THE FATE OF FETAL AND ADULT B-CELL PROGENITORS GRAFTED INTO IMMUNODEFICIENT CBA/N MICE*

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It has been clearly established that B lymphocytes, like other components of the hematopoietic system, arise from multipotential stem cells (1-3). In the mouse, stem cells are first detected in the yolk sac of 7-d-old fetuses (4). Subsequently, they migrate into the embryo and by day 10-12 have given rise to progeny, found in the fetal liver, capable of synthesizing immunoglobulin (5-7). Typically, these cells are large and contain detectable levels of cytoplasmic immunoglobulin (cIg),[1] but do not stably display surface immunoglobulin (sIg) (5-7). They are designated pre-B cells as they are thought to be the direct progenitors of smaller sIg^+ B cells which first appear in 16- to 17-d-old embryos (5-12). A similar cIg^+sIg^- cell has also been detected in adult bone marrow, the primary site of hematopoiesis in mature mice and may be an analogous intermediate along the stem cell to B-cell pathway (6, 13-15).

Although this framework for B-cell differentiation currently represents the most plausible interpretation of available data, it is neither complete nor necessarily correct. The factors which influence the transition of a stem cell into the B lineage, and even the site(s) of commitment, remain obscure. Furthermore, while it is tempting to assume that B-cell development in adult mice proceeds in a manner identical to fetal differentiation, this has not yet been established.

The present study was undertaken to evaluate the differentiative and proliferative potential of various B-cell precursors present during fetal and adult life. We monitored the emergence of B lymphocytes from progenitors found in yolk sac, fetal liver, and adult organs after injection into unirradiated or irradiated immunodeficient CBA/N recipients. The impetus for these studies was the discovery that CBA/N mice lack a class of B cells capable of forming colonies in agar, yet, nonetheless, provide an environment suitable for their development from less differentiated cells of normal donors (16, 17). Among the advantages of this protocol are: (*a*) It detects a functional B cell which is present in all populations of B lymphocytes except those in the CBA/N mouse; (*b*) small numbers of cells are easily monitored owing to both the sensitivity of the B-cell cloning assay and to the background-free environment of the CBA/N host; (*c*) colony formation is not dependent on accessory cells and is linear under the conditions employed; and (*d*) use of CBA/H-T6T6 cells enables us to

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[1] *Abbreviations used in this paper:* cIg, cytoplasmic immunoglobulin; CFU-B, colony-forming unit-B cell; Con A, concanavalin A; CSA, colony-stimulating activity; FCS, fetal calf serum; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PFC, plaque-forming cell; PHA, phytohemagglutinin; sIg, surface immunoglobulin; SRBC, sheep erythrocytes.

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compare B-cell reconstitution with reconstitution of other cell compartments by cytogenic analysis (16–18).

Materials and Methods

Mice. The mice used in these studies were all from our own colony and maintained in a laminar flow air-supplied room under specific pathogen-free conditions. CBA/N mice were originally obtained from the National Institutes of Health (NIH), Bethesda, Md., CBA/H-T6T6 from Dr. O. Stutman, Sloan-Kettering Institute, N. Y., and CBA/Cum mice were purchased from Cumberland View Farms, Clinton, Tenn.

Irradiation. Mice were exposed to variable amounts of radiation emitted from a ^{137}Cesium irradiator delivered at a dose rate of 113.5 rads/min (J. L. Shepherd, Glendale, Calif.). In most experiments mice received either a single dose of 1,050 rads or a split dose of 1,300 rads delivered in equal parts 3.5 h apart.

Preparation of Cell Suspensions from Fetal and Adult Mice. Female CBA/H-T6T6 mice were housed together with males and examined each morning for the presence of vaginal plugs. The day of appearance of a plug was designated day 0 and pregnant mice were sacrificed 9–14 d later. The uteri were removed, washed with medium and cleaned of all maternal blood. The embryos, along with the placenta and the surrounding endometrium, were removed by carefully cutting away the uterine membrane with fine scissors. These were again extensively rinsed to remove more maternal blood and the embryos, with yolk sac intact, were freed from surrounding tissue. After another washing the yolk sacs were separated from the embryos and allowed to settle through 4–5 ml of medium containing 5% fetal calf serum (FCS). Single cell suspensions of yolk sacs were prepared by gently drawing the tissue through a 26-gauge needle. After the cells were expelled, clumps were allowed to settle, single cells in the supernate were harvested, and viable cell counts were obtained using trypan blue. In older embryos, 12–14 d of gestation, livers were removed using fine needles, washed extensively, partially minced, and drawn through a 26-gauge needle. Single cell suspensions of either fetal liver or yolk sac were injected intravenously; when large numbers of cells were injected, heparin was added at a concentration of 5 U/ml. For yolk sac experiments, 10–20 pregnant mice in the 9th or 10th day of pregnancy were sacrificed, each yielding 6–9 embryos. Fetal liver was usually obtained from 3–10 mice 12–14 d pregnant.

Single cell suspensions of adult spleen or mesenteric lymph node were prepared by pushing partially minced tissue through a fine wire screen. Femoral bone marrow cells were obtained by flushing them out with 1 ml of medium. Clumps were dispersed by gentle passage through a 26-gauge needle. Viable nucleated cell counts were obtained in the presence of trypan blue.

B-cell Cloning Assay. B-cell cloning in soft agar was performed as described previously (18, 19). Single cell suspensions were plated with 1 ml of McCoy's medium (Grand Island Biological Co., [GIBCO], Grand Island, N. Y.), containing 15% fcs (Flow Laboratories, Inc., Rockville, Md.), 0.3% agar (Difco Laboratories, Detroit, Mich.) and supplemented as previously described (18) at concentrations which varied from 2.5–10 × 10^4/ml. The cultures were usually stimulated with either 10 μg lipopolysaccharide (LPS) (*Salmonella typhosa* W0901, Difco Laboratories) alone or with LPS + 0.05 ml of 20% sheep erythrocytes (SRBC) (Flow), which had been previously washed 5 times in 5 vol of phosphate-buffered saline (PBS). After the cultures had gelled, they were placed at 37°C in a humid atmosphere containing 7% CO_2 for 6 d. The cultures were examined under a dissecting microscope and the number of colonies consisting of 20 or more cells was determined. Cultures containing SRBC were fixed in 0.3% acetic acid to lyse the erythrocytes which otherwise obscure B-cell colonies.

Chromosome analysis. Chromosome analysis was done with single cell suspensions of spleen or bone marrow cultured in the presence of either LPS, phytohemagglutinin (PHA), concanavalin A (Con A), or colony-stimulating activity (CSA). For the analysis of lymphoid cells, host spleen was cultured at a concentration of 2 × 10^6/ml in RPMI + 5% FCS at 37°C in a humid atmosphere which contained 7% CO_2 for 3 d in the presence of either 10 μg LPS (+ 2-mercaptoethanol), 10 μg PHA, or 10 μg Con A. For the analysis of granulocyte-macrophage progenitors, spleen or bone marrow cells were cultured in McCoy's medium containing 15% FCS and supplemented as described previously for B-cell cloning (without 2-mercaptoethanol), at a concentration of 2 × 10^5/ml in the presence of 0.1 ml/ml of 10-times-concentrated CSA

obtained from the supernate of the WEHI-3 cell line. Liquid cultures were pulsed on the third day of culture with colcemid (0.2 μg/ml) 90 min before harvesting. The cells were spun down and resuspended in 1 ml of 0.95% sodium citrate and allowed to swell for 20 min. 1 ml of fixative, consisting of three parts ethanol and one part acetic acid, was layered over the cells, allowed to stand for 5 min, and then gently mixed with a Pasteur pipette. After 15 min, the cells were gently spun down and resuspended in 100% fixative. After an additional 15 min, the cells were again centrifuged and resuspended in one or two drops of 45% acetic acid. This was placed on a warm glass slide and allowed to slowly dry while manually spreading the cells over the surface of the slide by gentle rocking. Splenic foci, obtained 9 d after injection of 2.5×10^4 bone marrow cells into an irradiated syngeneic mouse, were also chromosome typed. The splenic foci were carefully removed with a scalpel and nonfoci area was trimmed away. A single cell suspension was made in RPMI by gently teasing the foci apart with two 26-gauge needles, and the cultures were then pulsed with colcemid for 90 min and prepared as described above. Alternatively, 50 μg of colcemid was injected i.p. 90 min before sacrifice and removal of spleen foci. In either case 10–50 metaphases were examined to determine the chromosome type of each focus.

B-cell Depletion. B lymphocyte-depleted bone marrow was obtained with two protocols. Treatment with anti-μ antibodies was initiated on the day of birth (0.5 mg/mouse injected i.p.) and repeated daily for 4 d, after which weekly injections were continued for 4 wk. Bone marrow was used 2 d after the final injection. In the second procedure, bone marrow cells were incubated at a concentration of 10×10^6/ml for 70 min at 4°C in 100 × 15-mm polystyrene Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) previously coated with anti-IgM antibodies.

Results

Absence of Clonable B Cells in Fetal and Adult CBA/N Mice. B lymphocytes capable of forming colonies in semisolid agar (CFU-B) are first detected in the liver and spleen of normal 16.5-d embryos and subsequently are present among all B-cell populations (18, 19, 22–24). Furthermore, the cloning efficiency of sIg^+ B cells in normal newborn spleen, adult bone marrow, spleen, lymph nodes, and peripheral blood was similar when LPS was used to potentiate the cultures (18). Immunodeficient CBA/N mice are exceptional in lacking B cells which function in this assay (16). As demonstrated in Table I, these are not detectable in CBA/N tissues at any stage of development. We have previously shown that clonable B cells emerge in irradiated CBA/N mice grafted with normal hematopoietic cells and all of these were of donor origin (17). Monitoring the emergence and expansion of colony-forming B cells in transplanted CBA/N mice thus offers a unique model for assessing the differentiative and proliferative potential of B lymphocyte precursors.

Generation of B Cells in Irradiated CBA/N Mice after Grafting. The kinetics of B-cell emergence in irradiated homozygous CBA/N recipients of 9–10 d fetal yolk sac, 12–13 fetal liver, or adult bone marrow are compared in Fig. 1. Recipients of either fetal liver or bone marrow had easily detectable numbers of clonable B cells in spleen within 8 d of grafting and these expanded to normal adult values within 4–6 wk. In contrast, CFU-B usually did not appear in recipients of yolk sac until after 9 wk and even then never approached normal levels. In all cases colony-forming B cells were simultaneously detected in the bone marrow although in lower frequency relative to total nucleated cells. Similar results were obtained using irradiated (CBA/N × CBA/H)F_1 male and female recipients. To assess the extent of chimerism achieved in irradiated CBA/N recipients, we undertook cytogenic analysis of various hematopoietic cell lineages including: in vivo spleen colony-forming units (multipotential stem cells), in vitro granulocyte-macrophage progenitors, and mitogen-responsive T

TABLE I
Incidence of B-Cell Colonies in CBA/H-T6T6 and CBA/N Mice of Different Ages

Age	Organ tested	Number of B-Cell Colonies*		
		CBA/H-T6T6		CBA/N
		Incidence/10^5 cultured cells‡	Total ($\times 10^{-3}$) per organ	Incidence/ 10^5 cultured cells
d				
−3	Spleen	1.7	0.002	0
	Liver	0.8	ND	0
3	Spleen	138.0	0.46	0
	Liver	36.0	ND	0
7	Spleen	113.0	28.7	0
	Liver	17.0	ND	0
56	Spleen	736.0	991.9	0
	BM	208.0	ND	0
	LN	364.0	ND	0
365	Spleen	1,180.0	1,123.3	0
	BM	185.0	ND	0
	LN	442.0	ND	0

* Mean number of B-cell colonies determined from three-five replicate cultures potentiated by LPS (10 μg/ml).
‡ It has been previously determined (18) that the cloning efficiency of sIg^+ B cells in newborn and adult tissues is similar when LPS is used to potentiate cultures.

and B cells. As demonstrated in Table II, grafting with 5×10^5 yolk sac cells consistently led to a low degree of chimerism in which donor cells were equally represented in each of the four cellular compartments tested. In contrast, grafting with 10^5 fetal liver cells resulted in variable degrees of chimerism except for LPS-stimulated cells which always were predominantly donor-derived. Grafting with larger numbers of fetal liver cells consistently resulted in nearly complete replacement of all compartments with donor cells.

Generation of B Cells in Unirradiated CBA/N Mice after Grafting. To further analyze the development of CFU-B from a potential precursor, reconstitution experiments were performed utilizing unirradiated CBA/N mice. It has been previously demonstrated that reconstitution of unirradiated CBA/N mice with spleen cells from normal donors overcomes the B-cell defect and, furthermore, results in chimerism restricted to the B lineage (25, 26). We confirmed and extended this finding using the cloning assay to measure the reconstitution potential of normal cells derived from various fetal and adult tissues. In preliminary experiments we established that injection of as few as 10^5 fetal liver cells led to small but variable numbers of clonable B cells 3 wk later, whereas larger numbers of cells led to reproducible levels of clonable B cells after a much shorter interval. Clonable B cells emerged in bone marrow and spleen of CBA/N mice 12–15 d after grafting with 2.5×10^7 fetal liver cells and were subsequently detected in lymph nodes as well (Fig. 2).

Clonable B cells were also detected in unirradiated CBA/N mice grafted with 5×10^7 bone marrow cells, although lower numbers of CFU-B were generated. This finding was confirmed in other experiments in which numbers of clonable B cells in spleens of fetal liver recipients were 2- to 4-fold higher than in recipients of twice as

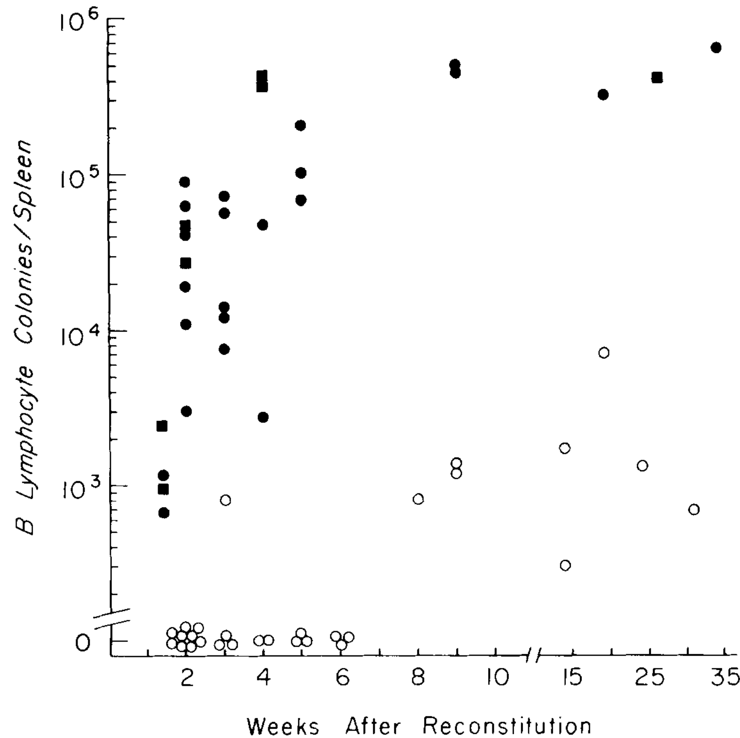

FIG. 1. Kinetics of colony-forming B-cell generation in irradiated CBA/N mice grafted with 5×10^4 bone marrow (■), 10^5 12- to 13-d fetal liver (●), or 5×10^5 to 2×10^6 9- to 10-d yolk sac (○) from CBA/H-T6T6 donors. Recipient mice were exposed either to 1,000–1,050 rads (single dose) or to 1,300 rads (split dose) 1 h before cell transfer. The number of cells injected were based on preliminary experiments which revealed that the mean CFU-s level in 12–15 irradiated recipients of adult bone marrow, 12- to 13-d fetal liver, or 9- to 10-d yolk sac was $11.2/10^5$, $4.5/10^5$, and $1.3/10^5$, respectively.

TABLE II

*Cytogenetic Analysis of Grafted CBA/N Mice**

Recipient	No. and origin of donor cells‡	No. of T6/total and % donor (range)			
		Spleen colonies§	Granulocyte-macrophage colonies‖	T lymphoblasts‖	B lymphoblasts‖
Irradiated	5×10^6 FL	27/28 96(93–100)	276/300 92(88–100)	332/362 92(88–97)	398/400 99(98–100)
CBA/N	10^5 FL	9/17 53(0–100)	39/65 60(38–100)	24/64 40(30–75)	107/113 95(85–100)
	5×10^5 YS	2/18 11	26/211 12(2–17)	18/137 13(10–16)	28/168 17(9–25)
Unirradiated	50×10^6 BM	2/31 6(0–33)	46/600 8(1–13)	31/600 3(3–8)	127/600 21(12–33)
CBA/N	25×10^6 FL	0/40 0	20/405 5(1–8)	55/658 8(3/18)	351/600 59(31–79)

* Experimental details described in Materials and Methods.

‡ Cells derived from either 12- to 13-d fetal liver, 10-d yolk sac, or adult bone marrow. Data presented was pooled from results obtained with three–five individual mice assayed for each group.

§ Spleen colonies were obtained from the spleen of heavily irradiated secondary recipients 9–10 d after grafting with 2.5×10^4 bone marrow cells from CBA/N primary recipients. 10–50 Individual metaphases were scored to determine the chromosome type of each spleen colony.

‖ Cell stimulated as described in Materials and Methods.

much bone marrow cells 14–16 wk after transplantation. In contrast to the generalized chimerism of hematopoietic tissues after grafting of irradiated mice, significant numbers of donor cells were only found among B cells in unirradiated, grafted CBA/N mice (Table II).

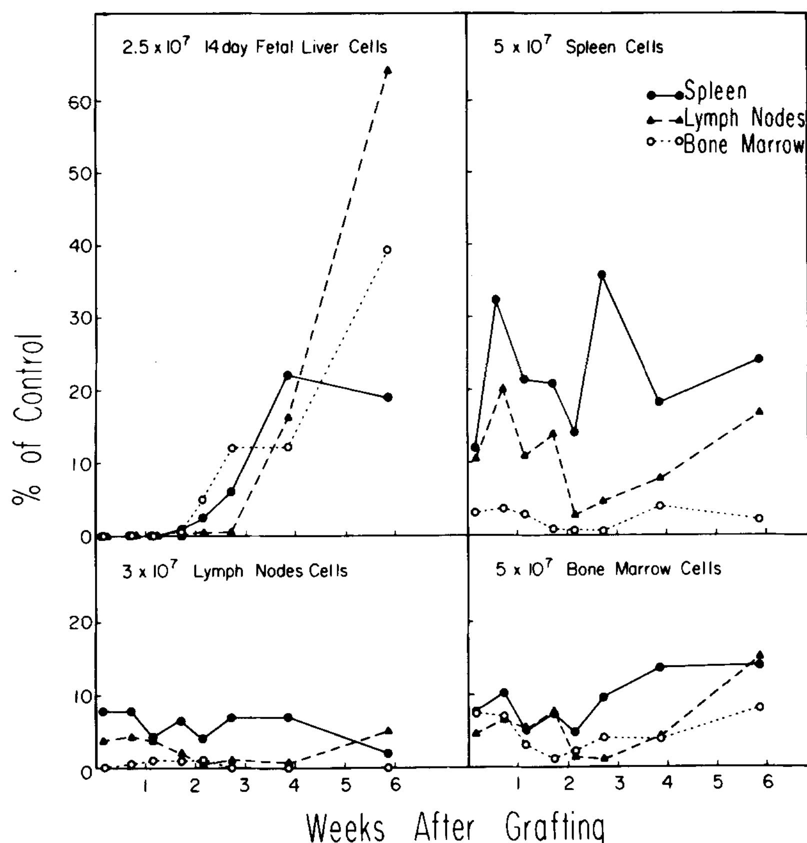

FIG. 2. Kinetics of colony-forming B-cell generation in unirradiated CBA/N mice grafted with 2.5 × 10^7 14-d fetal liver (a), 5 × 10^7 spleen (b), 3 × 10^7 lymph node (c), or 5 × 10^7 bone marrow (d) from CBA/H donors. Clonable cells were measured in the bone marrow (○), spleen (●), and lymph node (▲) of recipient mice. Each point represents the mean of 3–4 replicate cultures from 2–3 individual mice reported as the percentage of normal control cells plated at the same time. Control values (expressed as colonies/10^5 cultured cells) for the individual time points were: spleen, 319 ± 13, 878 ± 76, 1,058 ± 48, 518 ± 35, 570 ± 30, 688 ± 32, 611 ± 35, and 893 ± 36; lymph node, 128 ± 17, 247 ± 63, 380 ± 10, 87 ± 12, 265 ± 4, 248 ± 17, 238 ± 18, and 102 ± 18; and bone marrow, 278 ± 40, 312 ± 16, 284 ± 21, 419 ± 19, 445 ± 16, 265 ± 25, 180 ± 2, and 273 ± 8.

Transferred lymph node cells were inefficient in achieving B-cell chimerism of any of the recipient tissues. Recipients of 5 × 10^7 spleen cells had substantial numbers of clonable B cells in spleen and lymph nodes but the degree of chimerism obtained 18 h after grafting was essentially unchanged over a 40-d period.

Contribution of sIg$^+$ B Cells to B-Cell Generation. Donor cell suspensions transferred in the above experiments are heterogenous in their content of B cells and their progenitors. This is particularly evident in bone marrow where both mature B cells and cIg$^+$sIg$^-$ cells, which are assumed to be the immediate precursors of sIg$^+$ B lymphocytes, are frequent. The importance of sIg$^+$ cells in the transferred bone marrow suspensions was assessed in two ways. Fig. 3 shows the results of experiments in which CBA/N mice were grafted with bone marrow derived from either untreated control CBA/H-T6T6 mice or from CBA/H-T6T6 mice treated with anti-μ antibodies from birth. In 1-mo-old mice treated in this manner, sIg$^+$ cells were reduced by 95% in

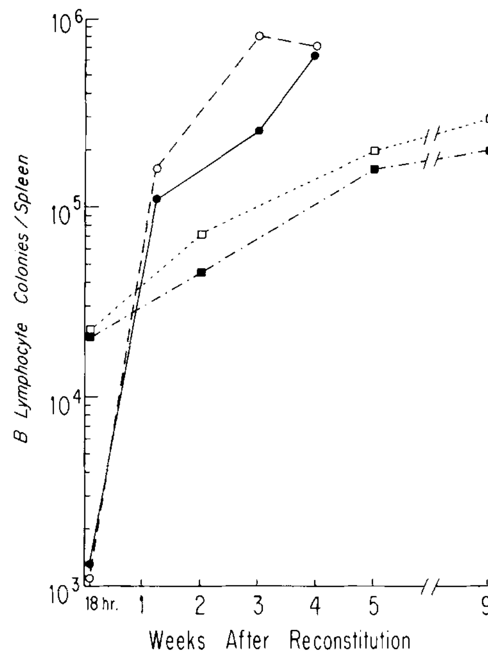

FIG. 3. Emergence of clonable B cells in irradiated (1,050 rads) (circles) or unirradiated (squares) CBA/N recipients of CBA/H-T6T6 bone marrow derived from normal (○, □) or anti-μ suppressed, B-cell depleted (●, ■) donor mice. Each point represents the mean of 3-4 replicate spleen cultures from 2-3 individual mice. Unirradiated mice were injected with 2.5×10^7 bone marrow cells. Irradiated mice were injected with 2.5×10^6 bone marrow cells.

spleen and 83% in bone marrow while clonable B cells were reduced by 99, 98, and 91% in spleen, lymph node and bone marrow, respectively. Such B-cell deficient marrow was equivalent to normal marrow in generation of clonable B cells in irradiated recipients and produced almost as many functional B cells after transfer to unirradiated CBA/N mice (Fig. 3).

An additional procedure, in which bone marrow was incubated on Petri dishes coated with anti-IgM antibodies, was also utilized to remove B cells. Like the previous experiment, CFU-B generation in unirradiated recipients of depleted marrow was similar to that in recipients of normal marrow, as demonstrated in Table III.

Contribution of Stem Cells to B-Cell Generation. Bone marrow and fetal liver contain multipotential stem cells as well as pre-B cells and it is not clear which of these populations are primarily responsible for B-cell generation in recipient mice. Because pre-B cells have not been detected in yolk sac, the results of experiments illustrated in Fig. 2, in which equal numbers of stem cells were injected from either fetal liver or yolk sac, suggest that pre-B cells are the major source of subsequent B-cell generation. An alternative explanation is that stem cells in yolk sac and other sites are intrinsically different and, therefore, a second protocol, illustrated in Fig. 4, was used to further assess stem cell contribution. We injected a mixture of CBA/N and chromosome-marked CBA/H-T6T6 bone marrow (50:50) into an irradiated CBA/N host. 8-14 d later, the resultant spleen foci, which are the clonal progeny of stem cells undergoing self-renewal and differentiation, were individually processed for cytogenetic analysis and assayed for colony-forming B cells. If the colony-forming B cells were the progeny

TABLE III

Colony-forming B Cells in Unirradiated CBA/N Mice Reconstituted with Normal or B-Cell Depleted CBA/H-T6T6 Bone Marrow

Potentiator used§	Organ tested	No. of B-cell colonies in CBA/N mice reconstituted with:*	
		Normal bone marrow	B-cell depleted marrow
		Incidence/10^5 cultured cells‡	
LPS	Spleen	814 $\times\div$ 1.17	703 $\times\div$ 1.43
	Lymph node	177 $\times\div$ 1.68	161 $\times\div$ 1.65
	Bone marrow	91 $\times\div$ 1.18	71 $\times\div$ 1.50
LPS	Spleen	1,086 $\times\div$ 1.09	1,060 $\times\div$ 1.29
+	Lymph node	579 $\times\div$ 1.29	541 $\times\div$ 1.30
SRBC	Bone marrow	236 $\times\div$ 1.12	198 $\times\div$ 1.40

* Unirradiated CBA/N mice were reconstituted with 5×10^7 CBA/H-T6T6 bone marrow cells. B-cell depleted marrow contained <0.5% sIg^+ cells compared to 10% sIg^+ cells in control marrow.

‡ Geometric mean of the incidence of colony-forming B cells in three replicate cultures from four individual recipients assayed 4.5–9 mo after reconstitution. A two-sample t test revealed no significant difference between recipients of normal or B-cell depleted marrow ($P > 0.8$ in all cases).

§ Cultures were potentiated by LPS (10 μg/ml) ± washed SRBC (1%).

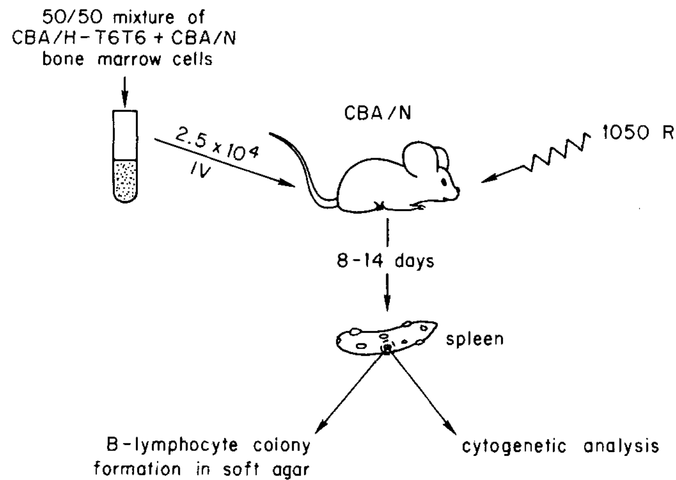

FIG. 4. Protocol utilized to determine relative stem cell contribution to the emergence of clonable B cells.

of donor stem cells, a proportion of the spleen foci derived from the normal CBA/H-T6T6 stem cells should contain colony-forming B cells. In contrast, the $T6^-$ spleen foci, derived from the CBA/N stem cells, should be uniformly negative for colony-forming B cells. As depicted in Table IV, 81 individual spleen foci derived from the 50:50 mixture were analyzed for CFU-B and 22 of these were also processed for chromosome typing. As expected, of those typed, approximately one-half were T6-derived, whereas the rest, lacking the T6 marker, were derived from CBA/N stem

TABLE IV
Colony-forming B Cells in Spleen Foci of Irradiated CBA/N Mice Given a Mixture of CBA/H-T6T6 and CBA/N Bone Marrow

Origin of spleen foci*	No. of foci positive for CFU-B/total No. of foci examined	Percentage positive	Clonable B cell/10^5 spleen colony cells‡	
			Arithmetic mean	SEM
		%		
CBA/H-T6T6	8/9	89	4.9	1.5
CBA/N	12/13	92	3.4	1.3
Untyped	54/59	92	3.4	1.2
Total	74/81	91	3.3	1.1

* Spleen foci were obtained 8–14 d after grafting irradiated (1,050 rads) CBA/N mice with 2.5–5 × 10^4 of an equal parts mixture of CBA/H-T6T6 and CBA/N bone marrow cells.
‡ B-cell colonies were potentiated by LPS (10 μg/ml) and SRBC (1%). Geometric means of the incidence of B-cell colonies in positive foci are presented. As determined using a two-sample t test, the difference between CBA/H-T6T6 foci and CBA/N foci was not significant ($P = 0.2$).

cells. However, 90% of all spleen foci, regardless of origin, contained colony-forming B cells. Furthermore, there was no significant difference in the incidence of CFU-B found in spleen colonies of different origin although intercolony areas contained ~5 times as many CFU-B (24.5 CFU-B/10^5 intercolony cells). Thus, we conclude that CBA/H-T6T6-derived pre-B cells present in the inoculum randomly seeded throughout the host spleen, expanded contiguously with stem cells, and were incorporated in the developing splenic foci. To exclude the unlikely possibility that the mixture of CBA/N and CBA/H-T6T6 cells conferred colony-forming ability on CBA/N cells, we recovered B-cell colonies grown from whole spleen reconstituted with the 50:50 mixture and found that 126/126 of these were T6T6.

To ensure that our results would be comparable to the observations of another group (27, 28), we performed additional experiments with a slightly modified experimental design. This included the use of CBA/H recipients instead of CBA/N and the use of SRBC instead of SRBC + LPS to stimulate CFU-B formation. In these experiments, 100% of the injected cells were normal CBA/H-T6T6. The results, shown in Table V, indicate: (*a*) that 90% of all spleen foci in either recipient contain CFU-B; (*b*) that intercolony areas always contained more CFU-B than did spleen foci; and (*c*) that unlike CBA/N recipients, normal irradiated uninjected mice have background levels of CFU-B. These colonies were morphologically lymphoid and inhibited by anti-μ antibodies.

Discussion

A major goal of these studies was to compare the differentiative and proliferative potential of various B-lymphocyte precursors. To accomplish this we utilized an adoptive transfer assay in which the emergence of B lymphocytes was monitored in CBA/N mice reconstituted with fetal or adult sources of B-cell progenitors derived from CBA/H-T6T6 donors. We determined that injection of small numbers of 12-d fetal liver or adult bone marrow cells into irradiated CBA/N recipients led to detectable numbers of CFU-B within 8 d and normal levels within 4–6 wk. The kinetics of B-cell recovery are similar to other studies in which functional or nonfunc-

TABLE V

Distribution of Clonable B Cells within and between Splenic Foci of Irradiated CBA/H-T6T6 or CBA/N Mice Given CBA/H-T6T6 Bone Marrow

Host	Agar culture stimulant	No. positive/total No. examined		Clonable B cells/10^5 cultured cells§		
		Foci	Intercolony	Foci	Intercolony	Uninjected spleen‖
CBA/H-T6T6	SRBC	18/19	5/5	1.3	4.4	3.0
	SRBC + LPS	19/19	5/5	5.2	13.4	8.9
CBA/N	SRBC	17/21	8/9	1.1	3.4	0
	SRBC + LPS	19/21	10/10	3.1	21.5	0

* CBA/H-T6T6 or CBA/N mice were exposed to 1,050 rads 1 h before grafting with 2.5×10^4 CBA/H-T6T6 bone marrow cells.

‡ B-cell colonies within splenic foci or in intercolony areas measured 12 d after reconstitution. Data from five individual mice were pooled for each group. Mean number of CFU-s in CBA/H-T6T6 recipients was 4.5/spleen, and in CBA/N recipients, 5.0/spleen.

§ Geometric mean of the incidence of B-cell colonies in the positive foci or intercolony areas. Within individual spleens the incidence of B-cell colonies within foci differed significantly from the incidence of B-cell colonies in intercolony areas ($P < 0.01$ using a paired t test). B-cell colonies were potentiated with 1% SRBC ± LPS (10 μg/ml).

‖ Geometric mean of the incidence of splenic B-cell colonies of six irradiated mice which were not reconstituted with donor cells.

tional assays were used to follow B-cell regeneration from fetal liver or bone marrow (8, 29–33). Because fetal liver of the age used in this and previous studies contained no sIg^+ cells, these experiments clearly indicate that the transition from sIg^- precursor cells to sIg^+ cells occurs in recipient mice. Extending this analysis to adult bone marrow sIg^- cells, CBA/H-T6T6 donors were treated from birth with anti-μ antibodies, a procedure which greatly reduces sIg^+ cells but does not alter the number of cIg^+sIg^- pre-B cells (15). When the reconstitution potential of bone marrow from mice treated in this manner was compared to bone marrow from normal controls, no difference in CFU-B generation was found, indicating that donor sIg^+ cells contribute little if anything to subsequent CFU-B formation. A similar result was obtained using a second procedure in which B cells were depleted by incubation in Petri dishes previously coated with anti-IgM antibodies. These data thus document the extensive proliferation of sIg^- B-cell precursors.

Because the role of pluripotent stem cells in these reconstitution experiments remains speculative, we critically examined their contribution in our system. In one approach, irradiated CBA/N mice were reconstituted with stem cells derived from 9- to 10-d fetal yolk sac as no cells with characteristics of the B lineage have been detected in this site, even after extensive organ culture (12, 34). For comparison, we injected control mice with 12- to 13-d fetal liver, a source of both stem cells and pre-B cells, and adjusted the cell numbers so that approximately equal numbers of stem cells were injected from either source. The inability of yolk sac cells to rapidly generate CFU-B in the host CBA/N mice, in contrast to fetal liver which lead to high levels of clonable B cells in <2 wk, suggests that pre-B cells and not stem cells are primarily responsible for B-cell reconstitution. This interpretation is supported by chromosome analysis of CBA/N mice after long-term reconstitution with either yolk sac or fetal liver. Yolk sac cells lead to approximately equal representation of donor cells in each

of the cellular compartments we examined, usually about 15% donor and 85% host. Fetal liver, however, was more variable, always leading to >90% donor in the B-cell compartment whereas other compartments ranged from 0–100%. These data reflect the efficiency of pre-B to B-cell transition in fetal liver grafts and, furthermore, support the contention that significant competition exists between donor and host cells even after lethal irradiation (35).

An alternative explanation for these data is that stem cells in yolk sac may differ from stem cells in other locations in their ability to generate B cells. Therefore, a second method was devised to determine the efficiency of the stem cell to CFU-B transition using adult bone marrow-derived stem cells. A 50:50 mixture of CBA/N and CBA/H-T6T6 was injected into an irradiated mouse and the resultant splenic foci, each of which is primarily composed of the clonal progeny of a hematopoietic stem cell undergoing self-renewal and differentiation, were assayed for CFU-B and typed for the presence of the T6 chromosome marker. As predicted, approximately one-half of the spleen foci were derived from CBA/H-T6T6 stem cells whereas the rest came from CBA/N stem cells. However, >90% of all spleen foci, regardless of origin, contained clonable B cells. This indicates that CBA/N spleen foci contained CBA/H-T6T6 B cells. To ensure that these were not in fact CBA/N B cells which had somehow acquired the ability to form colonies in soft agar as a result of the exposure to normal bone marrow, we chromosome typed the CFU-B from the spleens of mice injected with the 50:50 mixture and found that 100% of these were T6T6. Because the clonable B cells found within spleen foci derived from CBA/N stem cells were not of CBA/N origin, they must have been derived either from T6T6 pre-B cells present in the original inoculum or, alternatively, have migrated from the T6T6 spleen foci where the transition from stem cell to B cell could have occurred. If migration between spleen foci was significant, it could be argued that the number of foci positive for CFU-B should increase with time and, furthermore, that those foci producing colony-forming B cells might contain more of these than those foci positive as a result of migration. Our data refute both of these predictions as the same percentage of foci are positive at 8 d after injection as are positive at 14 d, and no significant difference in the number of CFU-B was found in foci derived from either CBA/H-T6T6 or CBA/N stem cells. These data suggest, therefore, that pre-B cells present in the inoculum randomly seeded throughout the spleen and that those expanding contiguous with hematopoietic stem cells were incorporated into the developing splenic foci. The finding that interfoci areas contained a higher incidence of CFU-B than was found within foci is consistent with this view, as these would not be diluted by the expanding number of nonlymphoid hematopoietic cells found within splenic foci. Whereas these data do not prove that stem cells are incapable of differentiating into B cells within splenic foci, they do imply that this transition, if it occurs, is not of the same magnitude as the pre-B to B-cell transition and thus is obscured.

This conclusion contrasts with the results of a similar investigation (27, 28). Because slightly different procedures were used we repeated our experiments using conditions which more closely approximated the other study. This included using CBA/H mice as recipients rather than CBA/N and the use of SRBC alone to stimulate colony-forming B cells rather than SRBC + LPS. With these modifications we still found that >90% of the splenic foci were positive with more CFU-B present in the interfoci

areas than within foci. In contrast, Lala and Johnson (28) reported that only ~40% of spleen foci contained clonable B cells and more were detected within foci than between them. In addition, the chromosome type of the CFU-B detected matched that of the spleen colony from which they were derived, although in 14 of 19 cases this claim was based on five or fewer CFU-B metaphases scored. In our study, chromosome analysis of CFU-B generated from the 50:50 mixture of CBA/N + CBA/H-T6T6 revealed that 100% (126/126) were T6T6. This protocol thus provides a far more sensitive method for detecting contamination of spleen foci by exogenous cells as we can demonstrate the presence of even one T6T6 CFU-B in an entire CBA/N-derived spleen colony. In addition, our results are in agreement with several other studies which demonstrate that spleen colonies often contain a small percentage of cells not derived from the colony itself (1, 36). It should also be noted that CFU-B were never detected in spleen colonies derived from CBA/H-T6T6 yolk sac cells injected into irradiated CBA/N recipients. The yolk sac, in contrast to fetal liver and bone marrow, is a source of multipotential stem cells in which committed B-cell progenitors have not been demonstrated. As we have previously mentioned, normal CBA/H recipients subjected to even 1,350 rads still possessed detectable numbers of clonable B cells (although no CFU-s) when assayed 10–14 d later. This result demonstrates the resiliency of endogenous hematopoietic precursors and emphasizes the importance of using background-free, CBA/N mice for these reconstitution experiments. It is essential to reiterate that our results do not disprove the possibility that stem cells can differentiate into lymphocytes within splenic microenvironments. However, they strongly suggest that the methodology employed thus far is insufficient for this determination.

To extend our analysis of B-cell differentiation, we have also utilized unirradiated CBA/N mice as recipients of fetal and adult CBA/H-T6T6 cells. We found that rapid and long-lasting engraftment was achieved with either fetal or adult donor cells as monitored by colony formation. Furthermore, in contrast to reconstitution of irradiated CBA/N recipients, donor cells were largely restricted to the B lineage. These results thus support a previous study of Volf et al. (26) which showed that unirradiated CBA/N mice can be reconstituted with adult spleen cells resulting in chimerism only in the B-cell compartment. Quintans et al. (37), measuring responsiveness to PC and TNP antigens, have demonstrated that unirradiated $(CBA/NxBALB/c)F_1$ ♂ mice can be reconstituted with normal adult cells but in their study neonatal liver was unable to colonize recipients unless these mice were first exposed to a low dose of radiation (32). As assessed by the colony-forming B-cell assay in our study, fetal liver consistently led to the highest degree of reconstitution when compared to adult donor cells, even bone marrow. This discrepancy in the grafting ability of bone marrow and fetal liver cells was only apparent when unirradiated recipients were used. Because B-cell depletion experiments demonstrated that sIg^- pre-B cells account for most of the observed B-cell reconstitution from bone marrow, these data may indicate that an intrinsic difference exists between fetal liver and bone marrow pre-B cells. Estimates of the number of pre-B cells found in these sites make this difference even more remarkable. For example, Burrows et al. (15) estimate that 3% of bone marrow cells are pre-B cells whereas similar methods reveal only 0.007–0.3% of 13-d fetal liver cells are pre-B cells (15). Other methods result in even lower estimates (38). Based on these figures, recipients of bone marrow receive 100–1,000 times more pre-B cells than do

recipients of fetal liver. Among the possible explanations for this difference are: (*a*) Pre-B cells in fetal liver have a much higher proliferative potential than bone marrow pre-B cells; (*b*) estimates of pre-B cells by cytoplasmic fluorescence may not accurately reflect the actual number of B-cell progenitors; and (*c*) the majority of adult pre-B cells are rejected by the CBA/N recipient by a mechanism which does not affect fetal cells.

The rationale for utilizing CBA/N mice for these experiments was to provide suitable environments for expansion and differentiation of stem cells and other precursors which were both functionally and cytogenetically marked. This was highly successful and all of our observations suggest that this process occurs as efficiently in irradiated CBA/N mice as in irradiated normal mice. Incidental to these studies were observations which help to delineate the selective humoral immunodeficiency of these mice. Firstly, the finding that yolk sac cells produced functional B cells in CBA/N recipients provides strong evidence that inductive microenvironments for B-cell development are intact in CBA/N mice. All previous grafting experiments with these mice employed donor cell suspensions which contained precommitted cells. Certain of our findings also address the question of which stage of development the mutant gene product is normally expressed. It has previously been suggested that the defect might occur relatively late in development such that certain immature functional cells are spared (25, 39, 40). The finding that normal but not CBA/N fetal liver contains clonable cells by 17 d of gestation is not consistent with this idea. Success in grafting hemopoietically deficient but not normal mice without prior irradiation indicates that you may only be able to engraft with cell types that are deficient in the recipient. According to this perhaps oversimplified logic, CBA/N mice must be particularly deficient in a category of pre-B cell which is prevalent in early fetal liver. Our results, therefore, support an alternate hypothesis that multiple lineages of B cells diverge early in development to yield functionally restricted progeny (41).

In summary, the demonstration that yolk sac cells are capable of developing into colony-forming B cells in irradiated CBA/N recipients confirms the pluripotentiality of these cells. Nonetheless, the finding that pluripotent stem cell contribution to B-cell development, though possible, is minimal in our systems raises the possibility that steady-state adult lymphopoiesis does not significantly involve multipotential stem cells. Furthermore, the results obtained from in vivo transfer experiments utilizing unirradiated recipients suggest a previously undetected degree of heterogeneity in the pre-B compartment and imply that lymphopoietic processes may differ significantly in adult and fetal life.

Summary

The relative ability of various precursors to generate functional B cells in vivo was assessed by transferring normal, chromosomally-marked CBA/H-T6T6 cells to irradiated or unirradiated immunodeficient CBA/N mice. Emergence of donor-derived B cells was monitored by means of a B-cell cloning assay (in which CBA/N cells are inactive), and by karyotypic analysis of lymphoid, myeloid, and stem cell metaphases. Grafts of lymph node, spleen, anti-μ surface immunoglobin suppressed bone marrow, sIg^+ cell-depleted marrow, normal marrow, fetal liver, and yolk sac suggest: (*a*) there is little self-renewal of sIg^+ B cells in these models; (*b*) pre-committed cells have extensive proliferative/differentiative potential and at least initially contribute most

of the newly-formed B cells; (*c*) populations or pre-B cells obtained from various sources differ in their regenerative ability; (*d*) CBA/N mice are deficient in a category of pre-B cells which are found in fetal liver; and (*e*) selective B-cell chimerism results from grafting of unirradiated CBA/N mice.

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