiation.

PNA binding by B lineage cells

In normal bone marrow certain cells bound PNA-FITC strongly, sometimes distributed uniformly around the cell circumference but often concentrated either in a diffuse cap or on a localized cell protrusion or uropod.

By double immunofluorescence labelling, combining PNA-FITC and anti-μ rhodamine binding in cell suspensions, the $s\mu^+$ cells were predominately (90%) PNA negative (Table 3). In contrast, when total μ-bearing cells ($c\mu+s\mu$) were labelled by exposing fixed cells to anti-μ rhodamine after PNA-FITC binding in cell suspension the μ-bearing cells included many PNA-binding cells (Table 3). By subtracting the incidence of $s\mu^+$ cells it may thus be concluded that a large majority of $c\mu^+s\mu^-$ cells showed PNA binding under the experimental conditions.

Regenerating $c\mu^+s\mu^-$ cells also bound PNA (Table 3). At 7 days after 150 rads X-irradiation the total μ-bearing cells ($c\mu+s\mu$) showed a considerable enrichment of PNA binding cells, suggesting that every $c\mu^+s\mu^-$ cell bound detectable PNA, the small number of μ^+ cells not binding PNA representing the incidence of $s\mu^+$ cells.

In one experiment, μ-bearing cells also bound PNA in 19 day foetal liver (Table 3). The labelling tended to be weaker than in the bone marrow, however. Some, but not necessarily all, $c\mu^+s\mu^-$ cells appeared to bind detectable PNA under the experimental conditions.

In contrast to the cytoplasmic μ-bearing cells, only a minority (11–15%) of the completely μ-negative cells in normal marrow, regenerating marrow or foetal liver showed PNA binding (Table 3), and this was often of relatively low intensity.

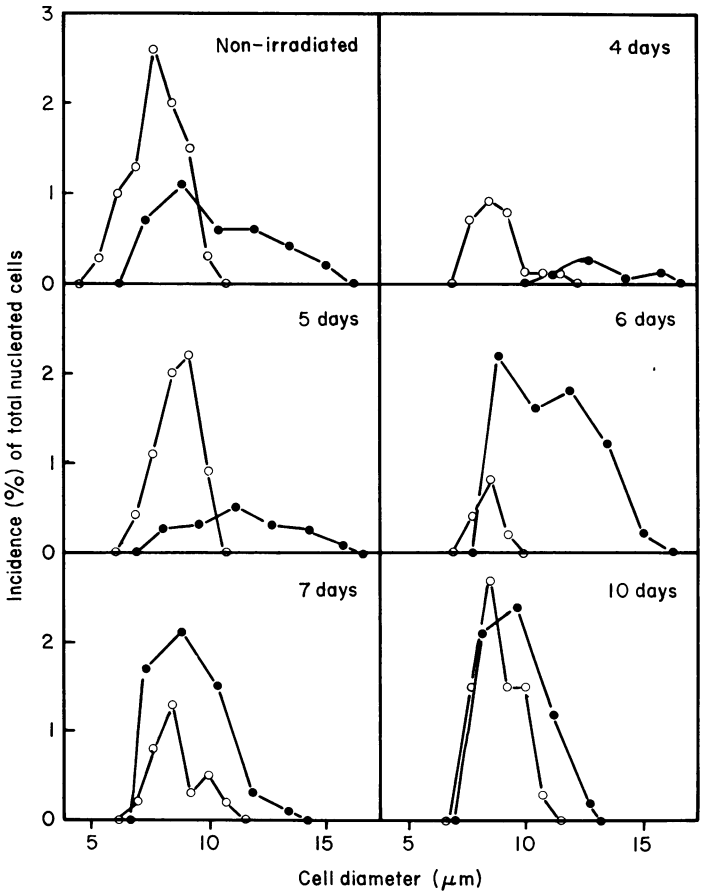

Figure 3. Size distribution of bone marrow cells bearing (●) cytoplasmic and (○) surface μ chains at various times after sublethal whole body irradiation (150 rads).

DISCUSSION

The present results provide a series of size distribution profiles for $c\mu^+s\mu^-$ pre-B cells in normal and developing bone marrow; they define cycling and non-cycling subsets and permit a correlation of immunofluorescence data with other marrow lymphocyte studies and pre-B cell assays in formulating a model of B lymphocyte genesis.

Many of the $c\mu^+s\mu^-$ pre-B cells in bone marrow are actually small cells coinciding in size distribution and morphology with $s\mu^+$ B small lymphocytes (Owen *et al.*, 1977a; Landreth *et al.*, 1981). Of the larger $c\mu^+s\mu^-$ cells, some are morphologically large lymphoid (transitional) cells, as previously described (Yoshida & Osmond, 1971; Rosse, 1971; Owen *et al.*, 1977a), while the largest are blasts that would not otherwise be recognized as of lymphoid lineage (Pearl *et al.*, 1978). The overall range in size of $c\mu^+s\mu^-$ cells is consistent with other reports (Owen *et al.*, 1977a; Landreth *et al.*, 1981). The bimodal size profile of $c\mu^+s\mu^-$ cells in normal marrow as well as the size distributions of cells accumulating in metaphase arrest and in regenerating marrow all define a subpopulation of cycling pre-B cells. The size distribution profiles of non-cycling and cycling subsets of $c\mu^+s\mu^-$ cells correspond with those established for small lymphocytes and large lymphoid cells, respectively, based on radioautographic analyses of DNA-synthesizing cells in conventionally stained cytocentrifuged marrow preparations (Yang *et al.*,

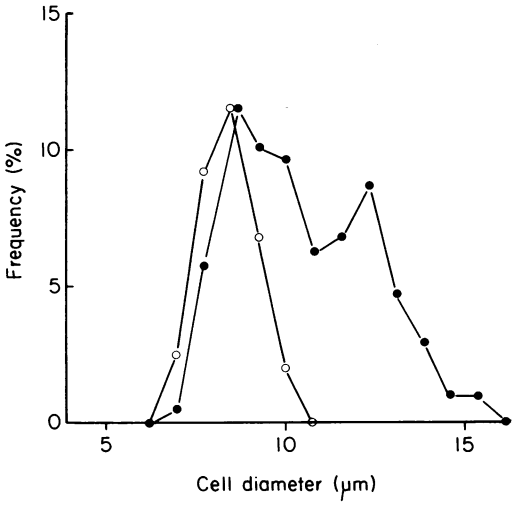

Figure 4. Size distribution of cells bearing (●) cytoplasmic and (○) surface μ chains in 19 day foetal mouse liver.

1978). Small $c\mu^+s\mu^-$ cells correspond with the $s\mu^-$ small lymphocytes previously detected by double radioautographic labelling in stained preparations, mainly the most newly formed, post-mitotic small lymphocytes (Osmond & Nossal, 1974).

Combining present and previous studies, the place of the $c\mu^+s\mu^-$ cells in marrow B cell genesis appears to be as shown in Fig. 5. A subset of large $c\mu^+s\mu^-$ cells, themselves formed from unknown precursors undergoing μ heavy chain gene rearrangement and expression, divide to give rise to a population of small lymphocytes which at first continue to exhibit $c\mu$ alone and subsequently express $s\mu$, thus becoming identifiable as B lymphocytes with no further division or change of cell size. Consistent with this model is the observed shift in size distribution after vincristine; the relative number of small $c\mu^+s\mu^-$ cells declines due to their continuing post-mitotic maturation into $s\mu^+$ small lymphocytes while not being replenished from the large dividing $c\mu^+s\mu^-$ cells, arrested in metaphase. The progressive increase in metaphase index with cell size among the large $c\mu^+s\mu^-$ cells resembles a comparable increase in incidence of DNA-synthesizing cells with size of morphologically defined large lymphoid cells in stained radioautographs (Miller & Osmond, 1973). Thus, the largest cells in these size distribution profiles are predominantly late in cell cycle. The present findings and proposed cell sequence reconcile previously disparate reports of the extent to which pre-B cells as a whole are cycling and the place of cell division in the transition of pre-B cells to B lymphocytes (Osmond & Nossal, 1974; Owen *et al.*, 1977a; Pearl *et al.*, 1978; Lau *et al.*, 1979; Rusthoven & Phillips, 1980; Landreth *et al.*, 1981; Paige *et al.*, 1981).

The size distribution profiles of $c\mu^+s\mu^-$ and $s\mu^+$ cells are compatible with sedimentation velocity analyses both of $c\mu^+s\mu^-$ cells and of functional assays of pre-B cells, respectively. From the data of Owen *et al.* (1977a) sedimentation velocity profiles for $s\mu^+$ and $c\mu^+s\mu^-$ murine marrow cells can be derived (Fig. 6). The profile of $s\mu^+$ small B lymphocytes and the relative position and shape of the profile of $c\mu^+s\mu^-$ pre-B cells resemble closely the present curves derived from direct measurements of cytocentrifuged cells. In turn, the profiles of size distribution and sedimentation velocity of $c\mu^+s\mu^-$ cells resemble the sedimentation velocity profiles of pre-B cells defined by

Table 3. Binding of peanut agglutinin by μ-bearing and μ-negative cells in normal bone marrow, regenerating bone marrow and foetal liver

		Incidence of cells (%)					
		$s\mu^+$		$(c\mu+s\mu)$		Others	
Tissue		PNA$^+$	PNA$^-$	PNA$^+$	PNA$^-$	PNA$^+$	PNA$^-$
Bone marrow							
Normal		0·6	5·6			15·8	78·0
				6·4	10·8	9·6	73·2
Regenerating*	6 days			7·3	6·7	8·7	77·3
	7 days			15·3	1·5	11·3	71·9
Foetal liver	19 days			5	16	8	71

* After 150 rads whole body X-irradiation.

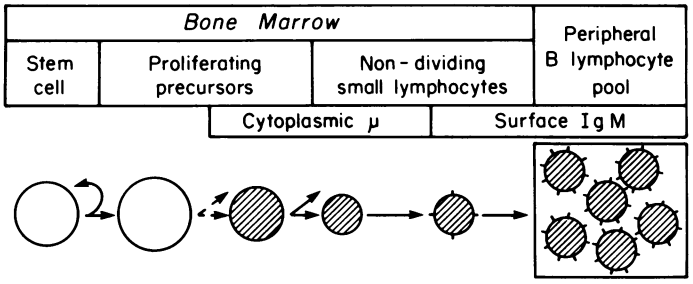

Figure 5. Scheme of cytoplasmic and surface μ-bearing cells in B lymphocyte genesis in bone marrow.

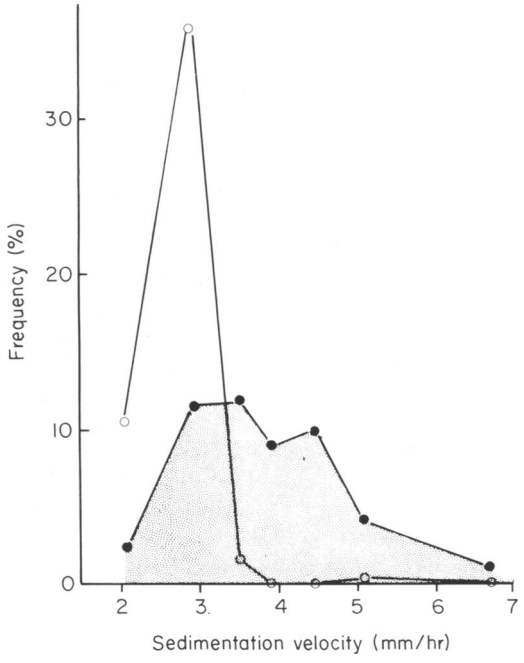

Figure 6. Sedimentation velocity distribution of cells bearing (●) cytoplasmic and (○) surface μ chains in the bone marrow of normal mice (derived from data of Owen *et al.*, 1977a).

functional assays (Lafleur *et al.*, 1972; Lau *et al.*, 1979). Mature, immediately reactive, B lymphocyte populations, giving a peak response *in vitro* to LPS in 4–5 days, present a single sharp profile of small cells. Cells reaching peak responses at longer intervals (7–8 days), however, show an additional broader profile of more rapidly sedementing, larger cells, deleted by hydroxyurea (Lau *et al.*, 1979; Rusthoven & Phillips, 1980; Freitas *et al.*, 1982). These are interpreted as being cycling pre-B cells which develop into LPS-responsive B lymphocytes during a maturation period *in vitro* of 3–4 days. The most rapidly sedimenting cell fractions have been selected experimentally as sources of pre-B cells (Lau *et al.*, 1979; Rusthoven & Phillips, 1980). It now appears that these would represent only a portion of the pre-B cell pool, which also may be partially synchronized late in cell cycle. The extent to which functionally defined pre-B cells are identical with $c\mu^+s\mu^-$ cells is unknown. Using cell size distribution as a common denominator, however, the present studies show a consistency between cytological and functional assays, and suggest that general comparisons between microscopic and sedimentation analyses of marrow lymphoid cells are justified.

The size distribution profiles of $c\mu^+s\mu^-$ cells in the normal steady state of cell renewal represent a summation of all cells with this μ chain status, from which it is not possible to distinguish individual generations or stages in development. To test some of the cell interrelationships implicit in the model (Fig. 5) and analyse the sequence of B lineage cells in relation to time the steady state was perturbed by sublethal X-irradiation and the marrow was examined during regeneration. Whole body X-irradiation (150 rads) produces severe depletion of the marrow lymphocyte population, followed by a rapid recovery and pronounced overshoot before returning to normal cell numbers (Osmond *et al.*, 1966; Osmond & Evoy, 1977). Small lymphocytes during this process show a sequential appearance of $s\mu^-$, weak $s\mu^+$ and strong $s\mu^+$ forms (Osmond & Evoy, 1977). The progressive shift in shape and area of the profiles of $c\mu^+s\mu^-$ and $s\mu^+$ cells with time now reveals an orderly wave of pre-B and B cell genesis, providing additional evidence for the developmental sequence; large cycling $c\mu^+s\mu^-$ small $c\mu^+s\mu^-$, small $s\mu^+$. This accords with observa-

tions based on post-cyclophosphamide (Burrows *et al.*, 1978) and ontogenic (Owen *et al.*, 1977a,b; Cooper & Lawton, 1979) sequences.

The number of times each $c\mu^+s\mu^-$ pre-B cell divides will determine the degree of expansion of B cell clones following the determination of their μ chain specificity. This may have implications both for the size of individual clones and the ultimate diversity of the B cell repertoire. The number of pre-B cell generations is unknown. The present metaphase arrest data indicate that the large $c\mu^+s\mu^-$ cells are cycling rapidly. The post-irradiation regenerative sequence suggests a lag of several (3–4) days between the first cycling $c\mu^+s\mu^-$ cells and B lymphocytes, time for several (4–8) divisions and thus a considerable expansion of each clone. Although their size distributions have not been fully analysed, foetal and post-cyclophosphamide studies show a similar time lag between pre-B and B cell development, as do functional assays *in vitro* (Melchers, 1977; Owen *et al.*, 1977b; Burrows *et al.*, 1978; Cooper & Lawton, 1979; Lau *et al.*, 1979), consistent with several pre-B cell generations. In contrast, from radioautographic data it has been suggested that $c\mu^+s\mu^-$ cells represent only the terminal mitosis of the B lineage (Landreth *et al.*, 1981). It cannot be excluded that the number of generations occurring in a progressive wave of B cell genesis may differ from those occurring in steady state equilibrium. Further studies are needed to test this point directly.

Of practical interest is the finding of a considerably increased incidence of large $c\mu^+s\mu^-$ cells with only a few $s\mu^+$ cells at a certain well defined stage (6–7 days) of post-irradiation (150 rads) regeneration. Such biologically enriched marrow provides a convenient source of cycling pre-B cells for experimental purposes (Osmond, submitted).

Foetal liver generally resembles adult bone marrow in the size distribution of $s\mu^+$ and $c\mu^+s\mu^-$ cells. The detailed size profile of $c\mu^+s\mu^-$ cells in 19 day foetal liver most closely resembles that of a particular phase of regenerating marrow, however, rather than normal marrow. This serves to emphasise that B lymphocyte genesis in mammalian foetal liver represents a limited number of generations producing a transient wave rather than a steady state of B cell production (Melchers, 1977). The composition of various B lineage cells may thus change markedly with foetal age, in contrast with the relatively stable populations maintained in dynamic equilibrium in post natal marrow.

PNA is a lectin binding specifically to glycoproteins with terminal galactose residues. Such binding sites are exposed on certain surface molecules of immature thymocytes (Reisner *et al.*, 1976), germinal centre cells (Rose *et al.*, 1980) and immature haemopoietic cells (Newman & Boss, 1980; Nicola *et al.*, 1980), but not peripheral lymphocytes on which the sites presumably are masked by sialic acid. Immunofluorescence double labelling now demonstrates that PNA binding normally characterises a small minority of $s\mu^+$ small lymphocytes and most of the $c\mu^+s\mu^-$ pre-B cells not only in normal marrow (Newman & Boss, 1980) but also in regenerating marrow and foetal liver. Thus, high intensity PNA binding consistently characterizes $c\mu^+s\mu^-$ cells under a variety of conditions, although it does not apparently discriminate between cycling and non cycling subsets or other developmental stages of pre-B cells. Because PNA binding to non lymphoid cells in the marrow is generally weak and of low incidence, however, this property is potentially useful as a surface marker to separate viable $c\mu^+s\mu^-$ cells from the bone marrow (Osmond, submitted).

ACKNOWLEDGMENTS

The generous gift of rhodamine conjugated, affinity column purified anti-μ chain antibody from Dr M. D. Cooper is gratefully acknowledged. The work was supported by the Medical Research Councils of the United Kingdom and Canada.

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