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CHARACTERIZATION OF MURINE COLONY-FORMING B CELLS

II. Limits to *in Vitro* Maturation, Lyb-2 Expression, Resolution of IgD$^+$ Subsets, and Further Population Analysis[1]

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Murine colony-forming B cells were further studied to determine the extent of their heterogeneity and to investigate events that take place within the cultures. Immature, surface Ig-negative (sIg$^-$) precursors of B cells were found to have little, if any, ability to proliferate in semisolid agar cultures. However, such cells from adult bone marrow acquired this capability within 24 hr of conventional liquid culture. Bone marrow B cells that were IgM$^+$, IgD$^-$ did not become sensitive to anti-δ antibodies during cloning. These results suggest that two known B cell maturation events do not occur efficiently in semisolid cultures. Most clonable B cells in fetal, neonatal, and adult tissues expressed detectable amounts of Lyb-2 alloantigen. A majority adhered to nylon wool but nonadherent B cells were enriched in colony-forming and mitogen-responding cells. Azathioprine sensitivity was similar to that of B cells that respond to mitogenic, T-independent antigens. Three categories of colony-forming B cells were distinguishable on the basis of expression and function of sIgD receptors. One type lacked sufficient IgD to adhere to anti-δ antibody-coated plastic dishes. A second category was sIgD$^+$ but proliferated in the presence of anti-δ antibodies, whereas a third, IgD$^+$ subset, was inhibited by very small concentrations of anti-δ. The relative frequencies of these populations in various tissues were calculated. All IgD positive cells in neonates and in adult bone marrow appeared to be sensitive to anti-δ antibody added to the cultures, whereas approximately one-third of the B cells in adult spleen were anti-δ resistant.

Murine B lymphocytes form colonies in semisolid agar cultures, and studies of the requirements for their optimal clonal proliferation have provided the basis for a reliable and simple functional assay for enumerating and characterizing B cells (1–5). In this system, individual B cells are dispersed in a semisolid gel matrix and divide in response to mitogens. This process is unaffected by helper or suppressor T cells. Macrophages elaborate enhancing and inhibitory substances, but their influence can be overcome with one of several culture variations such that colony formation is directly proportional to numbers of B cells cultured. It is known that clonable B cells are present in all lymphoid tissues and include phenotypically different subsets of B cells, e.g., IgM$^+$ Ia$^-$ δ$^-$; IgM$^+$ Ia$^+$ δ$^-$; and IgM$^+$ Ia$^+$ δ$^+$ cells (5, 6). The cells that respond in this assay are also heterogeneous in sensitivity towards capping of their surface Ig receptors, their size, buoyant density, density of their sIg, and C3 receptor expression (5, 7, 8). Partially immunodeficient CBA/N strain mice lack colony-forming B cells at all stages of their development, and since normal embryos have clonable B cells by day 17 of gestation, the CBA/N defect must affect the implementation of an early genetic program (9–11). The expansion and differentiation of functional B cells can be followed with the cloning assay after engraftment of normal hemopoietic cells in irradiated or unirradiated CBA/N mice, and this has made it possible to study progenitor populations in fetal and adult tissues (12). The utility and versatility of this system would be increased still further if more were known about responsive cell populations and the events that take place within the cultures. For certain applications, it would be useful to know if immature cells can be recruited to respond and continue their maturation during the 6-day culture period. Observations to be presented here indicate that cloning ability is acquired by immature B cells simultaneously with surface Ig expression, and that neither this maturation step nor acquisition of sIgD by δ$^-$ B cells occurs efficiently in semisolid cultures. In contrast, less mature precursors in adult bone marrow became functional within 24 hr in relatively crowded liquid cultures. Characterization with respect to sIgD and Lyb-2 expression as well as other parameters further illustrate the heterogeneity of clonable B cells, and two categories of IgD$^+$ B cells were resolved by using purified hybridoma anti-δ antibodies with a dish depletion technique.

MATERIALS AND METHODS

Animals. CBA/Cum or C57BL/6 Cum mice from Cumberland View Farms (Clinton, Tenn.) or CBA/H-T6T6 mice from our own colony were used for all experiments. For the purpose of staging embryos, the appearance of vaginal plugs after overnight matings was taken as day 0 of gestation.

Cell cultures. McCoy's modified 5a medium containing 15% fetal calf serum (FCS) and amino acids was used for all semisolid agar cultures as in previous studies (3–5). B lymphocytes were cloned in the presence of 50 μM 2-mercaptoethanol (2-ME)[2] and either 25 μg/ml *Salmonella typhosa* endotoxin (LPS)

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[2] Abbreviations used in this paper: CFU-B, colony-forming B cell; CFU-c, *in vitro* granulocyte-macrophage colony-forming cell (granulo-

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or 1.0% washed sheep red blood cells (SRBC). The importance of using LPS or SRBC for achieving reproducible colony numbers and a linear dose-response curve is discussed in previous reports (2–5). Cultures were examined with a dissecting microscope for the presence of colonies (aggregates of >20 cells) after 6 days of incubation at 37°C in a fully humidified atmosphere of 7% CO_2 in air. Granulocyte-macrophage progenitors (CFU-c) were similarly cloned in the absence of 2-ME in cultures containing WEHI-3 leukemia cell conditioned medium as a source of colony-stimulating activity. For mitogen-stimulated liquid cultures, RPMI 1640 medium with the same additives and 5% FCS were used. Triplicate cultures (0.2 ml of 2×10^6 cells/ml) were stimulated with 5 μg of LPS or 25 μg of agar extracted mitogens (3), cultured for 3 days, pulsed with ^{125}IudR[2] (0.2 μCi/well) in the presence of 10^{-6} M cold FUdR[2] for 4 hr, and finally harvested and assessed for ^{125}I incorporation, as previously described (3, 5, 9). Bone marrow cell suspensions depleted of B cells were held overnight in the same medium in 35 x 10 mm tissue culture dishes with 2×10^6 cells in 2 ml of medium.

Anti-immunoglobulin antibodies. Anti-IgM antibodies were collected from goat anti-mouse myeloma M 104E (μ, λ) serum on an absorbent of HPC-76 (μ, κ) and rendered specific for μ-chains by repeated passage over HOP-1 (γ_{2a}, λ), UPC 10 (γ_{2a}, κ), and MPC 11 (γ_{2b}, κ) immunoabsorbents. Goat anti-κ antibodies were prepared by immunizing with F(ab')$_2$ fragments of MOPC-460 (α, κ), eluting the resulting antibodies from the HPC 76 column and then cross-absorbing on the HOP-1 column. The 10-4.2 anti-IgD allotype hybrid cell line produced at Stanford (13) was obtained from the Salk Institute Cell Distribution Center and maintained in RPMI 1640 medium. Hybridoma culture supernatants were raised to pH 8.6 with concentrated Tris and passed through a column of *Staphylococcus* protein A-Sepharose (Pharmacia, Piscataway, N. J.). Approximately 3 μg protein/ml of culture fluid was eluted from the column with glycine-HCl buffer, pH 2.8, and this was neutralized, concentrated, and dialyzed. This preparation contained only murine IgG2a by Ouchterlony analysis and was subsequently referred to as pure 10-4.2 anti-δ. It was not cytotoxic for B cells with our rabbit complement (C) but inhibited cloning of B cells from CBA mice when added directly to the cultures. Cultures of B cells from A/J or C57BL/6 strain mice were unaffected by this preparation.

Anti-Lyb-2 serum. Antiserum specific for Lyb-2.1 was made by immunizing (C3H.I × B6)F_1 mice with the I strain spontaneous ascites tumor I.29. Repeated absorption with various Lyb-2.1 negative tissues was required to remove autoantibody reactive especially with thymocytes (14). Anti-Lyb-2.2 serum was prepared by immunizing (C3H/An × BALB/c)F_1 mice with CE/J spleen and by repeated absorptions with Lyb-2.2 negative tissues (including in particular tissues of HSFS/N strain mice) to remove autoantibodies and antibodies of unidentified specificities (15). Both anti-Lyb-2 sera were tested for specificity on the Lyb-2 congenic B6(Lyb-2.1) and B6(Lyb-2.2) strains.

Alloantiserum treatment of cells. Single cell suspensions were prepared, washed, and adjusted to 2 or 5×10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated FCS, mixed with equal volumes of anti-Lyb-2.1 or anti-Lyb-2.2 serum (diluted 1:20) and kept for 30 min at 4°C. The cells were then layered over and spun through heat-inactivated FCS, resuspended in rabbit C (preselected for low background cytotoxicity, absorbed with CBA spleen cells, and diluted 1:12), held at 37°C for 45 min, and washed once. Cultures were then set up on the basis of the starting number of cells.

Multipotential stem cell assay. Multipotential stem cells (CFU-s) were detected by injecting 7.5×10^4 bone marrow cells i.v. into irradiated (900 R) mice. After 8 days, spleens were removed, placed in Bouin's fixative, and macroscopic colonies were counted.

Cell separations. B cells from spleen, lymph nodes, or bone marrow were depleted by adherence to anti-Ig-coated Petri dishes according to the method of Wysocki and Sato (16). Purified anti-μ or anti-δ allotype antibodies at 5 μg/ml in 10 ml of 0.05 M Tris-HCl, 0.15 M NaCl, pH 9.5 buffer were placed in polystyrene 100 x 15 mm Petri dishes (Falcon No. 1001, Oxnard, Calif.). These were then allowed to stand at room temperature for at least 40 min before washing four times with phosphate-buffered saline (PBS) and once with 1.0% FCS in PBS. Then 5 to 10×10^6 cells in 3 ml of PBS with 5% FCS were added and held at 4°C for 70 min. Dishes were swirled at least once during this incubation to ensure even distribution. Nonadherent cells were then harvested with three gentle washes by using 5% FCS, PBS. Nylon wool column separations were performed according to the method of Julius *et al.* (17). The contents of three LP-1 Leuko-Pak filters (Fenwal Labs., Morton Grove, Ill.) were boiled six times each in 6 liters of triple-distilled H_2O, and dried at room temperature. This was then pulled apart and 6 to 8 ml were packed into 10 ml syringes and autoclaved. These were moistened with phosphate-buffered balanced salt solution and warmed to 37°C before adding 10^8 spleen cells in 2 ml of the same solution. This was held at 37°C for 45 min and then eluted with 25 ml of warm salt solution. The incidence of B cells in separated cell suspensions was estimated by immunofluorescence by using rhodamine-labeled anti-κ antibodies.

Anti-μ suppression of mice. Differentiation of sIg$^+$ B cells was inhibited in young CBA/Cum mice by giving four daily injections of 0.5 mg of purified goat anti-mouse μ-chain antibodies in 0.05 ml i.p. beginning at birth and weekly injections thereafter of 1.0 mg.

RESULTS

Requirement for surface immunoglobulin (sIg) expression. Injection of mice from birth with anti-μ antibodies aborts development of sIg$^+$ B cells but spares sIg$^-$, cytoplasmic immunoglobulin-positive (cIg$^+$) pre-B cells in bone marrow (18). B cells capable of proliferating in semisolid agar cultures were not detectable in 8-day-old anti-μ suppressed mice, and these were diminished by an average of 89%, 98%, and 99% in bone marrow, spleen, and lymph nodes, respectively, of older treated mice (Table I). The incidence of Ig$^+$ cells in these organs was determined by immunofluorescence, and these correlated with numbers of functional B cells. In other experiments, suspensions of bone marrow, lymph node, and spleen cells were held at 4°C in anti-μ-coated Petri dishes before being decanted and placed in culture (Table II, expt. I). In all cases, greater than 90% of the clonable B cells adhered to the dishes, and this often exceeded 99%. When immunofluorescence was performed on the same cell suspensions, the incidence of Ig$^+$ cells generally corresponded with relative numbers of colony-forming cells. However, if bone marrow suspensions depleted of sIg$^+$ cells were held overnight in conventional liquid cultures before plating in semisolid medium, substantial increases in numbers of clonable cells were demonstrable (Table III). In the six experiments shown, B cell depletion averaged 92%, and from 3 to 20 times

cyte-macrophage progenitor); CFU-s, *in vivo* spleen colony-forming cell (multipotential stem cell); sIg, surface immunoglobulin; cIg, cytoplasmic immunoglobulin; 2-ME, 2-mercaptoethanol; ^{125}IUdR, 5-iodo-2'-deoxyuridine; FUdR, 5-fluoro-2'-deoxyuridine; CSA, colony-stimulating activity.

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the residual numbers of functional cells were recovered after liquid culture. These results suggest that very few, if any, cells that lack surface IgM are capable of colony formation. Furthermore, it appears that although pre-B cells in adult bone marrow fail to mature into colony-forming cells after dispersion in semisolid cultures, they can undergo this maturation step in conventional liquid cultures. Other data in Table II illustrate that cell suspensions depleted of IgD^+ cells by adherence to anti-δ allotype-coated dishes do not become sensitive to anti-δ antibodies during semisolid cultures (see below).

Cycle status of bone marrow clonable B cells. The experiments described above suggest that sIg^-, cIg^+ cells do not proliferate in semisolid agar cultures. Such putative pre-B cells are known to be rapidly dividing (19, 20), and we tested the effect of hydroxyurea pretreatment on the cloning ability of bone marrow cells. Granulocyte-macrophage progenitors (CFU-c) are also know to be in cycle (21), and these were assayed in the same cell suspension in a duplicate set of cultures containing a source of colony-stimulating activity (CSA) and no mercaptoethanol. CFU-c numbers in these cultures were reduced by 33%, whereas the cloning potential of bone marrow B cells was unaffected (Table IV). Similar results were obtained by using high specific activity ^{3}H-thymidine to suicide dividing cells, but only when the cells were washed in 10 M cold thymidine before culture (data not shown). Failure to prevent thymidine reutilization in this way resulted in an erroneous conclusion drawn in a preliminary study (22).

TABLE I

Incidence of colony-forming B cells in anti-μ-suppressed mice

Experimental Group	8 Days of Age		31 Days of Age		
	Bone marrow	Spleen	Bone marrow	Spleen	Lymph nodes
Anti-μ suppressed	0[a]	0	52 ± 5	16 ± 2	1 ± 1
Control	70 ± 7	>500	406 ± 16	752 ± 27	749 ± 6

[a] Colonies/10^5 cultured cells ± S.E.

Expression and function of sIgD on clonable B cells. In our previous studies, we added a noncytotoxic rabbit anti-mouse IgD serum directly to cultures in order to assess IgD expression and found that extremely small amounts of antiserum were sufficient to achieve a plateau of inhibition (5). We have now confirmed this result with purified hybridoma antibodies to IgD allotype and found that as little as 50 ng/ml of anti-δ specifically inhibited colony formation (23). Substantially larger quantities of goat anti-μ or anti-κ antibodies were required for essentially complete suppression of all colony-forming cells (24). The maximum inhibition obtainable with anti-δ added to spleen cell cultures was on the average 63%, whereas immunofluorescence studies suggest that greater than 80% of all splenic B cells are δ^+ (25–27). This discrepancy suggests that clonable B cells are either not typical of B cells as a whole or that a fraction of IgD^+ cells is resistant to anti-δ antibodies. To investigate this further, we determined numbers of δ^+ cells by allowing them to adhere to anti-δ coated dishes and compared the results with direct addition of anti-δ to the cultures (Table II). In the example shown, about 90% of the colony-forming cells from CBA spleen adhered to anti-δ coated dishes, and the nonadherent cells from

TABLE III

Apparent maturation of bone marrow pre-B cells in liquid cultures

Cells Cultured	Total Clonable B Cells					
	Expt. I	Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI
Unfractionated bone marrow	6,460[a]	10,780	10,200	9,000	2,720	7,620
B cell-depleted bone marrow	1,220	940	300	580	60	880
Depleted marrow after liquid culture	3,741	3,741	1,036	3,257	1,171	8,483

[a] An aliquot of cells from each fraction was placed in LPS-potentiated semisolid culture. The incidence of colonies was then multiplied by the total number of nucleated cells per fraction. In experiments I to V liquid cultures were for 24 hr and in experiment VI for 36 hr.

TABLE II

Adherence of clonable B cells to antibody-coated dishes

Expt. No.	Cells from	Strain	Separation Procedure	Total Nucleated Cells Recovered × 10^{-6}	Clonable B Cells Recovered	
					Total	Anti-δ resistant[a]
I	Spleen	CBA/H		3.65	12,012	4,897
			Control (plain dish)	3.89	11,356	5,148
			Anti-μ-coated dish	1.33	71	1
			Anti-δ-coated dish	2.34	1,182	1,068
		C57BL/6		3.95	52,367	54,538
			Control (plain dish)	3.35	46,626	48,048
			Anti-μ-coated dish	1.50	240	16
			Anti-δ-coated dish	3.86	38,303	37,367
II	Bone marrow	CBA/H		5.04	13,558	4,133
			Control (plain dish)	4.87	14,854	8,620
			Anti-δ-coated dish	4.62	7,762	7,808
	Lymph nodes	CBA/H		8.19	91,400	18,346
			Control (plain dish)	7.63	84,693	18,236
			Anti-δ-coated dish	5.64	5,527	3,440
	Bone marrow	C57BL/6		4.86	28,237	29,986
			Control (plain dish)	4.76	23,562	24,562
			Anti-δ-coated dish	4.60	20,516	20,424

[a] Total number of B cells per fraction that were clonable in the presence of 2 μg/ml 10-4.2 hybridoma anti-δ antibody. Details of the fractionation procedure are given in *Materials and Methods.*

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TABLE IV

Resistance of bone marrow colony-forming B cells to hydroxyurea

	Control	Hydroxyurea	% Inhibition
B cell colonies	110 ± 18[a]	107 ± 12	3
Granulocyte-macrophage colonies	164 ± 10	110 ± 7	33

[a] Mean number of colonies/10^5 initial cells ± S.E. Cells were pretreated for 1 hr at 37°C with 2×10^{-3} M hydroxyurea or medium and washed before culturing.

TABLE V

Composition of clonable B cell populations

	Surface IgD$^+$			Surface IgD$^-$
	Anti-δ sensitive	Anti-δ resistant	Total	
		%		%
Bone marrow	51[a]	0[b]	46[c]	54[b]
Spleen	59	33	92	8
Lymph nodes	79	18	97	3

[a] Average inhibition by direct addition of hetero or allo anti-δ antibodies in 10 experiments.

[b] Calculated from data in the other columns.

[c] Average of 3 anti-δ-coated dish depletion experiments.

this suspension contained the same number of functional cells when assayed in the presence or absence of anti-δ. This is contrasted to the 57% reduction of colony numbers by addition of anti-δ to cultures of unselected CBA spleen cells. Therefore, only sIgD$^+$ B cells are sensitive to anti-δ added to the cultures, and furthermore, some sIgD$^+$ B cells are insensitive to the continuous presence of large amounts of anti-δ antibodies. Only a small fraction of the total nucleated cells and clonable cells were nonspecifically lost in uncoated dishes, whereas anti-μ-coated plates depleted 99% of either CBA/H or C57BL colony-forming B cells. Allotype specificity of the anti-δ was demonstrated by its inability to deplete C57BL cells or to inhibit C57BL cultures on direct addition. These data resolve three major categories of clonable B cells whose approximate frequencies in bone marrow, spleen, and lymph nodes are calculated in Table V. Cells that lack detectable amounts of sIgD are frequent in bone marrow, and comprise a small fraction of peripheral B cells. These are insensitive to anti-δ antibodies. A second subset is sIgM$^+$ and sIgD$^+$, and is particularly sensitive to anti-δ antibodies. All of the marrow sIgD$^+$ B cells are of this type. An additional category of B cells is apparent in other organs, particularly spleen, and these IgD$^+$ cells are refractory to the inhibitory effects of anti-δ on their proliferative responses. These calculations and conclusions derive from the assumption that various B cell populations have roughly equivalent cloning efficiencies in LPS-potentiated cultures (5) and were only possible because of the very low nonspecific cell losses obtained with the dish separation technique.

Adherence of clonable B cells to nylon wool. Nylon wool columns are widely used for preparative separation of B and T cells (17). We noticed that although numbers of clonable B cells were reduced in column effluents, the reduction did not parallel the depletion of sIg$^+$ cells. In the example shown in Table VI, approximately 43% of the initial number of spleen cells were recovered in the effluent, and these were 89% depleted of B cells. However, when the cloning potential of these cells was expressed on a per B cell basis, the incidence of cells with this functional capability was enriched approximately 3-fold as compared with unfractionated B cells. Colony formation was found to be linear and completely sensitive to anti-μ antibodies (data not shown), and these cells were enriched with respect to mitogen responsiveness in liquid cultures as well. We conclude that the majority of clonable B cells are nylon adherent, but that nonadherent populations are preferentially enriched for cells with this capability.

Azathioprine sensitivity. B and T lymphocyte populations have been characterized with respect to sensitivity to the immunosuppressive drug azathioprine (28, 29), and we have done the same for clonable B cells. From 4 to 6 μg/ml of the drug was sufficient for complete inhibition of B lymphocyte cloning with the drug present throughout culture, and we saw no consistent difference in the sensitivity of bone marrow, spleen, or lymph node cell cultures potentiated with either LPS or SRBC (Fig. 1). The dose-response curves did not suggest heterogeneity of clonable B cells in this respect, and would allow them to be classified as moderately resistant to azathioprine. Granulocyte-macrophage progenitors were even more resistant, and greater than 10 μg/ml was required for complete inhibition.

Expression of Lyb-2. The data illustrated in Tables VII and VIII show, with two target strains representing two of the three known Lyb-2 alleles, that this alloantigen is uniformly expressed on immature and mature colony-forming B cells. Preliminary experiments with different target cells established the specificity of the antisera and the requirement for C for cell lysis. Also shown in these tables is the fact that conditions that completely eliminate Lyb-2-bearing, colony-forming B cells spare both granulocyte-macrophage progenitors (CFU-c) and multipotential stem cells (CFU-s) in bone marrow.

TABLE VI

In vitro responsiveness of nylon wool column-passaged spleen cells

	% κ$^+$ Cells	Semisolid Cultures Colony-Forming Cells/10^5 Cultured B Cells		Liquid Cultures cpm ^{125}IUdR Incorporated/10^5 Cultured B Cells	
Unseparated spleen	42.0	Unpotentiated	928	Background	364
		LPS potentiated	2,200	LPS	37,426
				Agar mitogens	19,757
Nylon wool-passaged spleen	4.5	Unpotentiated	2,830	Background	3,111
		LPS potentiated	7,780	LPS	200,061
				Agar mitogens	57,100

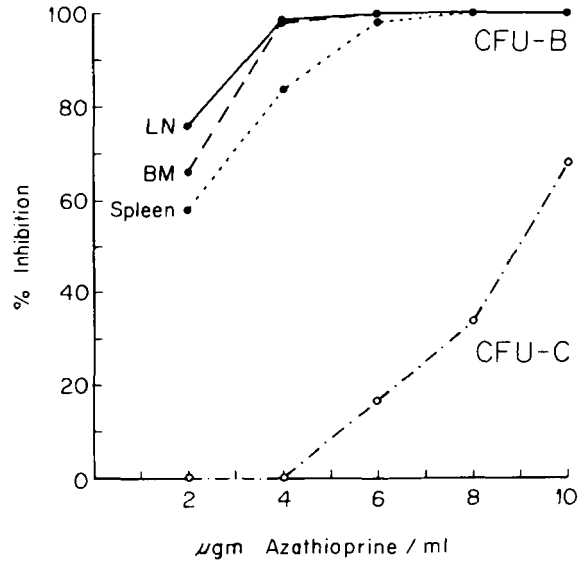

Figure 1. Sensitivity of CFU-c in bone marrow and CFU-B in various adult tissues to azathioprine.

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TABLE VII

Expression of Lyb-2.2 on clonable B cells in CBA/H mice

Source of Cells	C Alone	Antiserum + C	% Reduction
18-day fetal liver	10 ± 2[a]	1 ± 1	90
Newborn spleen	83 ± 7	2 ± 1	97
Adult bone marrow	289 ± 12	11 ± 3	96
Adult spleen	616 ± 108	56 ± 24	91
Adult lymph nodes	128 ± 12	0	100
Bone marrow CFU-c	155 ± 8	148 ± 1	[b]
Bone marrow CFU-s	14 ± 1	12 ± 1	[b]

[a] Mean number of colonies/10^5 initial cells ± S.E.

[b] Not significant.

TABLE VIII

Expression of Lyb-2.1 on clonable B cells in CBA/Cum mice

Source of Cells	C Alone	Antiserum + C	% Reduction
19-day fetal liver	25 ± 3[a]	3 ± 1	88
Newborn spleen	71 ± 5	13 ± 3	82
Adult bone marrow	236 ± 11	8 ± 1	97
Adult spleen	745 ± 69	78 ± 7	90
Adult lymph nodes	213 ± 13	0	100
Bone marrow CFU-c	87 ± 10	103 ± 7	[b]
Bone marrow CFU-s	8 ± 3	19 ± 5	[b]

[a] Mean number of colonies/10^5 initial cells ± S.E.

[b] Not significant.

DISCUSSION

Cloning in semisolid agar is a simple means of enumerating and characterizing functional B cells, and the studies described here provide additional insight into the populations of cells that can be detected. Essentially all were defined as being $sIgM^+$ and Lyb-2^+. In adult tissues some are δ^-, and two types of δ^+ cells can be distinguished by their sensitivity to hybridoma anti-δ. Dividing, sIg^- precursors of B cells had little, if any, cloning potential, and there was no indication that $sIgD^-$ cells could become $sIgD^+$ within semisolid cultures. However, some pre-B cells in adult bone marrow appear to be capable of maturing within short-term liquid cultures and acquiring cloning potential. Most clonable B cells adhered to nylon wool, but nonadherent sIg^+ populations were enriched in colony-forming and mitogen-responding cells. Azathioprine sensitivity was similar to that of B cells that respond to mitogenic, T-independent antigens.

Observations in these and previous studies suggest that conditions of semisolid culture are not sufficient for differentiation of immature cells, and B cells must acquire sIg before being capable of clonal proliferation. Colony-forming cells and sIg^+ cells appear simultaneously at 16.5 days gestation in fetal liver, whereas cytoplasmic Ig^+, sIg^- cells are detectable much earlier (10, 30). Similarly, anti-μ suppression *in vivo* aborts development of sIg^+ as well as clonable B cells, but spares pre-B cells (18). Finally, pre-B cells in adult marrow are known to be cycling (19, 20), whereas there was little indication of hydroxyurea sensitivity of colony-forming cells from that tissue. Since depletion of colony-forming cells on anti-μ coated dishes often exceeded 99% and corresponded with depletion of Ig^+ cells, null cells such as those described in spleen must have little, if any, cloning potential. The $sIgD^-$ cells that did not adhere to anti-δ-coated dishes cloned equally well in the presence or absence of anti-δ. It thus appears that large numbers of $sIgD^-$ cells do not mature to an $sIgD^+$, anti-δ-sensitive stage within the semisolid cultures. In contrast, there was some indication that sIg^-, pre-B cells become functional during overnight liquid culture. Many previous studies have demonstrated *in vitro* maturation in conventional cultures (19, 31–36), and this process may be favored by high cell density conditions (18). Depending on the stage of development, pre-B cells from fetal tissues may require much longer than 24 hr of *in vitro* culture for functional maturation, and we have found other differences in fetal and adult pre-B cells (12, 35). It remains to be determined if mitogens or other factors in fetal calf serum or cell-cell interactions "induce" pre-B cell maturation in these cultures. Alternatively, a population of precursors may have reached a stage where they can spontaneously mature to functional capability within crowded liquid cultures. Combining cell separation techniques with the short-term liquid culture procedures used here should provide insight into this question and make it possible to compare pre-B cells in fetal, adult, and aged tissues.

There are three known alleles of Lyb-2, and these have been mapped to chromosome 4 (14, 15, 37, 38). The gene product is apparently expressed on both immature and mature clonable B cells. As with anti-Ia and C cytotoxicity (5), bone marrow B cells were killed without the incidence of stem cells or granulocyte-macrophage progenitors being affected. The incidence of cells expressing Lyb-2 as determined by cytolysis or rosetting suggests that this alloantigen may not only be expressed on all sIg^+ B cells but on some additional cells as well (14). For example, Lyb-2+ cells were less depleted than Ig^+ cells and clonable B cells in anti-μ suppressed animals. In addition, established B cell lines that are sIg^- partially express Lyb-2 (M. Scheid and C. J. Paige, unpublished observations). This alloantigen could therefore be a particularly useful surface marker for detecting and manipulating sIg^- progenitors of B lymphocytes.

Much has been written about the possibly unique function of IgD receptors in the activation of B cells. A special role in responsiveness to nonmitogenic and T-dependent antigens, regulation of tolerance sensitivity, eliciting anti-idiotype production, and in expansion of memory from virgin cells have all been posited (39–43). Three types of clonable B cells can be discriminated on the basis of surface IgD expression and function, and their approximate incidence in bone marrow, spleen, and lymph nodes is given in Table V. The first subset is not adherent to anti-δ-coated dishes, and its proliferative ability is not affected by anti-δ antibodies added directly to the cultures. Their frequency was estimated by the proportion of functional cells not depleted by the dish technique, and this approximates published determinations of $sIgD^-$ B cells made by immunofluorescence techniques (25–27). Surface IgD may be lost as a consequence of mitogen or antigen stimulation (44, 45), so this category may include some memory B cells as well as virgin B cells that have not yet acquired sIgD. A second category is $sIgD^+$ and is highly sensitive to anti-δ antibodies added to the cultures (5, 6, 23). In this respect, anti-δ presents a unique and artificial negative signal to B cells that is unlike the effects of either antigen or anti-idiotype. The difference between adherence to anti-δ-coated dishes and sensitivity to anti-δ in cultures reveals a third population of clonable B cells. These cells apparently possess sIgD receptors that do not function in receiving an inhibitory stimulus. This population is not demonstrable in adult bone marrow, and comparison of immunofluorescence studies with the ontogeny of anti-δ sensitivity (5, 27, 46) suggests that it must also be lacking in neonates. From this distribution one could conclude that they are not immature and these cells could correspond to the effectors of *in vitro* responses to TNP-*Brucella* or TNP-LPS in adult spleen (47). It has been recently shown that such cells are actually $sIgD^+$ even though they are insensitive to anti-δ antibodies and are functionally

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unaffected by selective cell surface δ removal. It was proposed that the delivery of a second signal through mitogen receptors by mitogenic carriers makes the IgD receptors on such cells unnecessary for an antibody response (47). We have found that two categories of δ^+ cells behave quite differently in the presence of anti-δ during a mitogen-driven, proliferative response. It remains to be determined whether the surface densities of μ and δ, the ratio of these, or other intrinsic differences correlate with the functional differences in these cells. It is noteworthy that this third population of cells may be absent from bone marrow and comparisons of δ^+ cells from bone marrow and spleen in sensitivities of their immune responses to anti-δ could be informative.

Nylon wool columns are widely used for the preparative separation of murine B and T cells (17). No doubt the effluent from such columns is sufficiently depleted of B cells for many applications. In our hands the depletion corresponded to that obtained in the original published description, i.e., approximately 90%, and the nonadherent cells were not removed by an additional passage (17). The incidence of clonable B cells and cells responsive to LPS and agar mitogens were, however, increased among the nonadherent B cells. That these were B cells is clear from the fact that their clonal proliferation was anti-μ sensitive. Others have found that B cells present in nylon passaged suspensions preferentially respond to certain T-independent antigens (M. B. Rittenberg, K. L. Pratt, and T. V. Tittle, personal communication).

Sensitivity to azathioprine has been employed to discriminate populations of functional T lymphocytes, and it has been reported that B cell responses to T-dependent and T-independent antigens are differentially sensitive to azathioprine (28, 29). We found that cultures of B cells from bone marrow, spleen, and lymph nodes with either SRBC or LPS used as potentiators were all similarly inhibited by this drug. The complete inhibition obtained with 4 to 6 μg of azathioprine/ml of culture medium is comparable to the sensitivity of B cells responding to mitogenic, T-independent antigens (48).

We routinely add either LPS or SRBC to semisolid cultures or use an underlayer of adherent cells from peritoneal exudate. This is done to optimize numbers and size of colonies and to achieve a linear relationship between numbers of cultured B cells and numbers of proliferating foci (2–4). All of the data presented here were obtained with LPS-potentiated cultures, but in most cases parallel assays were done by using SRBC, and similar results were obtained. On the other hand, numbers of colonies in cultures containing both LPS and SRBC are often close to the sum obtainable with each used alone (4), and we have noted several cases where responses under the two conditions were not the same. An equivalent fraction of the B cells in newborn spleen, adult bone marrow, spleen, lymph nodes, and peripheral blood are clonable in LPS-containing cultures (5), whereas bone marrow cells clone less well in SRBC cultures than do spleen cells (8). An unusual type of anti-μ-resistant B cell found in tissues of NZB strain mice can be detected in LPS-containing, but not in SRBC-containing-cultures (23). Also, SRBC-potentiated cultures are more sensitive to inhibitors found in normal mouse serum than are LPS-containing cultures (P. W. Kincade, unpublished observations). Practical considerations may influence the choice of culture techniques employed (49), but we caution that small variations in methodology can occasionally greatly influence the results obtained.

The diversity of colony-forming cells described in this and preceding reports might imply that all sIg^+ B cells are potentially clonable. However, with the most optimal of culture conditions that we have used, more than half of the B cells were totally unresponsive. Also, partially immunodeficient CBA/N strain mice have no clonable cells. The residual B cells in these mice are unresponsive in cultures where the cell density is low, whereas they are capable of certain other humoral immune functions (9). The possible significance of the CBA/N defect with respect to definable B cell subpopulations has been discussed in some detail elsewhere (11). The major uncertainty at this point is whether CBA/N B cells are truly representative of some categories of lymphocytes from normal strains of mice, or alternatively whether all of their B cells are at least partially affected by the X-linked mutation.

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