

MATURATION OF BONE MARROW LYMPHOCYTES  
II. Development of Fc and Complement Receptors and Surface  
Immunoglobulin Studied by Rosetting and Radioautography\*

BY W. C. YANG, S. C. MILLER AND D. G. OSMOND

*From the Department of Anatomy, McGill University, Montreal, Quebec, Canada*

Many small lymphocytes in mouse bone marrow display surface IgM molecules and receptors for Fc and complement (1-3). Cyto centrifuge rosetting studies demonstrate well-defined Fc and complement receptors on certain marrow small lymphocytes, but the incidence of these cells relative to IgM-bearing lymphocytes is lower than in the peripheral lymphoid tissues (1, 2). These findings suggest that the Fc and complement receptor-bearing cells may form part of the population of young, B small lymphocytes<sup>1</sup> produced continuously in the marrow (4). If so, the timing and sequence of receptor development on small marrow lymphocytes would define stages in the maturation of primary B lymphocytes, allowing a correlation to be made with the onset of functional responsiveness. In addition, in the light of the kinetic and functional heterogeneity of marrow lymphocytes (4, 5), some Fc and complement receptor-bearing cells may represent lymphocyte subtypes other than immature B lymphocytes.

A majority of marrow small lymphocytes are newly formed cells, renewed rapidly by local cell production (4-7). After a postmitotic delay, many of these cells develop readily detectable surface IgM molecules increasing in apparent density with time (8). Other young, marrow small lymphocytes, so-called null cells, lack both surface IgM and Thy.-1 antigen (2-5, 8). It remains to be verified whether all or only some of the null marrow lymphocytes actually belong to the B lineage, destined to develop surface IgM and other B-lymphocyte receptors as they mature. Newly formed marrow small lymphocytes in general migrate rapidly to the spleen and lymph nodes (9, 10) where their maturation may be completed (4, 11). In contrast, a minority of small lymphocytes in the marrow are long-lived slowly renewed cells, mainly recirculating immigrants, which increase in numbers throughout postnatal life (4-6). Their receptor subtypes are unknown. Numerically, many of the observed Fc and complement receptor-bearing lymphocytes in the marrow could be long-lived recirculating B lymphocytes, rather than newly formed indigenous cells.

Double-labeling experiments have now been performed to relate the surface receptor status of individual marrow lymphocytes to cell age and renewal, combining surface rosetting, and radioautographic DNA-labeling techniques. The aims of the studies were, (a) to compare the development of Fc and complement receptors on newly formed marrow small lymphocytes with that of surface IgM and to examine the receptor status of proliferating large lymphoid cells, as shown by the rosetting

\*Supported by the Medical Research Council of Canada.

<sup>1</sup> *Abbreviations used in this paper:* B lymphocyte, lymphocyte bearing readily detectable surface immunoglobulin, potential antibody-forming cell precursor; CR, complement receptors; FcR, Fc receptors; FCS, fetal calf serum; MEM, minimum essential medium; SRBC, sheep erythrocytes.

properties of cells labeled during a continuous infusion of [ $^3\text{H}$ ]thymidine in vivo, (b) to characterize the surface receptors on long-lived marrow lymphocytes prelabeled by [ $^3\text{H}$ ]thymidine in vivo 3–5 wk previously, (c) to measure the degree to which marrow small lymphocytes may simultaneously express Fc or complement receptors as well as surface IgM, using double-surface labeling by rosetting and radioautographic anti-globulin-binding, respectively, (d) to determine whether young [ $^3\text{H}$ ]thymidine-labeled marrow lymphocytes can continue to develop surface IgM, Fc, and complement receptors after homing into the spleen of syngeneic recipients. The results contribute to a model of primary B lymphocyte maturation, define some surface properties of presumptive marrow lymphocyte progenitors and recirculating cells, and provide evidence for the production in mouse bone marrow of some null lymphocytes which may not be destined to become B cells.

### Materials and Methods

*Animals.* Male, C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) were used when 9–10-wk old.

*[ $^3\text{H}$ ]Thymidine Labeling In Vivo.* To label newly formed cells, groups of four to five mice were injected intraperitoneally with 25  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (sp act 6.7 Ci/mM; New England Nuclear Corp., Boston, Mass.) followed immediately by a subcutaneous infusion of [ $^3\text{H}$ ]thymidine (2  $\mu\text{Ci}$  in 0.04 ml sterile water/g body wt) as described (6). Mice were killed at eight intervals from 1 h to 4 days. To label a fraction of long-lived cells, groups of five mice were given a daily intraperitoneal injection of 25  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine for 14 days and killed after a further lag of 3 wk to allow the disappearance of labeled rapidly renewed cells.

*Cells.* Bone marrow cells from groups of mice were flushed from the femoral diaphysis with cold Hepes-buffered Eagle's minimal essential medium (MEM) and 10% fetal calf serum (FCS), pooled, and washed, as described (1).

*Rosetting and Cytocentrifugation.* Fc and complement receptors and surface IgM were detected on marrow lymphocytes by rosetting techniques, detailed elsewhere (1). Briefly, marrow cell suspensions were mixed (1:30) either with washed sheep erythrocytes (SRBC) coated with one of the following: (a) mouse anti-SRBC antibody (for Fc receptors), (b) rabbit anti-SRBC stroma antibody (Cordis Laboratories Inc., Miami, Fla.) plus normal mouse serum (for complement receptors), (3) goat anti-mouse IgM antibody (Meloy Laboratories Inc., Springfield, Va.) (for surface IgM), or with uncoated SRBC (controls). The cell mixtures were incubated in centrifuged pellets for 30 min at either 37°C (Fc and complement receptors), or 0°C (IgM), resuspended, and cytocentrifuged. Erythrocytes attached to individual small lymphocytes were counted, four or more erythrocytes being scored as rosettes. The incidence of any rosettes in control preparations (usually four to five erythrocytes) was subtracted from the values for rosettes of similar size in experimental preparations, to give the specific rosette incidence and size distribution.

*Radioautographic Analysis.* Cytocentrifuged rosette preparations were methanol-fixed, coated with NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.), exposed at 4°C for 28 days and stained through the fixed emulsion with MacNeal's tetrachrome. After [ $^3\text{H}$ ]thymidine infusion, marrow small lymphocytes, identified as described elsewhere (1–8), were examined to record radioautographic grains over the nucleus and the number of surface-bound erythrocytes. Positive [ $^3\text{H}$ ]thymidine labeling was set at four or more grains per cell, background counts over erythrocytes being negligible. Most labeled small lymphocytes had grain counts considerably higher than the threshold value. After control rosette correction, the [ $^3\text{H}$ ]thymidine labeling index could thus be derived separately for the total marrow small lymphocyte population, and for the receptor-bearing (rosetting) and receptor-negative (nonrosetting) small lymphocytes, respectively. At each [ $^3\text{H}$ ]thymidine infusion interval, 1,700–4,200 (mean 2,300) marrow small lymphocytes were examined for the total labeling index, the labeling indices of receptor-bearing cells being based on counts of ~800 (650–1,000) small lymphocytes and 250 (150–500) rosettes. In the long-lived small lymphocyte experiments, sets of cytocentrifuge preparations were scanned completely for labeled small lymphocytes and their specific rosette incidence and rosette size distribution determined.

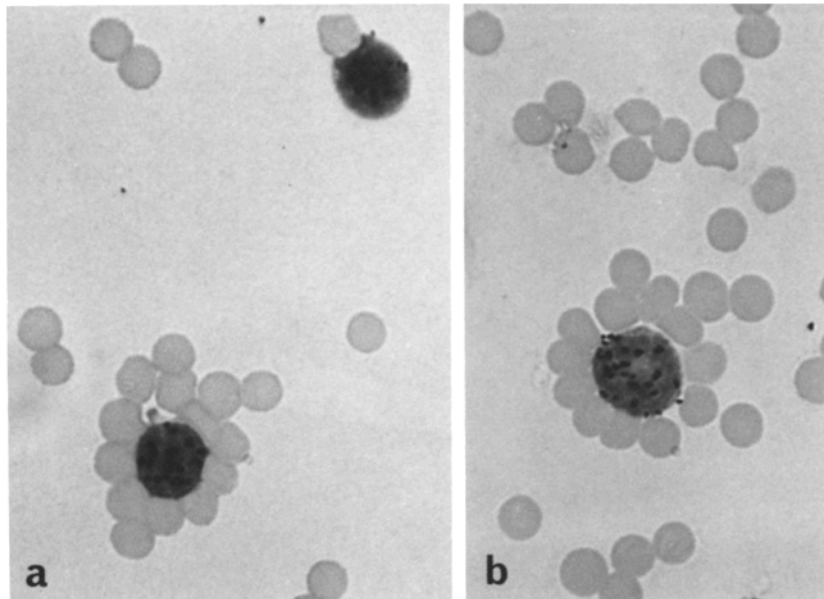


FIG. 1. Small lymphocytes from bone marrow (a) and spleen (b) of mice infused with  $^3\text{H}$ -thymidine showing  $^3\text{H}$ -thymidine labeling of the nucleus and rosettes of adherent erythrocytes indicating surface Fc receptors. ( $\times 1,280$ ).

*Double-Surface Labeling.* Pooled femoral marrow cells from groups of 5 mice were mixed in aliquots of  $2 \times 10^6$  nucleated cells in 0.1 ml MEM-10% FCS for 30 min at  $0^\circ\text{C}$  with equal volumes of goat anti-mouse IgM serum (Meloy Laboratories Inc.), previously labeled with  $^{125}\text{I}$ , as described (2). After washing through two discontinuous FCS gradients, the cells were mixed with coated SRBC for Fc and complement receptor rosettes, as described above, cytocentrifuged, and examined in radioautographs. Small lymphocytes were examined for surface IgM ( $\leq 10$  grains) and either Fc or complement receptors ( $\geq 4$  SRBC). The proportion of small lymphocytes expressing one, both or neither surface marker was calculated.

*Homing of Newly Formed Marrow Small Lymphocytes to the Spleen.* Groups of five donor mice were infused subcutaneously with [ $^3\text{H}$ ]thymidine for 24 h, as described above. Doses of  $20 \times 10^6$  nucleated donor marrow cells, suspended in 0.5 ml Hanks balanced salt solution, were injected into the tail veins of groups of 5 recipient mice, killed at 1, 2, and 3 days thereafter. Recipient spleen cells were suspended in MEM-10% FCS, rosetted for surface IgM, Fc, and complement receptors and examined radioautographically, as above.

In each donor marrow, 1,000–2,000 labeled small lymphocytes were examined. Sets of 10 cytocentrifuge preparations of each recipient spleen ( $\approx 2,000$  nucleated cells) were scanned completely for labeled small lymphocytes.

Segments of the spleen from 1 day marrow recipients were also fixed in Bouin's fluid, embedded in Epon (Shell Chemical Co., Division of Shell Oil Co., N.Y.), serially sectioned ( $1 \mu\text{m}$ ), stained with toluidine blue, prepared for radioautography, and exposed for 2 mo. Cells with five or more overlying grains were scored as labeled, background grains being negligible.

## Results

*Development of Fc and Complement Receptors and Surface IgM on Newly formed Marrow Small Lymphocytes.* Young small lymphocytes produced over a 4-day period were identified by radioautographic DNA-labeling during an infusion of [ $^3\text{H}$ ]thymidine, while their surface receptor status was simultaneously revealed by SRBC rosettes, as illustrated in Fig. 1. The contrasting cell markers were readily distinguished from one another, and the morphology of the rosette-forming cells was well preserved.

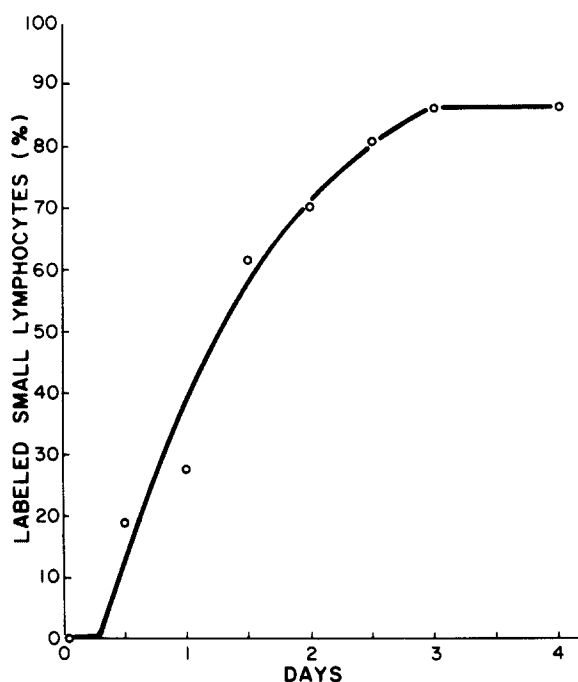


FIG. 2. Percentage of labeled small lymphocytes in the bone marrow during continuous [ $^3\text{H}$ ]-thymidine infusion in vivo.

Throughout the infusion period, the percentage of marrow small lymphocytes forming rosettes for surface IgM ( $39.2 \pm 2.0\%$ ), Fc ( $25.2 \pm 2.2\%$ ), and complement receptors ( $20.2 \pm 1.5\%$ ) remained unchanged and closely comparable with values in a previous nonradioautographic study (1).

Fig. 2 shows the [ $^3\text{H}$ ]thymidine labeling of the total small lymphocyte population, resembling previous findings in conventional smears of mouse marrow (6-8) and demonstrating a rapid replacement of nondividing small lymphocytes by newly formed cells derived from proliferating progenitors (4, 5).

Fig. 3 shows the [ $^3\text{H}$ ]thymidine labeling index determined separately for surface IgM-rosetting and IgM-negative marrow small lymphocytes, respectively. The IgM-negative cells were the first to show labeling, which increased rapidly to 89-95% after 2.5 days. IgM-bearing small lymphocytes showed an initial lag before undergoing a similarly rapid increase in labeling to 74% at 3 days. These cells showed a sequential increase in rosette size with age (Fig. 4). The youngest small lymphocytes formed rosettes of near-threshold size (4-5 SRBC) with only a minimal initial lag in their appearance. The capacity to form larger rosettes developed at progressively later times, a lag of more than a day preceding the detection of any IgM-rosettes  $> 10$  SRBC per small lymphocyte.

The [ $^3\text{H}$ ]thymidine labeling of Fc and complement receptor-bearing small lymphocytes resembled that of IgM-bearing cells (Figs. 5, 6). After a short initial delay, the labeling increased rapidly to 80-83% at 3 days. In each case, receptor-negative small lymphocytes were the first to become labeled (Figs. 5, 6). However, no correlation between cell age and rosette size could be demonstrated. Fig. 7 shows the rosette size distribution of labeled small lymphocytes during [ $^3\text{H}$ ]thymidine infusion. In contrast

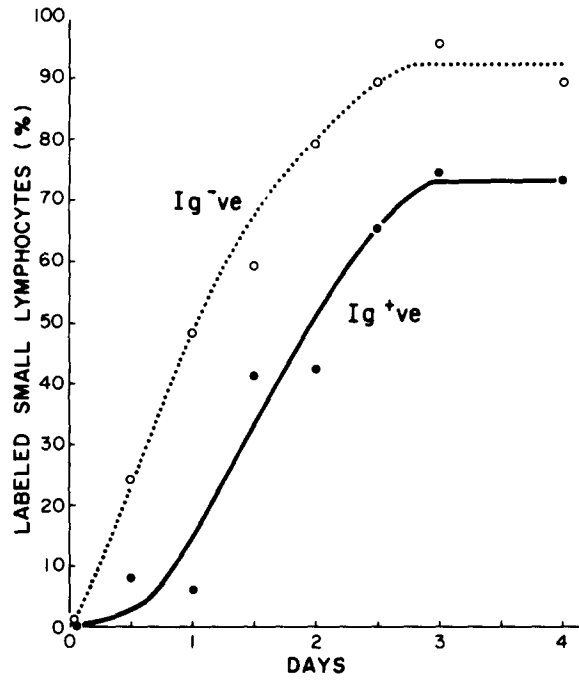


FIG. 3. [<sup>3</sup>H]Thymidine labeling of surface IgM-negative and IgM-bearing small lymphocytes in the bone marrow during continuous [<sup>3</sup>H]thymidine infusion in vivo.

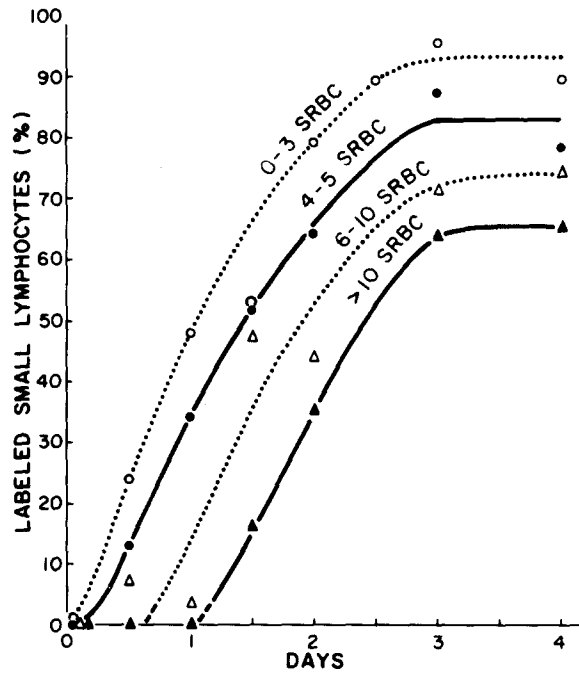


FIG. 4. [<sup>3</sup>H]Thymidine labeling of bone marrow small lymphocytes forming surface IgM rosettes of various sizes (SRBC per lymphocyte) during continuous [<sup>3</sup>H]thymidine infusion in vivo.

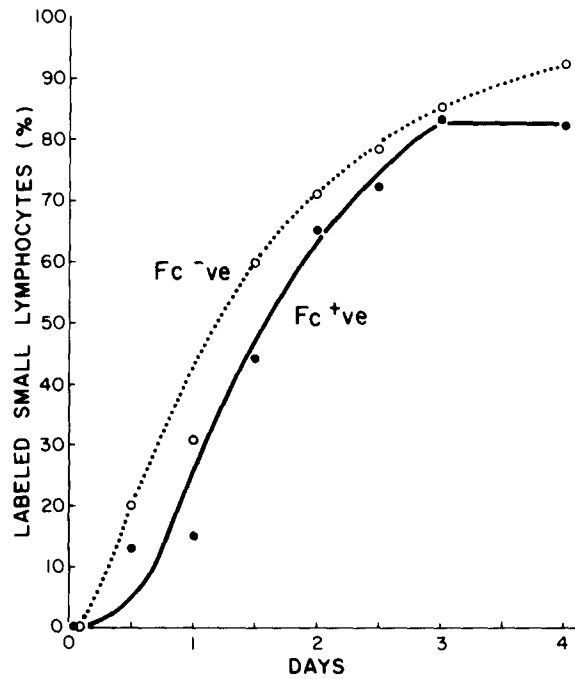


FIG. 5. [<sup>3</sup>H]Thymidine labeling of bone marrow small lymphocytes defined by the presence or absence of surface Fc receptors during continuous [<sup>3</sup>H]thymidine infusion in vivo.

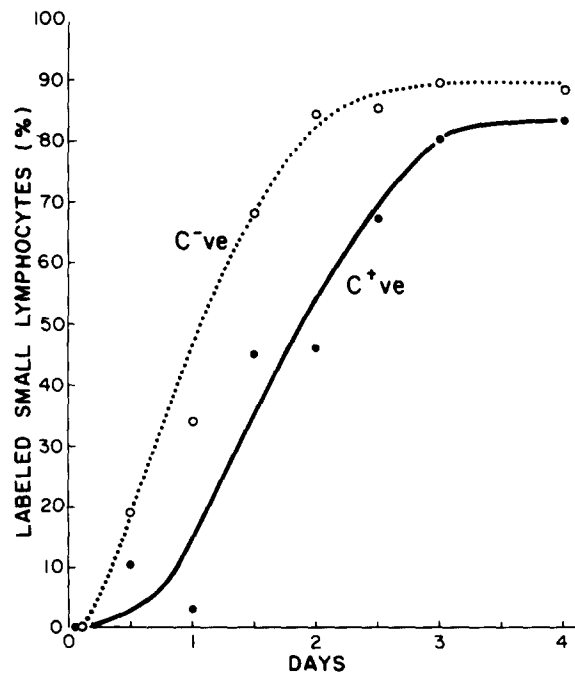


FIG. 6. [<sup>3</sup>H]Thymidine labeling of bone marrow small lymphocytes defined by the presence or absence of surface complement receptors during continuous <sup>3</sup>H-thymidine infusion in vivo.

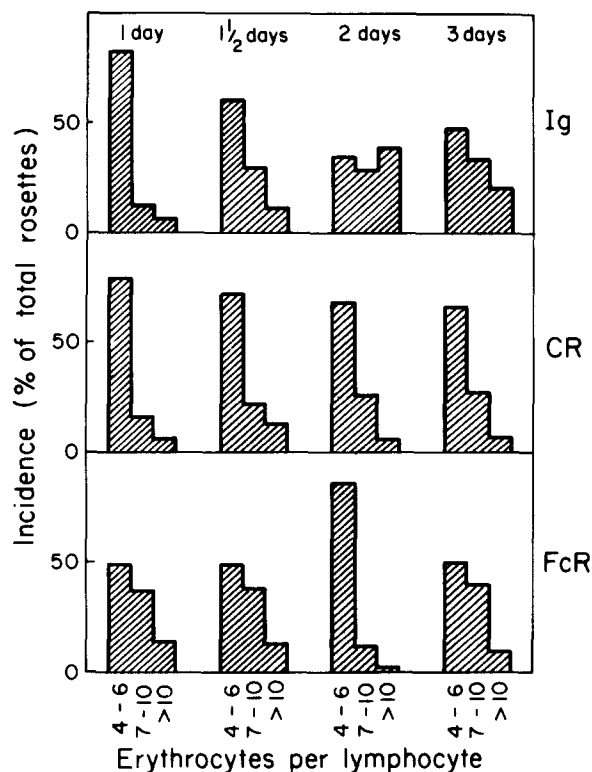


FIG. 7. Size distribution of rosettes for surface IgM (Ig), CR and FcR on [ $^3\text{H}$ ]thymidine-labeled small lymphocytes in the bone marrow during [ $^3\text{H}$ ]thymidine infusion in vivo.

with the IgM-bearing cells, the labeled Fc and complement receptor-bearing small lymphocytes showed no progressive increase in rosette size distribution with increasing labeling duration.

In Fig. 8 the [ $^3\text{H}$ ]thymidine labeling data are expressed semilogarithmically as the disappearance of unlabeled cells from the marrow. The labeling indices for each receptor-bearing and receptor-negative subset of marrow small lymphocytes conformed to exponential curves during the first 3 days of [ $^3\text{H}$ ]thymidine infusion, as demonstrated previously for total and Ig-bearing marrow small lymphocytes (6-8). The one-half-renewal times for the total small lymphocytes and IgM-negative small lymphocytes were 23 and 17 h, respectively. The IgM-, Fc-, and complement receptor-bearing small lymphocytes lagged in their initial appearance behind that of the respective receptor-negative cells by 7, 7, and 9 h; their one-half-renewal times were 32, 24, and 27 h, respectively.

*Fc and Complement Receptors and Surface IgM on Long-Lived Marrow Small Lymphocytes.* Table I presents the incidence and size distribution of rosettes formed by a fraction of the long-lived small lymphocytes in the bone marrow, prelabeled by [ $^3\text{H}$ ]thymidine in vivo 3-5 wk previously. Counts performed by Dr. F. P. H. Chan (McGill University, Montreal, Canada) revealed that many labeled cells had surface IgM, Fc, and complement receptors. In each case, the proportion of rosette-forming cells was actually higher among the long-lived small lymphocytes than the total marrow small lymphocytes, especially in the case of complement-receptor bearing

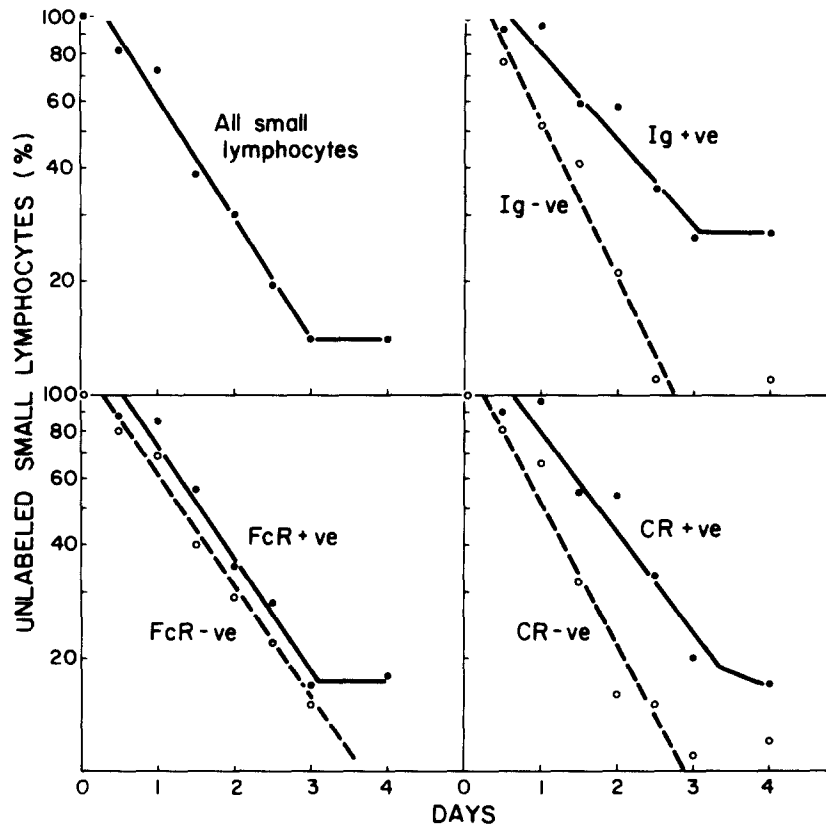


FIG. 8. Disappearance of cells unlabeled by [ $^3\text{H}$ ]thymidine from the bone marrow populations of all small lymphocytes and of small lymphocytes defined by the presence or absence of surface FcR, and CR during continuous [ $^3\text{H}$ ]thymidine infusion in vivo. The regression lines were calculated by the method of least squares.

TABLE I

*Long-Lived Small Lymphocytes\* in Bone Marrow: Incidence and Rosette Size Distribution of Cells Forming Rosettes for Surface IgM, Fc, and Complement Receptors*

Surface marker	Incidence of rosettes (%)‡ formed by:		Size distribution of rosettes (%)§ formed by long-lived small lymphocytes		
	Long-lived small lymphocytes*	Total small lymphocytes	4-6 SRBC	7-9 SRBC	>10 SRBC
IgM	42.8	36.7	35.2	24.2	40.6
FcR	37.0	30.2	57.9	18.1	24.0
CR	37.6	17.1	53.6	26.2	20.2

\* Small lymphocytes prelabeled with [ $^3\text{H}$ ]thymidine 3-5 wk previously.

‡ For each surface marker, 1,500-4,000 total marrow small lymphocytes, including 115-240 labeled long-lived marrow small lymphocytes, were examined in cytocentrifuge rosette preparations using both coated and uncoated (control) SRBC.

§ Adjusted with reference to a standard rosette size distribution profile of total marrow small lymphocytes common to this and the foregoing [ $^3\text{H}$ ]thymidine infusion study; the values may be compared with those of newly formed marrow small lymphocytes in Fig. 7.



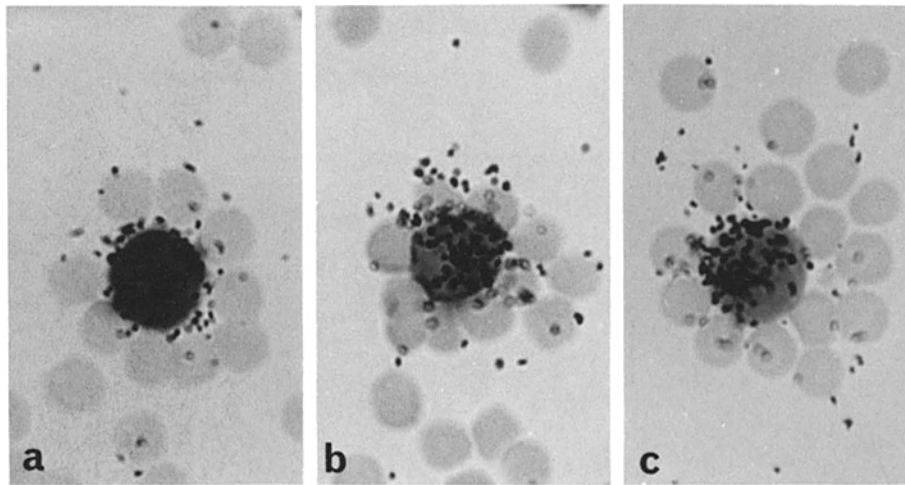


FIG. 9. Small lymphocytes from bone marrow (a, b) and spleen (c) showing surface IgM (radioautographic grains), plus receptors for Fc (a, c) and complement (b) (SRBC rosettes) after sequential exposure to  $^{125}\text{I}$ -anti-IgM antiserum and cyto centrifuge rosetting procedures. ( $\times 1,600$ ).

cells (Table I). Long-lived small lymphocytes bearing receptors for either Fc or complement were almost as numerous as those having surface IgM, unlike the total marrow small lymphocyte population in which complement receptor-bearing cells were less than one-half as numerous as IgM-bearing cells (Table I) (1). The size of the rosettes formed by the long-lived small lymphocytes (Table I) was consistently somewhat greater than those formed by either the total marrow small lymphocytes in the same experiment or the labeled newly formed marrow small lymphocytes during [ $^3\text{H}$ ]thymidine infusion (Fig. 7).

*Double-Surface Labeling of Bone Marrow Small Lymphocytes.* Some IgM-bearing marrow small lymphocytes concomitantly showed Fc or complement receptors when examined by combined  $^{125}\text{I}$ -antiglobulin binding and surface rosetting. Examples are illustrated in Fig. 9. However,  $\approx 55$  and 20% of the Fc and complement receptor-bearing small lymphocytes, respectively, did not show surface IgM, whereas  $\approx 60$ -70% of the IgM-bearing small lymphocytes had no receptors for Fc or complement (Fig. 10).

*Rosette Formation by Large Lymphoid Cells in Bone Marrow.* Measurements of marrow lymphocytes of all sizes in cyto centrifuge rosetting preparations showed that approximately one-quarter (24-27%) of all the rosettes for surface IgM, Fc, and complement receptors were formed by large lymphoid cells ( $>10\ \mu\text{m}$  nuclear diameter) (1), which include the progenitors of small lymphocytes (4, 5). As presented in Table II, the various rosettes were formed by substantial proportions (31-62%) of large lymphoid cells, including cells in DNA synthesis and lymphoid cells in the largest subgroups ( $>13\ \mu\text{m}$  nuclear diameter). Although some rosettes were of borderline size (4-5 SRBC), many were larger ( $\approx 6$  SRBC) (Table II).

*Development of Surface Markers on Marrow Lymphocytes after Homing into the Spleen.* In donor marrow, one-third of the young labeled lymphocytes (0-24-h-old) showed some surface IgM by rosetting, whereas only 10% formed Fc receptor rosettes, and complement receptor rosettes were almost absent (Fig. 11). When recovered from the spleens of normal syngeneic recipients, the proportion of labeled small lymphocytes bearing

## MATURATION OF BONE MARROW LYMPHOCYTES

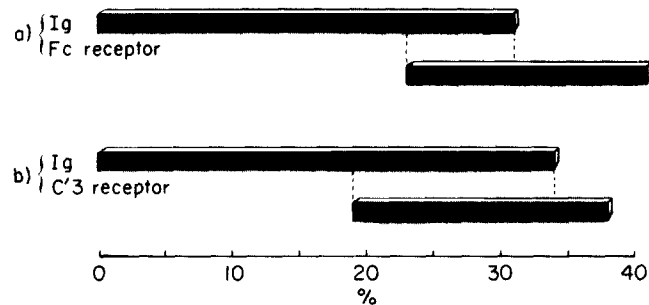


FIG. 10. Double-surface labeling of bone marrow small lymphocytes. Overlap of bone marrow small lymphocyte populations showing the incidence of surface IgM and of receptors for either Fc or complement.

TABLE II  
*Large Lymphoid Cells in Bone Marrow Forming Rosettes for Surface IgM, Fc, and Complement Receptors*

Large lymphoid cells*	Rosette size threshold (SRBC/lymphoid cell)	Proportion of large lymphoid cells forming rosettes for:		
		Surface IgM	FcR	CR
			%	
Total	>4	62	31	54
	>6	54	22	43
DNA-synthesizing cells‡	>4	56	21	46
	>6	42	16	34
Nuclear diameter:				
10-13 $\mu$ m	>4	53	35	59
>13 $\mu$ m	>4	79	23	32

\* Lymphoid cells >10  $\mu$ m nuclear diameter in cytocentrifuge preparations.

‡ Cells labeled in radioautographs 1 h after [<sup>3</sup>H]thymidine injection in vivo: samples of 150-260 total marrow lymphocytes, including 39-50 labeled cells, were measured individually in radioautographic cytocentrifuge preparations, to correlate surface rosette incidence with the size and DNA synthetic phase of marrow large lymphoid cells.

each surface marker increased progressively with time (Fig. 11), IgM-bearing cells increasing most rapidly, followed by Fc receptor-bearing cells. By 2 days, surface IgM and Fc receptors were demonstrable on 72 and 63% of labeled small lymphocytes, respectively, whereas each of the small number of labeled cells detected at 3 days showed surface IgM and Fc receptors. The incidence of complement receptor-bearing cells plateaued at 47-53% at 2-3 days.

The size of the rosettes formed by labeled marrow small lymphocytes increased progressively with time (Fig. 12). Initially, most IgM rosettes were small (4-5 erythrocytes) but, subsequently, the proportion of medium size rosettes (6-10 erythrocytes) increased, whereas large rosettes (>10 erythrocytes) appeared for the first time at 2 days. Similarly, Fc and complement receptor-bearing cells showed increasing proportions of medium size rosettes and a delayed appearance of large rosettes after 1-2 days (Fig. 12).

In control preparations of donor marrow cells mixed with uncoated erythrocytes, the labeled small lymphocytes showed a customarily low incidence of small rosettes

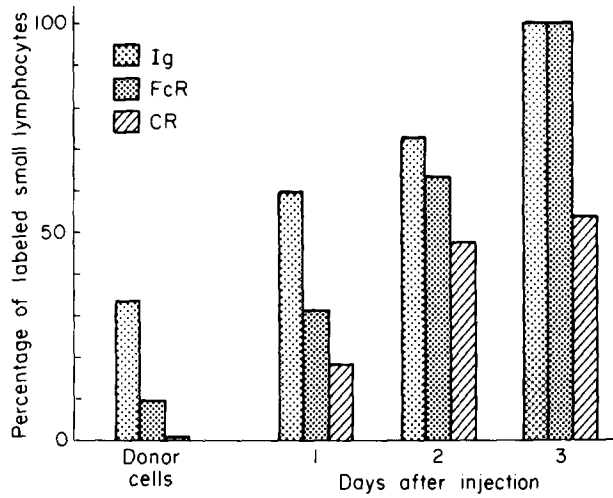


FIG. 11. Percentage of young [<sup>3</sup>H]thymidine-labeled small lymphocytes (0 to 24-h-old) forming rosettes for surface Ig, FcR, and CR in donor bone marrow, and in recipient spleens, 1, 2, and 3 days after injecting the labeled bone marrow suspensions into normal syngeneic mice.

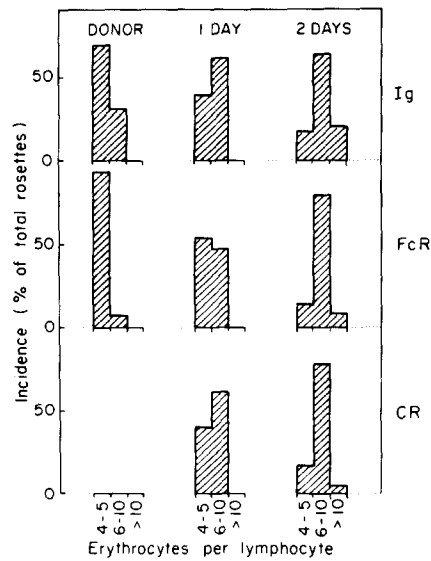


FIG. 12. Size distribution of rosettes for surface IgM(Ig), FcR, and Cr formed by young [<sup>3</sup>H]-thymidine-labeled small lymphocytes (0-24-h old) in donor bone marrow, and in recipient spleens, 1 and 2 days after injecting the labeled bone marrow suspensions into normal syngeneic mice.

(mean, 2.5%). After recovery from recipient spleens, the control rosettes on labeled small lymphocytes were increased in incidence (mean, 17.1%), though not in size (mainly 4-5 erythrocytes).

The fraction of injected labeled lymphocytes recovered from the spleen declined progressively with time (Table III). However, the absolute number of rosette-forming labeled cells recovered at 1-2 days actually exceeded the number of comparable cells in the initial marrow inoculum (Table III). In the case of IgM-bearing cells, the

TABLE III  
*Number of Labeled Small Lymphocytes Forming Rosettes for Surface IgM Molecules, Fc, and Complement Receptors in Donor Marrow and Recipient Spleen*

Labeled small lymphocytes	Number of [ <sup>3</sup> H]thymidine-labeled small lymphocytes ( $\times 10^4$ ) in:			
	Bone marrow inoculum	Recipient spleen after bone marrow injection:		
		1 day	2 days	3 days
Total	120*	91‡	50‡	10‡
Rosette-forming:				
Surface Ig	40	54	36	10
FcR	12	28	32	10
CR	0.7	16	22	5

\* Calculated from the total number of nucleated marrow cells in the inoculum, together with the incidence and 24 h [<sup>3</sup>H]thymidine labeling index of marrow small lymphocytes.

‡ Calculated from the percentage of labeled small lymphocytes in each preparation and the total number of small lymphocytes per spleen ( $101.2 \pm 1.3 \times 10^6$ ).

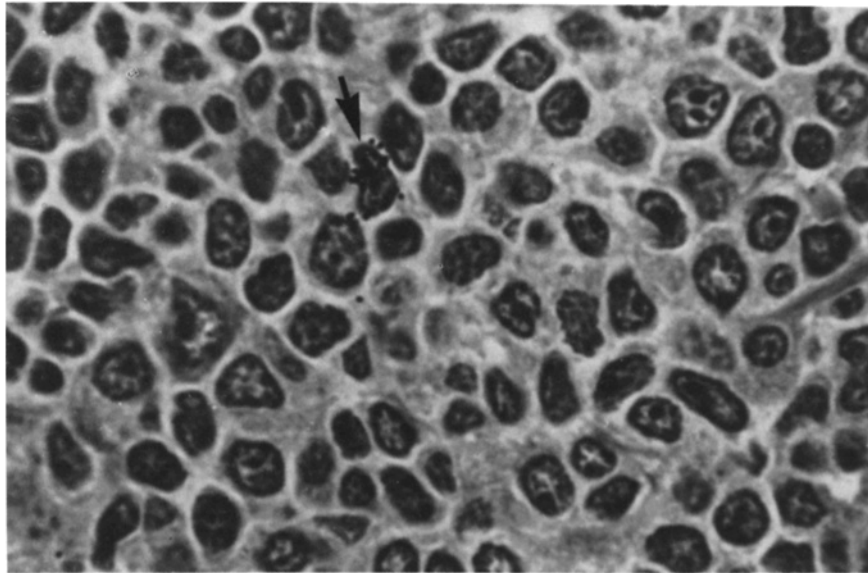


FIG. 13. [<sup>3</sup>H]Thymidine labeled small lymphocyte (arrow) in splenic red pulp 1 day after transfusion of labeled bone marrow cells. ( $\times 1,280$ ).

absolute increase at 1–2 days was particularly marked for medium size rosettes, originally few in number. The appearance in the spleen of labeled small lymphocytes forming large IgM rosettes, medium and large size Fc receptor rosettes, and complement receptor rosettes of all sizes was entirely an absolute phenomenon because the total numbers of such cells were all negligible in the donor marrow.

The initial homing of labeled marrow-derived small lymphocytes into the spleen occurred almost exclusively into the red pulp, as seen in radioautographic sections at 1 day after marrow transfusion (55/57 cells observed) (Fig. 13).

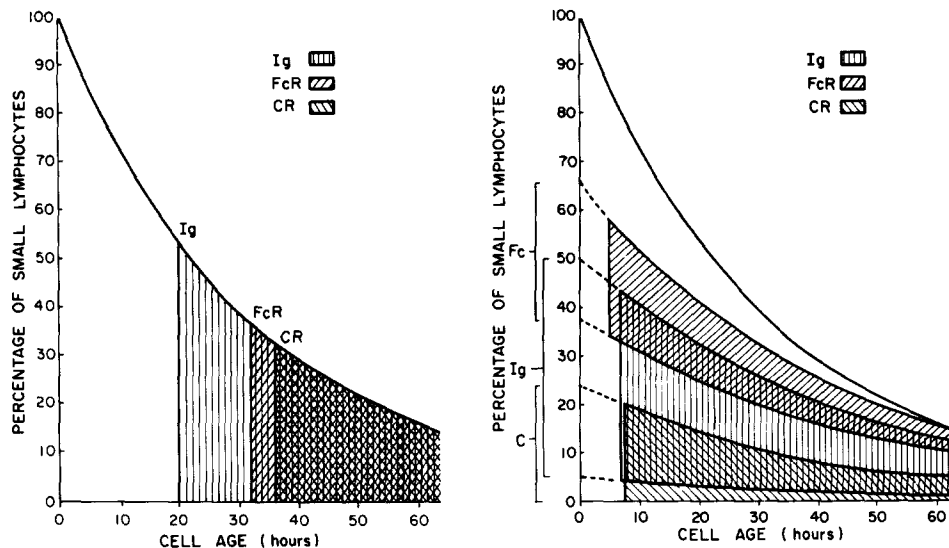


FIG. 14. Two hypothetical maturation models of newly formed small lymphocytes in mouse bone marrow: (A) a single developmental pathway with sequential appearance of surface Ig, FcR, and CR (left) or (B) a parallel maturation of multiple subtypes. The diagrams depict the exponential renewal of small lymphocytes and the expression of surface receptors with time after the production of these cells from proliferating progenitors in the marrow. See text for further details.

### Discussion

*Production of Small Lymphocytes bearing Fc and Complement Receptors and Surface IgM in Mouse Bone Marrow.* The present kinetic study demonstrates that bone marrow small lymphocytes bearing receptors for Fc and complement form part of the major population of indigenous rapidly-renewed marrow cells, and are not solely long-lived immigrants from the recirculating lymphocyte pool. At least 75–85% of the marrow small lymphocytes showing surface IgM, Fc, and complement receptors are newly formed cells which display one or more of these surface components within 3 days of their production. In mice (7), as in other species (4, 5, 12), the newly formed marrow small lymphocytes are continuously generated from locally situated progenitors in the marrow.

The findings confirm the large-scale production of IgM-bearing small lymphocytes in the marrow, previously demonstrated by radioautographic double-labeling techniques (8). IgM-bearing small lymphocytes in the marrow generally tend to have lower densities of surface IgM than those in the spleen, as shown by binding of radiolabeled (2) and fluoresceinated (13) anti-IgM antisera. A progressive increase in the effective density of surface IgM during cell maturation in the marrow is suggested in the present work by the progressive increase in the number of anti-IgM coated SRBC bound by marrow small lymphocytes with increasing cell age, paralleling a similar increase in their capacity to bind anti-IgM antiserum.

The production in the marrow of small lymphocytes with Fc and complement receptors raises the question of their interrelationships with IgM-bearing cells. The simplest interpretation would be that all the small lymphocytes produced in the marrow represent only one cell lineage, developing into IgM-bearing B lymphocytes. A model of such a single population is depicted in Fig. 14 (A), based on the observed

exponential renewal kinetics of the small lymphocyte population and the incidences of small lymphocytes bearing surface IgM, Fc, and complement receptors, respectively. Each small lymphocyte would be apparently receptorless when first produced and would express surface IgM, Fc, and complement receptors sequentially as it matured. The exponential renewal implies that cells may leave the marrow at random with respect to age, frequently before the surface receptors could be fully expressed (4, 8). For IgM-bearing cells alone, this model fits all previous data (4). However, for Fc and complement receptor-bearing small lymphocytes two predictions which would follow from the model could not be substantiated in the present work. (a) The lag before Fc and complement receptors first appear should be appreciably longer than that for surface IgM. In fact, although the data do not permit precise kinetic measurements, no difference in the mean lag times for the respective receptor-bearing populations could be demonstrated. (b) All cells having either Fc or complement receptors should simultaneously bear surface IgM. However, whereas 30–40% of IgM-bearing small lymphocytes do show concomitant Fc or complement receptors, in accord with a progressive maturation of IgM-bearing B lymphocytes, many Fc receptor-bearing small lymphocytes are IgM-negative and thus appear to be a separate population. These observations argue in favor of more than one small lymphocyte maturation pathway in the marrow.

Fig. 14 (B) depicts a hypothetical model showing the development of various marrow small lymphocyte subtypes, incorporating the time lag and receptor overlap data, as well as the exponential renewal and incidences of the three types of receptor-bearing cells. In this model, the IgM-negative small lymphocytes fall into two main categories. (a) Some are newly formed cells which are destined to develop surface IgM as well as Fc and complement receptors, representing the youngest, least mature small lymphocytes in the B lymphocyte lineage. This accords with evidence that some null small lymphocytes in the marrow (2, 8) and blood (14) rapidly develop surface IgM, in apparently increasing density (14), when cultured in vitro. (b) Other IgM-negative small lymphocytes apparently do not develop surface IgM although they are in the marrow, though many of them do rapidly exhibit either Fc receptors or, in some cases, complement receptors. Fc and complement receptor-bearing cells have also been reported among the null small lymphocytes in human blood (15) and adult mouse spleen (16, 17).

The two extreme models presented in Fig. 11 are not mutually exclusive and current evidence indicates that the development of marrow small lymphocyte populations includes features of both. (a) One lineage does develop Fc and complement receptors as well as surface IgM in a sequential fashion, representing the maturation of newly generated B lymphocytes. This concept is strongly supported by the present spleen homing study, discussed below. (b) Other newly formed marrow small lymphocytes develop Fc receptors and, to a lesser extent, complement receptors, without concomitant surface IgM, at short intervals after their production and apparently abruptly with respect to their rosette-forming capacities. The identity and potentials of this apparently large and rapidly produced population of null cells are being verified.

*Maturation of Newly Formed, Marrow Derived Small Lymphocytes in the Spleen.* Young marrow small lymphocytes, which initially lack surface IgM molecules, Fc, and complement receptors, sequentially express these surface components after migration into the spleen. Although all such cells appear to develop surface IgM molecules and

Fc receptors by 3 days, only approximately one-half develop complement receptors. This receptor sequence is also shown by labeled newly formed small lymphocytes normally appearing in the spleen of mice infused with [<sup>3</sup>H]thymidine *in vivo* (15). Parish and Hayward (16) reported in rat peripheral lymphoid tissues that some lymphocytes bearing both surface Ig and Fc receptors lacked complement receptors. The present studies indicate that such a receptor combination, shown by some lymphocytes in the marrow (1), blood (17), and spleen (16), typifies young B lymphocytes.

A similar receptor sequence is shown by developing B lymphocytes as they first appear in ontogeny (18–21) during the repopulation of lymphoid tissues of adult irradiated mice given syngeneic bone marrow (18) and during lipopolysaccharide induction of B lymphocyte surface markers (22). In each case, complement receptors are expressed later than surface IgM and Fc receptors. However, the ontogenic development of B lymphocytes and their continuing postnatal genesis in the marrow are not necessarily identical processes. Apparent differences include the timing of the receptor sequence and the present observation in the marrow of two subpopulations of maturing IgM-bearing lymphocytes, distinguishable by their development or lack of complement receptors.

The capacity of marrow-derived small lymphocytes in the spleen to form rosettes of increasing size for surface IgM, Fc, and complement receptors suggests that maturing B lymphocytes undergo a progressive quantitative development of surface features, including receptor density or turnover. For surface IgM, these findings are consistent with those in the marrow itself, as already noted. Regarding Fc and complement receptors, however, newly formed small lymphocytes within the marrow show no overall increase in rosette size with increasing cell age. Current studies aim to verify whether in the marrow the development of small rosettes for Fc and complement receptors on IgM-bearing lymphocytes may be overshadowed by an earlier appearance of large rosettes on a separate lineage of IgM-negative lymphocytes. The present findings suggest that most, if not all, of the labeled marrow null lymphocytes which home into the spleen are committed to B-lymphocyte development. Null small lymphocytes normally present in the blood and spleen (2, 14) include immature B small lymphocytes (2, 3, 14), many of which would be newly formed cells migrating from marrow to spleen. However, apparently not all null small lymphocytes in the spleen are of this type, because some show a low turnover rate by [<sup>3</sup>H]thymidine labeling (23).

Marrow lymphocytes normally migrate preferentially to the spleen. The high recovery of transfused young marrow small lymphocytes from the spleen in the present experiments accords with their homing in irradiated guinea pigs (24) and the migration of marrow lymphocytes under physiological conditions *in vivo* (9, 10). *In vitro* manipulation of transfused labeled cells may modify the cell surface, as suggested by a tendency to form small nonspecific rosettes with unsensitized SRBC, not shown by labeled young small lymphocytes in the spleens of mice receiving [<sup>3</sup>H]thymidine infusion (15). Nevertheless, the homing behavior of the cells and the use of non-irradiated syngeneic recipients, suggests that the present experiments mimic the normal traffic of marrow lymphocytes.

The extent to which the splenic environment is obligatory for the maturation of marrow-derived small lymphocytes is not clear. Although the maturation of B lymphocytes from transfused progenitors can proceed in the spleen after marrow

destruction by  $^{89}\text{Sr}$  (25, 26), splenectomy has little effect on the development of mouse B lymphocytes (27, 28), and marrow lymphocytes show a progressive maturation of surface IgM (3, 8) and functional responsiveness (29, 30) even when cultured in vitro. Newly formed marrow small lymphocytes may be precommitted to a maturation sequence which, although normally proceeding during cell migration in vivo, is independent of the local environment. The converse possibility remains, viz. that cell migration depends upon cell surface development. The homing of marrow lymphocytes into the splenic red pulp and the concomitant rapid development of surface receptors suggests that a surface maturation phase in the red pulp may be necessary before the cells can migrate into the white pulp (9, 24).

The development of surface receptors strongly suggests that the young, marrow-derived small lymphocytes which migrate into the spleen are virgin B lymphocytes. This view is supported by the similarity in the biological and kinetic properties of marrow lymphocytes and of virgin B lymphocytes, as defined by functional assays. Thus, many virgin B lymphocytes in the spleen are small cells (11, 31, 32), newly formed from proliferating precursors (11, 28) and having a short functional one half-life (33). The marrow itself contains few cells immediately responsive to primary antigens (27, 29-31), but such cells rapidly appear when marrow cells are either cultured (29, 30) or transferred to adoptive hosts before antigenic challenge (29). Similarly, the response of marrow cells challenged with antigen continuously in adoptive hosts lags behind that of spleen cells by  $\cong 2-4$  days (11, 34). The newly formed lymphocytes in the marrow apparently require another 1-4 days before they become immunologically responsive, corresponding with the period of receptor development in the present work and in vivo migration to peripheral lymphoid tissues.

The brief persistence of labeled marrow lymphocytes in the spleen, as in previous intramyeloid labeling studies (9, 10), may reflect their short life span, dilution of label by cell activation and division, or cell efflux. Possibly, the cells may be activated for a limited period after maturing in the spleen, otherwise being lost by spontaneous cell death (4, 11, 28). Alternatively, the findings would not exclude a postulated two-stage development of virgin B lymphocytes (32), an antigen-independent genesis of small lymphocytes being followed by nonspecific stimulation by environmental antigens into large proliferating cells (11, 28, 32). Some labeled large lymphoid cells appeared in the spleen in the present work, as in intramyeloid labeling and transfusion studies (9, 24). The extent to which such cells may represent either the proliferation of transfused lymphocyte progenitors or the amplification of virgin small B lymphocytes by environmental antigens remains to be established.

*Long-Lived Small Lymphocytes bearing Surface IgM, Fc, and Complement Receptors in Mouse Bone Marrow.* The fact that 15-25% of the various receptor-bearing marrow small lymphocytes remain unlabeled during [ $^3\text{H}$ ]thymidine infusion for 3-4 days suggests that these cells belong to the population of long-lived small lymphocytes. This is confirmed by the selective labeling of a sample of long-lived cells in the marrow, nearly one-half of which show surface IgM, an appreciably greater proportion than that of newly formed (0-4-day-old) small lymphocytes ( $\cong 35\%$  IgM + ve under identical rosetting conditions). From the total incidence of long-lived small lymphocytes in the marrow of the mice used ( $\cong 12\%$ , (6)), and the total incidence of IgM-bearing small lymphocytes (39%) it may be calculated that at least 15% of the IgM-bearing small lymphocytes in the marrow belong to the long-lived population. Many of the cells forming large IgM rosettes fall into this category.



The incidence of both Fc and complement receptors on long-lived marrow small lymphocytes resembles that of surface IgM, suggesting that all three surface markers are present simultaneously on these cells. This contrasts with the relatively low incidence of complement receptors on newly formed small lymphocytes in the marrow or spleen (15), suggesting either that the receptor appears subsequently, or that receptor-bearing cells are selected out during the development of the long-lived small lymphocyte pool from virgin B lymphocytes. The resemblance in receptor status between long-lived small lymphocytes in the marrow and spleen (15) accords with evidence that these cells form part of the same recirculating small lymphocyte pool (4, 5).

In peripheral lymphoid tissues the long-lived, recirculating small lymphocytes include memory cells mediating secondary immune responses (11). The present demonstration of both IgM+ve and IgM-ve long-lived small lymphocytes in the marrow provides a cellular basis for the observations of Benner et al. (35) that the development of numerous antibody-forming cells in the marrow during secondary immune responses depends upon immigrant cells, including T lymphocytes, which originate in the spleen after primary antigenic stimulation and circulate to the marrow (27, 35).

*Surface IgM, Fc, and Complement Receptors on Proliferating Lymphoid Cells in Mouse Bone Marrow.* Surface receptor rosettes are formed by some marrow lymphoid cells larger than the defined small lymphocyte size range. Some of these cells may represent nondividing lymphocytes partially overlapping the large lymphoid cell population in size. However, because rosettes are formed by cells in DNA synthesis and by some of the largest cells in the whole series, this must also be a property of some of the proliferating large lymphoid cells, known to include the progenitors of small lymphocytes (4, 5, 36).

Previous antiglobulin-binding studies in mice have consistently shown marrow large lymphoid cells to be surface IgM-ve (2, 4, 8). On the other hand, Melchers et al. (37) described large cells located in the marrow but not in the spleen which synthesize 7S IgM but release it rapidly, with little accumulation of IgM molecules in the cell membrane. Such cells may correspond with the marrow large lymphoid cells in the present work. If so, the current rosetting technique affords a sensitive means of detecting transient surface IgM molecules, possibly because the multiplicity of contacts between the SRBC and the lymphocyte surface can maintain intercellular binding while IgM molecules are simultaneously shed and inserted in the cell membrane. The results raise the possibility that some marrow small lymphocyte progenitors already express B-lymphocyte surface markers at certain stages in their development.

### Summary

Radioautographic DNA labeling and rosetting techniques were combined to study the development of surface IgM, Fc, and complement receptors (FcR, CR) on small lymphocyte populations in mouse bone marrow. [<sup>3</sup>H]thymidine was either infused continuously to label newly formed cells for periods up to 4 days, or injected daily, 21–35 days before use, to label a sample of long-lived cells. Bone marrow cells were incubated with sensitized sheep erythrocytes to detect surface IgM, FcR, and CR, respectively, and examined radioautographically after cytocentrifugation. During [<sup>3</sup>H]thymidine infusion, marrow small lymphocytes lacking surface markers were the first to show [<sup>3</sup>H]thymidine labeling. Most of these cells became labeled by 4 days

(IgM-ve, 89%; FcR-ve, 92%; Cr-ve, 88%). Labeling of small lymphocytes bearing surface IgM, FcR, and Cr began after an initial lag and increased to high values by 4 days (IgM+ve, 73%; FcR+ve, 82%; CR+ve, 83%). Labeled IgM+ve small lymphocytes formed progressively larger rosettes as cell age increased. Some proliferating large lymphoid cells formed rosettes for IgM, FcR, and CR. Labeled long-lived small lymphocytes expressed surface IgM, FcR, and CR, the incidence of each receptor being uniformly high (38-43%) and the rosettes tending to be larger than those formed by newly formed lymphocytes. In double-surface marker studies, FcR and CR rosettes were formed by some IgM-ve small lymphocytes as well as IgM+ve cells in the marrow. After transfusion of marrow cells from donor mice infused with [<sup>3</sup>H]thymidine for 24 h, many labeled newly formed lymphocytes homed into the splenic red pulp of unlabeled syngeneic recipients. Subsequently, these cells showed a rapid increase in the incidence of rosettes for surface IgM, FcR, and CR, together with a progressive enlargement of each type of rosette. Although all the labeled small lymphocytes recovered from the spleen developed both surface IgM and FcR by 3 days, only approximately one-half developed CR. The results demonstrate that most of the small lymphocytes bearing FcR, CR, and surface IgM in mouse bone marrow are newly formed indigenous cells. Each receptor becomes detectable by rosetting soon after the small lymphocytes are first produced. The newly formed, marrow-derived small lymphocytes are able to continue their development of surface IgM, FcR, and CR after migrating into the spleen, consistent with a maturation of primary B lymphocytes. In addition, the data indicate the genesis in mouse marrow of a non-B lineage of lymphocytes (notably, IgM-ve FcR+ve cells.). A minority of small lymphocytes bearing IgM, FcR, and CR in mouse marrow are long-lived cells, presumptive recirculating immigrants, differing in receptor status from the newly formed cells. The results are discussed with regard to the heterogeneity of marrow lymphocytes and proposed models of primary B lymphocyte and null lymphocyte production.

Dr. F. P. H. Chan is thanked for performing the rosette counts on long-lived marrow lymphocytes. The technical assistance of Mrs. P. Young is gratefully acknowledged.

Received for publication 3 January 1978.

### References

1. Yang, W. C., and D. G. Osmond. 1978. Maturation of bone marrow lymphocytes: I. quantitative rosette methods of detecting Fc and complement receptors and surface immunoglobulin. *J. Immunol. Methods*. In press.
2. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. I. Quantitative radioautographic studies of antiglobulin-binding by lymphocytes in bone marrow and lymphoid tissues. *Cell. Immunol.* **13**:117.
3. Ryser, J.-E., and P. Vassalli. 1974. Mouse bone marrow lymphocytes and their differentiation. *J. Immunol.* **113**:719.
4. Osmond, D. G. 1975. Formation and maturation of bone marrow lymphocytes. *J. Reticuloendothelial Soc.* **17**:97.
5. Rosse, C. 1976. Small lymphocyte and transitional cell populations of bone marrow; their role in the mediation of immune and hemopoietic progenitor cell functions. *Int. Rev. Cytol.* **45**:155.
6. Miller, S. C., and D. G. Osmond. 1975. Lymphocyte populations in mouse bone marrow: Quantitative kinetic studies in young, pubertal and adult C3H mice. *Cell Tissue Kinet.* **8**:97.

7. Miller, S. C., M. Kaiserman, and D. G. Osmond. 1978. Small lymphocyte production and lymphoid cell proliferation in mouse bone marrow. *Experientia (Basel)*. **34**:129.
8. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. 2. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. *Cell. Immunol.* **13**:132.
9. Brahim, F., and D. G. Osmond. 1970. Migration of bone marrow lymphocytes demonstrated by selective bone marrow labeling with thymidine- $H^3$ . *Anat. Rec.* **168**:139.
10. Brahim, F., and D. G. Osmond. 1976. Migration of newly-formed small lymphocytes from bone marrow to lymph nodes during primary immune responses. *Clin. Exp. Immunol.* **24**:515.
11. Strober, S. 1975. Immune function cell surface characteristics and maturation of B cell subpopulations. *Transpl. Rev.* **24**:84.
12. Osmond, D. G., and N. B. Everett. 1964. Radioautographic studies of bone marrow lymphocytes in vivo and in diffusion chamber cultures. *Blood*. **23**:1.
13. Scher, I., S. O. Sharrow, R. Wistar, Jr., R. Asofsky, and W. E. Paul. 1976. B-lymphocyte heterogeneity: ontogenic development and organ distribution of B-lymphocyte populations defined by their density of surface immunoglobulin. *J. Exp. Med.* **144**:494.
14. Chess, L., H. Levine, R. P. MacDermott, and S. F. Schlossman. 1975. Immunologic functions of isolated human lymphocyte subpopulations. VI. Further characteristics of the surface Ig negative, E rosette negative (null cell) subset. *J. Immunol.* **115**:1483.
15. Chan, F. P. H., and W. C. Yang. 1977. Turnover of small lymphocytes bearing surface immunoglobulin molecules, Fc and complement receptors in mouse spleen. *Anat. Rec.* **187**:548.
16. Parish, C. R., and J. A. Hayward. 1974. The lymphocyte surface. I. Relation between Fc receptors, C'3 receptors and surface immunoglobulin. *Proc. R. Soc. Lond. B. Biol. Sci.* **187**:47.
17. Ross, G. D., E. M. Rabellino, M. J. Polley, and H. M. Grey. 1973. Combined studies of complement receptor and surface immunoglobulin-bearing cells and sheep erythrocyte rosette-forming cells in normal and leukemic human lymphocytes. *J. Clin. Invest.* **52**:377.
18. Gelfand, M. C., G. J. Elfenbein, M. M. Frank, and W. E. Paul. 1974. Ontogeny of B lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. *J. Exp. Med.* **139**:1125.
19. Owen, J. J. T., M. C. Raff, and M. D. Cooper. 1975. Studies on the generation of B lymphocytes in the mouse embryo. *Eur. J. Immunol.* **5**:468.
20. Sidman, C. L., and E. R. Unanue. 1975. Development of B lymphocytes. I. Cell populations and a critical event during ontogeny. *J. Immunol.* **114**:1730.
21. Rosenberg, Y. J., and C. R. Parish. 1977. Ontogeny of the antibody-forming cell line in mice. IV. Appearance of cells bearing Fc receptors, complement receptors and surface immunoglobulin. *J. Immunol.* **118**:612.
22. Hämmerling, U., A. F. Chin, and J. Abbott. 1976. Ontogeny of murine B lymphocytes: sequence of B-cell differentiation from surface-immunoglobulin-negative precursors to plasma cells. *Proc. Natl. Acad. Sci. U.S.A.* **73**:2008.
23. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface  $\theta$  or immunoglobulin determinants. *J. Exp. Med.* **138**:71.
24. Yoshida, Y., and D. G. Osmond. 1978. Homing of bone marrow lymphoid cells: localization and fate of newly-formed cells in lymphocyte-rich marrow fractions injected into lethally X-irradiated recipients. *Transplantation (Baltimore)*. **25**:246.
25. Phillips, R. A., and R. G. Miller. 1974. The marrow environment is not required for differentiation of B lymphocytes. *Nature (Lond.)*. **251**: 444.
26. Rozing, J., W. A. Buurman, and R. Benner. 1976. B lymphocyte differentiation in lethally irradiated and reconstituted mice. I. The effect of Strontium-89 induced bone marrow aplasia on the recovery of the B cell compartment in the spleen. *Cell. Immunol.* **24**:79.
27. Benner, R., and A. van Oudenaren. 1975. Antibody formation in mouse bone marrow. IV.

- The influence of splenectomy on the bone marrow plaque-forming cell response to sheep red blood cells. *Cell. Immunol.* **19**:167.
28. Strober, S. 1975. Maturation of B lymphocytes in the rat. II. Subpopulations of virgin B lymphocytes in the spleen and thoracic duct lymph. *J. Immunol.* **114**:877.
  29. Stocker, J. W., D. G. Osmond, and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. 3. The adoptive response of bone marrow cells to a thymus cell-independent antigen. *Immunology.* **27**:795.
  30. Nossal, G. J. V., and B. L. Pike. 1975. Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J. Exp. Med.* **141**:904.
  31. Lafleur, L., R. G. Miller, and R. A. Phillips. 1973. Restriction of specificity in the precursors of bone marrow-associated lymphocytes. *J. Exp. Med.* **137**:954.
  32. Nossal, G. J. V., K. Shortman, M. Howard, and B. L. Pike. 1977. Current problem areas in the study of B lymphocyte differentiation. *Immunol. Rev.* **37**:187.
  33. Elson, C. J., K. F. Jablonska, and R. B. Taylor. 1976. Functional half-life of virgin and primed B lymphocytes. *Eur. J. Immunol.* **6**:634.
  34. Rosenburg, Y. J., and A. J. Cunningham. 1976. Ontogeny of the antibody-forming cell line in mice. II. Maturation of B cells during fetal development. *J. Immunol.* **117**:1618.
  35. Benner, R., A. van Oudenaren, and H. de Ruiter. 1977. Antibody formation in mouse bone marrow. VIII. Dependence on potentially circulating memory cells: a study with parabiotic mice. *Cell. Immunol.* **33**: 268.
  36. Yoshida, Y., and D. G. Osmond. 1971. Identity and proliferation of small lymphocyte precursors in cultures of lymphocyte-rich fractions of guinea pig bone marrow. *Blood* **37**:73.
  37. Melchers, F., H. von Boehmer, and R. A. Phillips. 1975. B-lymphocyte subpopulations in the mouse. Organ distribution and ontogeny of immunoglobulin synthesizing and of mitogen-sensitive cells. *Transpl. Rev.* **25**:26