MONOCLONAL ORIGIN OF B LYMPHOCYTE COLONY-FORMING CELLS IN SPLEEN COLONIES FORMED BY MULTIPOTENTIAL HEMOPOIETIC STEM CELLS*

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Since the original description by Till and McCulloch (1) of a class of multipotential hemopoietic stem cells (CFU-S)[1] that can produce colonies in the spleen of irradiated, reconstituted mice, a large number of studies have been performed to define the potential of CFU-S for generating different blood cell types. While erythroid cells, granulocytes, and megakaryocytes are consistently observed in spleen colonies, lymphoid cells have not been described (2). Wu et al. (3) used radiation-induced unique chromosome markers to provide conclusive evidence that each spleen colony was a clone; they further showed that erythroid cells, granulocytes as well as multipotential stem cells contained in the individual colony were progeny of the CFU-S. A number of studies (4–8) have provided evidence indicating a close link between CFU-S and functional lymphoid cells; however the evidence for a precursor-product relationship is circumstantial rather than direct in such studies. More recently Abramson et al. (9), with the aid of radiation induced unique chromosome markers have identified several classes of adult bone marrow stem cells: pluripotential stem cells capable of generating all blood cell types; stem cells restricted for myeloid potentials i.e. capable of generating erythrocytes, granulocytes and megakaryocytes; and T-restricted stem cells capable of producing only T lymphocytes. This study did not detect (albeit could not exclude) the presence of a B-restricted stem cell. While it was shown that B cells, T cells, and CFU-S could originate from a common ancestral cell in the marrow, this study was not designed to examine whether B or T cells can be produced by the CFU-S itself. Recently Metcalf and Johnson (10) reported that a certain proportion of spleen colonies at day 7 or later after reconstitution with fetal liver or adult bone marrow cells contained a variable number of B-lymphocyte colony-forming cells (BL-CFC), detectable when grown in agar in the presence of 2-mercaptoethanol and sheep erythrocytes (11). Their study, however, did not address itself to the question whether BL-CFCs found within the individual spleen colony were monoclonally derived from the CFU-S generating the colony or whether they were immigrant cells generated

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[1] *Abbreviations used in this paper:* BL-CFC, B-lymphocyte colony-forming cell; CFU-S, spleen colony-forming unit synonymous with multipotential hemopoietic stem cell; DME, Dulbecco's modified Eagles medium; EBSS, Eisen's balanced salt solution; FCS, fetal calf serum; SRBC, sheep erythrocytes.

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elsewhere.

The present study was designed to determine the origin of the BL-CFC in spleen colonies generated by CFU-S, by using mixtures of CBA and CBA T_6/T_6 CFU-S, growing B-lymphocyte colonies from spleen colonies of known karyotype and determining the karyotype of the pooled B-lymphoid colony cells.

Materials and Methods

Mice. CBA, CBA/T_6T_6, C57B1/6 or (CBA × C_{57}B1/6)F_1 mice of either sex maintained at Walter and Eliza Hall Institute animal house under specific pathogen-free conditions for 8 wk and then conventionalized, were used at 10–12 wk of age throughout this study. To calculate the duration of gestation in the donors of fetal liver cells, the day of observation of the vaginal plug was taken as day 0.

Irradiation. Mice were subjected to 800–850 rads whole body irradiation before injection with fetal liver cells and 850 rads for mice injected with adult marrow cells. Irradiation was performed using a Philips RT250 machine, 250 kV, 15 mA: half value layer. 0.8 mm Cu at a dose rate of 80 rads/min.

Preparation of Cell Suspensions and Spleen Colony Assay. Fetal liver cells were obtained from 12-day fetuses, as described previously (12). Adult bone marrow cells were obtained from the femurs and pooled from at least two animals of each strain. Viable nucleated cell counts were determined by eosin exclusion.

Irradiated mice were injected with 0.8–2 × 10^5 viable nucleated fetal liver cells or 0.75–1 × 10^5 viable nucleated bone marrow cells and spleen colonies were harvested on days 6–15 after reconstitution for harvesting spleen colonies.

Individual spleen colonies were carefully dissected free of surrounding spleen tissue using a pair of cataract knives with the aid of a dissection microscope. All visible colonies were removed from each spleen. Colonies were gently dispersed into a single cell suspension using 22 gauge needles fitted to a syringe containing 1 ml Eisen's balanced salt solution (EBSS). Viable and total nucleated cell counts were determined for each spleen colony cell suspension.

BL-CFC Assay. As few as 4 × 10^5 spleen colony cells or as many as half of the cell population contained in a single spleen colony were used to prepare four 1-ml cultures and on some occasions two 1-ml cultures for B-lymphocyte colonies in semisolid agar in the presence of 5 × 10^{-5} M 2-mercaptoethanol and 3% sheep erythrocytes. Details of this technique have been published (10, 11). Cultures were scored 6–7 days later. Colonies were defined as aggregates of 50 or more cells.

Experimental Protocol used to Determine Origin of BL-CFC within Spleen Colonies. The protocol is summarized in Fig. 1. Irradiated CBA mice were injected with an equal number of CBA and CBA/T_6T_6 12 day fetal liver (1.2–2.0 × 10^5) or adult bone marrow (1 × 10^5) nucleated cells. Because spleen colonies are known to be clones (3, 13, 14), this procedure was expected to generate spleen colonies of pure T_6+ve or T_6-ve karyotypes. 7–15 day old colonies were individually dispersed, 40% of the cell suspension was used to prepare chromosome spreads while varying proportions of the remainder were used for the BL-CFC assay. 6 days of culture was found to be optimal for obtaining maximal numbers of mitotic B-lymphocyte colony cells. B-lymphocyte colonies derived from single spleen colonies were pooled and their karyotypic constitution determined. B-colony karyotypes were then compared with the karyotypic constitution of the spleen colony from which the B-lymphocyte colonies were grown. Of the three possible results of this comparison: (*a*) a 100% matching of the two karyotypes would indicate a monoclonal origin of BL-CFC from the CFU-s (*b*) a 100% mismatch would indicate that they must be immigrants (*c*) a partial mismatch would mean either they were all immigrants, or a mixture of immigrants and CFU-S descendants.

Chromosome Analysis

(1) SPLEEN COLONIES. To identify the karyotic constitution of each spleen colony, 0.4 ml of the freshly isolated colony cell suspension in EBSS (containing 40% of the cells from a single colony) was mixed with 0.8 ml of a prewarmed (37°C) Dulbecco's modified Eagle's medium (DME) fetal calf serum (FCS)-Colcemid medium which was prepared by mixing the following:

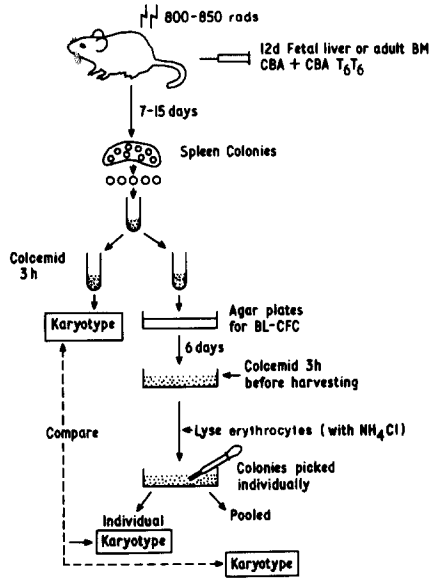

FIG. 1. Experimental procedure followed for determining spleen colony and B-lymphocyte colony cell karyotypes derived from single spleen colonies. (Details in Materials and Methods).

1 vol Colcemid (Ciba Pharmaceuticals, Melbourne) stock solution (10 μg/ml EBSS) + 3 vol Dulbecco's modified Eagle's medium (DME) containing 15% fetal calf serum (FCS). The Colcemid-treated cell suspension was then incubated for 3 h at 37°C in a humidified atmosphere containing 10% CO_2 to cause metaphase arrest in dividing cells. After this, a modification of the method described by Rothfels and Siminovitch (15) was used to make air dried preparations. In brief, cells were gently spun down, and the pellet was resuspended in 2 ml of 1% sodium citrate. 20 min later, 2 ml of fixative, containing 1:3 glacial acetic acid: methanol (vol/vol), was poured down the tube to allow the cells to be fixed in suspension before spinning. This step improved recovery and reduced the probability of breakage of metaphase plates. Cells were then gently pelleted by centrifugation and resuspended in fresh fixative for 30 min. This procedure was repeated twice, but for 10-min periods each, before the final centrifugation and removal of the supernate. Cells were gently resuspended in a drop of fresh fixative and then dropped with a fine Pasteur pipette onto clean grease-free glass slides prechilled in ice cold distilled water. The spreads were then quickly dried over a flame and stained with 10% Giemsa for 10 min. Slides were assigned blind codes and scored before the outcome of the BL-CFC assay was known. Metaphases were examined under oil immersion at × 1,200 magnification. Only well spread metaphases in which all the chromosomes were visible were scored. To establish the karyotypic constitution of a spleen colony, usually 20–25 metaphases were scored, with a minimum of 15 and a maximum of 35. A karyotype was called T_6+ ve when double T_6 markers were identified; A T_6-ve karyotype meant that no T_6 marker was present. When all the karyotypes in a spleen colony were T_6 + ve, the colony karyotypic constitution was named as pure T_6 + ve. Similarly in pure T_6-ve colonies, all were T_6-ve. A colony was considered to have a mixed karyotypic constitution, when there was a mixture, even when contaminated by a single metaphase of the minority karyotype.

(2) B-LYMPHOCYTE COLONIES. A 0.2-ml solution of Colcemid (10 μg/ml in EBSS) prewarmed to 37°C was added to the agar culture plates which were then reincubated for another 3 h. Sheep erythrocytes (SRBC) were then lysed by adding 1 ml of 0.168 M NH_4Cl solution. 10–15 min later the supernate containing the lysed SRBC in NH_4Cl solution was gently removed by pipetting. Occasionally, when colony cells appeared to float into this supernate, the supernate was used separately for chromosome spreads; otherwise it was discarded. For preparing chromosome spreads from B-lymphocyte colonies, colonies were individually picked

off using a fine Pasteur pipette under a dissection microscope and subjected to either of the two following methods, depending upon the number of colonies available: (a) slide method: this was employed occasionally for a single colony, and more often for a small number (two to six) of pooled colonies derived from a single spleen colony. Picked colonies were gently but thoroughly dispersed in a drop of distilled water placed on a clean slide. 10 min later, one or two drops of fixative containing 1:3:9 distilled water: acetic acid: methanol (vol/vol) was added to the suspension. Water present in this fixative helped mixing of the fixative and the cell suspension; straight addition of acid-alcohol usually tends to disperse the cell suspension off the slide. A drop or two of fresh fixative (1:3 acetic acid:methanol) was then repeatedly added at 10-min intervals. After a minimum fixation for 30 min, the slide was then placed on a hot plate to allow quick evaporation of the fixative. (*b*) Tube method: colonies derived from a single spleen colony were pooled into 1–1.5 ml EBSS. They were then thoroughly dispersed by pipetting up and down a 1-ml narrow tipped measuring pipette. 1 ml of FCS was then layered underneath the cell suspension and the tubes were centrifuged at 1,000 rpm for 5 min. This allowed the cells to pellet leaving most of the agar floating on top of the FCS. The supernate was discarded by suction, and the cells were resuspended in 1% sodium citrate. Subsequent procedures were as described for spleen colony cells. Usually cell numbers in these preparations were too small to make more than one preparation.

Membrane Immunoglobulin on B-Lymphocyte Colony Cells. B-lymphocyte colonies were mass harvested from 7-day cultures of 10 day old CBA spleen colony cells, dispersed by gentle pipetting, and centrifuged through FCS before washing in EBSS to remove any adherent agar. Colony cells were incubated for 30 min at 4°C with antisera at a concentration of 160 μg/ml. The antisera to mouse Ig used was affinity purified from a sheep anti-M86 (IgG2b, kappa) serum, and fluorescein-conjugated. The conjugate was fractionated on DEAE-Sepharose and the final product had a molar fluorescein/protein ratio of 2.6. The cells were washed three times before examination at × 1,000 magnifications using both phase contrast and incident light fluorescence using a Zeiss III RS fluorescence attachment.

Results

Development of BL-CFC within Spleen Colonies. Previous work has established that spleen colonies obtained from either 12 day fetal liver or adult bone marrow cells, contain BL-CFC (10). The present results extend and confirm the previous data. A total of 437 individual spleen colonies have been assayed between 6 and 13 days after reconstitution; of these, 182 (42%) were found to contain BL-CFC. BL-CFC-positive spleen colonies were first detected among 7–8 day colonies (1 out of 35 and 18 out of 75, respectively) and at all later ages the proportion of positive colonies ranged between 18 and 45%. A significant increase in frequency of positive spleen colonies was not observed with increasing spleen colony age. At all ages tested the absolute number of BL-CFC per spleen colony varied considerably, ranging between 1 and 10,318. However the majority of spleen colonies contained fewer than 200 BL-CFC. The frequency distribution of the BL-CFC number detected in BL-CFC positive spleen colonies is shown in Fig. 2. The number of BL-CFC have been grouped in increasing increments of 200 and it can be seen that most colonies contained fewer than 200 BL-CFC (from 182 positive colonies, 92 [53%] contained less than 200 BL-CFC). Not shown on Fig. 2 are the data from five colonies that contained more than 4×10^3 BL-CFC.

With increasing spleen colony age the mean number of nucleated cells per spleen colony increased from 8.8×10^3 at day 6 to 10.3×10^6 by day 14, but at each time point the range of colony sizes showed considerable variation and by day 14, colonies contained from 2×10^4 to 46×10^6 cells. The proportion of spleen colonies containing BL-CFC did not increase significantly with increase in spleen colony size although colonies with fewer than 200,000 cells (22% of those assayed) were less likely to contain

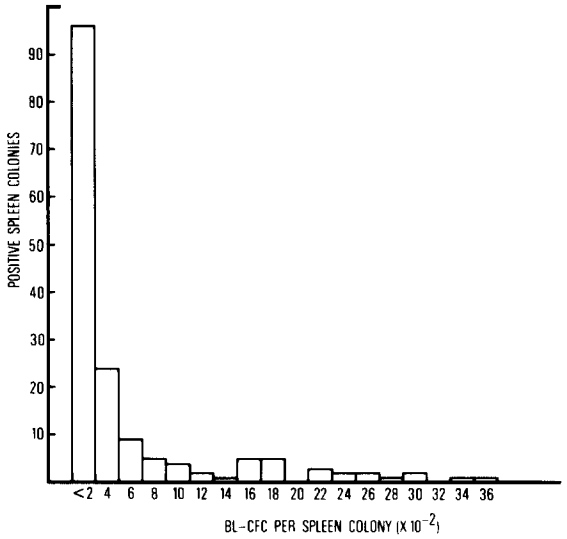

FIG. 2. Frequency distribution of the number of 7–13 day spleen colonies containing B-lymphocyte colony forming cells (BL-CFC- versus the absolute number of BL-CFC per spleen colony. Total BL-CFC numbers per colony have been grouped in increasing increments of 200.

BL-CFC than larger colonies (Table I). However an inverse relationship was observed between the frequency of BL-CFC and the number of nucleated cells in individual spleen colonies (Table I).

Membrane Immunoglobulin on B-Lymphocyte Colony Cells. To verify that the culture conditions used permitted the growth of B-lymphocyte colonies, the membrane immunoglobulin status of pooled colony cells was determined. B-lymphocyte colony cells were mass harvested from 7-day cultures of 10-day old spleen colony cells and analyzed using fluoroscein-conjugated sheep-anti-mouse Ig. In two separate experiments the percentage of immunofluorescent cells was found to be 57 and 98%. No immunofluorescent cells were observed with thymus cells harvested ½ h after suspension in agar-medium and 97% of B-lymphocyte colony cells grown from normal spleen cells were immunofluorescent.

Organ Cultures of Individual Spleen Colonies. The above data may indicate that the development of BL-CFC within spleen colonies is followed by their rapid emigration out of the spleen colony resulting in a decreased frequency of these cells as the colony size increases. Experiments to answer this question were attempted by performing organ cultures. Individual 6–7 day spleen colonies were either placed whole or as three to four fragments on a Millipore membrane resting at the gas-medium interphase on a piece of gel foam sponge soaked in Dulbecco's modified Eagle's medium containing 10% FCS with or without 5×10^{-5} M 2-mercaptoethanol. However, these organ cultures did not support spleen colony growth as indicated by a rapid fall in the viable cell count (from $0.2–0.3 \times 10^6$ cells at day 0 to $1.4–1.5 \times 10^3$ viable cells at day 7–8 of culture). Although the frequency of small IgM+ve cells within these colonies (as judged by immunofluorescence) increased 100-fold (from 0.8–0.9% at day 0 to 8–9% by day 7–8), their absolute number fell by a factor of 10 or more. The incidence of IgM+ve cells at day 0 was similar in both colony and intercolony portions of the spleen. No BL-CFC were detected in these organ cultures of spleen

TABLE I

Correlation between Spleen Colony Size and the Frequency of BL CFC within the Colonies

Total nucleated cells in spleen colony	Spleen colonies containing BL-CFC/number tested	Mean number of BL-CFC per 10^5 spleen colony cells
<200,000	21/86 (24%)	379
200,001-400,000	18/47 (47%)	1,074
400,001-600,000	12/24 (50%)	258
600,001-800,000	10/20 (50%)	117
800,001-1,000,000	12/19 (63%)	158
1,000,001-2,000,000	42/64 (66%)	31
2,000,001-4,000,000	20/49 (41%)	15
4,000,001-6,000,000	15/26 (58%)	5
6,000,001-8,000,000	8/13 (62%)	9
8,000,001-10,000,000	5/12 (42%)	62
10,000,001-12,000,000	4/6 (67%)	8
12,000,001-14,000,000	5/11 (45%)	45
14,000,001-16,000,000	3/5 (60%)	5
16,000,001-18,000,000	0/0	
18,000,001-20,000,000	1/1 (100%)	3
>20,000,001	5/8 (63%)	5

colonies.

BL-CFC Incidence in Splenic Inter-Colony Areas. After the dissection of all visible spleen colonies the remaining portion of each spleen was also cultured for BL-CFC. From 41 spleen intercolony areas assayed, a mean of 19.8 BL-CFC per 1×10^5 viable nucleated cells was obtained and a maximum of 656 BL-CFC/10^5 cells was obtained in one instance. The corresponding data, from the 437 spleen colonies assayed, were a mean of 82.8 BL-CFC per 1×10^5 viable colony cells, the maximum frequency in one colony being 16,920/10^5 cells.

The data are consistent with the hypothesis that BL-CFC develop within individual spleen colonies and, to prove this, comparative karyotypic analysis of individual spleen colonies and of the B-lymphocyte colonies obtained from individual spleen colonies was performed.

Karyotypic Constitution of Spleen Colonies. This was determined for a large number of spleen colonies and permitted (*a*) a verification of the clonal nature of spleen colonies, (*b*) an assessment of the competitive growth potential of CBA and CBA/T_6T_6 cells in CBA mice.

Table II summarizes the results on spleen colonies generated by fetal liver cells. Out of 133 spleen colonies karyotyped, 90% showed pure karyotypes. The remaining 10% had mixed karyotypes most of which were dominated by one karyotype or the other. This suggested that the mixture may have resulted from contamination by confluent growth of two adjacent colonies rather than from a polyclonal origin of these colonies. The former possibility was supported by the observation that mixed karyotypes were mainly derived from colonies which were 9 days or older. The almost equal frequency of pure T_6+ve (44%) and pure T_6-ve (46%) colonies indicated that neither type of stem cell enjoyed a growth advantage over the other in the CBA spleen.

TABLE II

Karyotypic Constitution of Spleen Colonies Generated by Fetal Liver Cells

Exp.	Spleen colony age	Number karyotyped	Pure T_6 + ve	Pure T_6 − ve	Mixed karyotype
	days				
1	7–14	46	22	21	3
2	7–9	23	9	12	2
3	10–13	43	20	17	6
4	12–13	8	2	4	2
5	12–13	8	4	4	0
6	13–15	5	2	3	0
		—	—	—	—
		133	59	61	13
		(100%)	(44%)	(46%)	(10%)

TABLE III

Karyotypic Constitution of Spleen Colonies Generated by Adult Bone Marrow Cells

Exp.	Spleen colony age	Number karyotyped	Pure T_6 + ve	Pure T_6 − ve	Mixed karyotype
	days				
1	9	6	3	3	0
2	10	5	2	3	0
3	11	4	1	2	1
4	15	5	2	2	1
5	8–14	52	24	24	4
6	14	4	1	3	0
7	12	4	2	2	0
		80	35	39	6
		(100%)	(43.8%)	(48.7%)	(7.5%)

Table III summarizes the results from karyotypic analysis of spleen colonies generated by adult bone marrow cells. Similar conclusions to the above can be drawn from the data.

Comparison of Spleen Colony Karyotypes with the Karyotypic Constitution of B-Lymphocyte Colonies Grown from these Colonies. A total of 234 spleen colonies generated by fetal liver cells and 140 spleen colonies generated by adult bone marrow cells were used for this study. Spleen colony ages ranged between 7 and 15 days. Detectable BL-CFC were found in 8% of the fetal liver-generated colonies and some 10% of the bone marrow-generated spleen colonies in this series. In part the lower observed frequency of positive spleen colonies (compared with that obtained previously) was due to incomplete lysis of the sheep erythrocytes by NH_4Cl which did not allow scoring of colonies at the edge of the cultures. In this series of experiments approximately ⅛th of a spleen colony (equivalent to 1×10^5–2×10^6 cells) was placed in a culture dish compared with 1×10^5 or fewer cells in earlier cultures. It was possible that the high cell concentration may have been inhibitory for B-lymphocyte colony formation. Mixing experiments were therefore performed by adding 50,000 adult spleen cells to cultures of spleen colony cells. No significant inhibition of BL-CFC numbers was seen in such mixed cultures, the number of BL-CFC varying between 80–100% of that seen in control spleen cell cultures.

TABLE IV

Comparison of the Karyotype of Individual Spleen Colonies with that of the B-Lymphoid Colony Cells Grown from these Spleen Colonies

Reconstituting cells	Spleen colony cells			BL-Colony cells (6 day)		
	Age	Metaphases		Metaphases		Karyotype match
		T_6 + ve	T_6 − ve	T_6 + ve	T_6 − ve	
Fetal liver	8	20	0	1	0	Yes
	13	0	20	0	4	Yes
	13	0	17	0	5	Yes
	13	19	0	8	0	Yes
	13	11	4	10	5	Yes
	13	0	20	0	2	Yes
	13	20	0	2	0	Yes
	13	0	20	0	1	Yes
	14	22	0	10	0	Yes
	14	20	1	20	3	Yes
	14	16	1	29	5	Yes
	15	0	20	0	2	Yes
Bone marrow	12	0	15	0	1	Yes
	12	20	0	3	0	Yes
	13	15	0	1	0	Yes
	13	20	0	2	0	Yes
	14	0	20	0	5	Yes
	14	21	0	3	0	Yes
	15	0	20	0	2	Yes

Successful karyotypes were obtained from B-lymphocyte colonies grown from 12 fetal liver cell derived spleen colonies and 7 spleen colonies derived from adult marrow cells. The results are presented in Table IV. It can be seen that the total number of scorable metaphases from B-lymphocyte colony cells was very small, ranging from 1 to 34. For the spleen colonies themselves, reasonable numbers of metaphases (15–22) were scorable. Despite this limitation, the karyotypic constitution of B-lymphocyte colonies on each occasion matched the karyotypic composition of the spleen colony from which B-lymphocyte colonies were grown. In 3 of 19 cases, mixed karyotypes were observed both in the spleen colony as well as in the B-lymphocyte colony. However this incidence was not significantly higher than that found in the overall survey of spleen colonies (Tables II and III). In the case of the three mixed karyotypes, the proportion of T_6+ ve versus T_6-ve metaphases recorded from B-lymphocyte colony karyotypes was very similar to that observed in spleen colony karyotypes; again consistent with the conclusion that BL-CFCs were progeny of the CFU-s generating the spleen colony.

Discussion

The present study conclusively demonstrates that B-lymphocyte colony-forming cells can be generated within a spleen colony by the initiating multipotential stem cell (CFU-S). Where the composition of the spleen colony cells was monoclonal, the BL-CFCs grown from that spleen colony were also monoclonal. Because BL-CFCs themselves are now found to be a heterogeneous population embracing nearly the

whole postmitotic B-cell lineage,[2] BL-CFC + ve spleen colonies can be considered to be B cell + ve even though such B cells cannot be expected to be identified morphologically in such colonies because of the small numbers of cells involved. Thus B cells can now be added to the list of other blood elements e.g. erythrocytes, granulocytes, and megakaryocytes known to be produced by the CFU-S (3). A similar capacity of CFU-S to generate T cells remains to be demonstrated directly.

Early fetal liver cells were used as one source of CFU-S in the present study to prevent the possible development of BL-CFC from cells precommited to B-lymphocyte development. At 12 days of gestation the fetal liver lacks mature B cells characterized by the presence of surface IgM, has very few pre B-cells identified by the presence of cytoplasmic IgM (16) and contains no BL-CFC (12). Spleen colonies that are primarily erythroid appeared to have the same probability of containing BL-CFC as granulocytic colonies (10) perhaps indicating that the decision to produce B-lymphoctes within a spleen colony is a random event. This view is supported by preliminary data in which primary spleen colonies were transferred into secondary recipients. BC-CFC were detectable in many secondary spleen colonies which were generated by primary spleen colonies that were BL-CFC negative (G. R. Johnson, unpublished data). However further experiments are needed to distinguish conclusively between preprogrammed and stochastic events underlying the generation of BL-CFC from CFU-S.

Likewise, the reasons for a delay of 7–8 days before BL-CFC differentiate within spleen colonies requires further investigation, as does the observation that considerable differences exist between individual mice in the extent of BL-CFC development (10).

The present demonstration of the clonal origin of BL-CFC, detected within relatively small spleen colonies generated by individual stem cells provides a powerful tool for the study of the factors controlling the development of B lymphocytes from multipotential hemopoietic stem cells as well as the events leading to B-lymphocyte diversification.

Summary

Spleen colonies produced by transplanting lethally irradiated mice with either 12 day fetal liver or adult bone marrow cells were found to contain B-lymphocyte colony-forming cells (BL-CFC). The proportion of BL-CFC positive spleen colonies did not increase substantially between 8 and 14 days after transplantation, the range being 18–45%. However, the absolute number of BL-CFC per spleen colony varied considerably (between 1 and 10,318), although the majority of colonies contained less than 200 BL-CFC. Irrespective of the time after transplantation, smaller spleen colonies were found to have a higher frequency of BL-CFC than larger spleen colonies.

To determine the possible clonal origin of BL-CFC from spleen colony-forming unit (CFU-S), CBA mice were injected with equal numbers of CBA and CBA T_6/T_6 fetal liver or adult bone marrow cells. Analysis of 7–15-day spleen colonies demonstrated that 90% were either exclusively T_6 positive or T_6 negative and approximately equal numbers of both colony types were observed. B-lymphocyte colonies were grown and successfully karyotyped from 19 spleen colonies. When compared with the original spleen colony karyotype the B-lymphocyte colony cells karyotype was identical in all 19 cases. In 3 of the 19 colonies analyzed a mixture of T_6 positive and T_6 negative

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karyotypes was present and identical proportions of the karyotypes were present in the pooled B-lymphocyte colony cells and spleen colony cells.

The data indicate that the B-lymphocyte colony-forming cells detected in spleen colonies are genuine members of the hemopoietic clone derived from the initiating hemopoietic stem cell (CFU-S).

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