THE "LY-1 B" CELL SUBPOPULATION IN NORMAL, IMMUNODEFECTIVE, AND AUTOIMMUNE MICE*

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Since the distinction was made between immunoglobulin-bearing (B) lymphocytes that give rise to antibody forming cells and thymus derived, Thy-1-bearing (T) lymphocytes responsible for a host of other immune functions, substantial effort has been directed toward finding individual cell surface markers that subdivide these populations. In the mid-1970s, the Lyt-1, Lyt-2, and Lyt-3 antigens were shown (with the assays then available) to be represented exclusively on T cells (1, 2) and to identify functionally distinct T cell subpopulations. Lyt-1 appeared to be restricted to the helper-amplifier subset, and Lyt-2 and Lyt-3 defined the suppressor-cytotoxic subset (3–6).

The development of monoclonal anti-Lyt reagents increased the sensitivity with which these antigens could be measured and significantly changed the status of the Lyt-1 (Ly-1)[1] marker: quantitative immunofluorescence studies with the fluorescence-activated cell sorter (FACS)[2] revealed that all Thy-1-bearing cells have some Ly-1 (8); that lower levels of Ly-1 on cytotoxic-suppressor cells explains the previous failure to detect this antigen on the Lyt-2,3 subset with cytotoxic depletion assays; and that the frequency of Ly-1^+ cells in normal spleen, in fact, is usually slightly greater than the frequency of Thy-1^+ cells in the same organ (9).

Recent studies (10, 11) demonstrating the existence of Ly-1-bearing B cells (Ly-1 B) indicate that such cells account for at least part of this excess. These findings, documented by FACS analyses on cell populations from normal animals, are consistent with a variety of previous observations: small numbers of Ly-1^+ cells are present in B cell areas of stained tissue sections in normal spleen (8); certain mouse B cell lymphomas synthesize and coexpress Ly-1 and IgM (7); old NZB mice tend to have increased numbers of Ly-1 bearing cells (12) that we have now shown to be IgM-positive and Thy-1-negative (unpublished observations); human B cell chronic lymphocytic leukemias tend to carry IgM and Leu-1 (13), a human cell surface molecule whose properties are homologous to mouse Ly-1 (14). Furthermore, a subpopulation

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[1] Several years after its initial description as the first in a series of lymphocyte differentiation antigens, Ly-1 was renamed Lyt-1 to reflect its apparently exclusive expression on T cells. We return here to the original usage since this antigen was recently demonstrated on B cell tumors (7) and we now report its presence on a subpopulation of normal B cells. No other cell surface antigen is presently called Ly-1.

[2] *Abbreviations used in this paper*: Bi, biotin; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; Fl, fluorescein; Ly-1 B, Ly-1-bearing B cell; PFC, plaque-forming cells; PI, propidium iodide; RIA, radioimmunoassay; TR, Texas red.

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of human B cells has been shown to carry Leu-1 and IgM (15, Hardy, R. R., T. Kipps, and L. A. Herzenberg. Manuscript in preparation), and a subpopulation of mouse B cells bearing Ly-1 has been shown to play a role in regulating antibody reponses (16).

In this work, we used multiparameter FACS analysis and sorting (10, 17) to define the basic characteristics (cell surface markers, frequency, organ location, ontogeny) of the Ly-1 B population in normal and immunodefective mice. In addition, we showed that, although sheep erythrocyte immunization does not generate Ly-1 B cells that secrete IgM antibody, the Ly-1 B population in NZB mice secretes large amounts of IgM in vitro and accounts for virtually all of the well-studied "spontaneous" IgM secretion by spleen cells from these autoimmune mice.

Materials and Methods

Mice. NZB/BinJ, NZB/N, (NZB × NZW)F_1, DBA/2, (CBA/N × DBA)F_1, and NZB.CBA/N (NZB X-id congenic mice carrying the CBA/N X-linked immunodeficiency) mice were provided by Dr. A. D. Steinberg (National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health). CBA/H-T6 and CBA/N mice provided by Dr. J. J. Haaijman (Institute of Experimental Gerontology, TNO, Netherlands) were bred at our animal facilities. BALB/c, SJA/9, and a C57BL/6 Ig congenic strain (C57BL/6.Igh-a) produced by Dr. Noel Warner (Becton-Dickinson Monoclonal Center, Mountain View, CA) were also bred in our facility. Nude (*nu/nu*) mice were provided by Dr. I. Weissman (Stanford University) and Dr. H. Wortis (Tufts Medical School). MRL/l mice were provided by Dr. D. Wofsy and Dr. W. Seaman (Veteran's Hospital, San Francisco, CA).

Monoclonal Antibodies. Reagents used in this study are shown in Table I.

Fluorochrome Coupling. Biotin (Bi) and fluorescein (Fl) labeling procedures have been described elsewhere (26, 27). Reagents were conjugated at low label:protein ratios to minimize backgrounds. Texas red (TR)-labeled avidin was prepared by adding 400 μg of TR reagent

TABLE I

Monoclonal Antibody Reagents

Clone name	Specificity	Isotype	Reference
53-7.3	Ly-1	Rat IgG2a	18, 19
53-6.7	Lyt-2	Rat IgG2a	18, 19
53-2.1	Thy-1.2	Rat IgG2a	18, 19
331.12	IgM	Rat IgG2a	20
30-H12	Thy-1.2	Rat IgG2b	18, 19
30-E2	"E2"	Rat IgG2b	18, 19
53-9.2	ThB	Rat IgG2c	18, 19, 21
10-4.2	IgD^a	Mouse IgG2a	22
MK-D6	Ia^d	Mouse IgG2a	23
11-5.2	Ia^k (Ia 2)	Mouse IgG2b	22
K10.6	Ly-19	Mouse IgG2b	24
K7.234	Ly-20	Mouse IgM	25

Rat monoclonal antibodies were isolated from culture supernatants by goat anti-Rat Ig affinity chromatography (18). Mouse monoclonal antibodies were purified from ascitic fluid by ammonium sulfate precipitation followed by ion-exchange chromatography.

(sulfonyl chloride rhodamine derivative; Molecular Probes, Junction City, OR) dissolved in 40 μl of dimethylformamide to 2 ml of a 5 mg/ml solution of avidin (affinity purified, Sigma Chemical Co., St. Louis, MO.) in carbonate buffer (pH 9.5). After rotating 4 h at room temperature, the conjugate was isolated on a 10-ml G25 column and the concentration was adjusted to 1 mg/ml. The dye-to-protein ratio was determined by measuring the absorbances at 280 and 595 nm. All reagents were stored at 4°C with 0.1% sodium azide and deaggregated at 100,000 *g* in a Beckman Airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) before use.

Staining for FACS Analysis and Sorting. Single-cell suspensions (from which the erythrocytes had been lysed by 0.165 M ammonium chloride) were stained in microtiter wells. For two-color staining, 0.5–1 million target cells were put into the antibody mixture (Fl-antibody A and Bi-antibody B) at a concentration of one million cells per 100 μl. After 30 min of incubation on ice, cells were washed three times. TR-avidin was added, and cells were further incubated on ice for 30 min. During the last 5 min of incubation, propidium iodide was added (50 μl per well at 1 μg/ml) to label dead cells (28). Cells were then washed as before and analyzed. A special staining medium (deficient RPMI 1640 that does not contain biotin, phenol red, and riboflavin; Irvine Scientific, Santa Ana, CA) supplemented with 3% heat-inactivated newborn calf serum, 10 mM Hepes, 0.1% sodium azide, pH 7.2, was used throughout the procedure. Larger numbers of cells (for sorting) were stained in conical centrifuge tubes.

To minimize nonspecific staining due to Fc receptor interactions, we deaggregated the staining reagents just before use. We included in all experiments separate control wells in which we "stained" with monoclonal antibodies of the same isotype (but of different specificity) as the staining reagent and subtracted this "background" frequency from the overall frequency of "positive" cells to determine the actual frequency of stained cells.

Two-color Immunofluorescence Analyses. To achieve the level of resolution required for these studies, we used a dual-laser FACS equipped with logarithmic amplifiers (for the fluorescence channels) to measure light scatter (size) and the amounts of pairs of fluorochrome-labeled monoclonal reagents bound to individual cells (29). In addition, we used propidium iodide (PI) staining (measured with a third fluorescence detection system) to eliminate the few (PI-stained) dead cells that were not rejected by the normal scatter-gating method for excluding dead cells (28). During the analysis, we used a VAX computer (VAX-11780; Digital Equipment Corp., Maynard, MA) to collect and store individual measurements on 30,000 (live) cells as list mode data for later analysis. Thus, we took into account all controls when establishing the boundaries ("gates") within which a particular subpopulation lies and when performing integrations to determine the frequency of cells within the gate.

Two-color staining data are presented as "contour plots" that can be viewed as representations of three-dimensional surfaces in which the levels of green and red fluorescence per cell define the location of cells on a 64 × 64 grid, and the frequency of cells at each location defines the elevation at that location. After smoothing this surface, contour lines are drawn to represent equal step changes in the elevation (frequency).

Immunizations and Antibody Responses. Donors were primed with 2×10^8 sheep red blood cells 4 d before sorting and assay. Plaque-forming cell (PFC) estimations were performed by the Cunningham method (30).

In Vitro Cultures. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 4×10^6/ml in microtiter wells. Usually, 4×10^5 cells were cultured in 0.1 ml. When smaller numbers of cells were cultured, culture volume was decreased proportionately.

Measurement of Secreted Immunoglobulins. Culture supernatants were cleared by centrifugation to avoid contamination by cellular debris, and IgM levels were estimated by a modified solid-phase competitive radioimmunoassay (RIA) (31) using monoclonal anti-IgM antibody coated at 10 μg/ml on microtiter wells. 20 μl of ^{125}I-labeled IgM (HPC-76) myeloma protein at a concentration of ~0.2 μg/ml was mixed with an equal volume of culture supernatant fluid (appropriately diluted) in the coated well and incubated for 1 h. The amount of IgM present in the supernatant was determined by comparing the counts bound to individual wells with a standard curve generated with unlabeled HPC-76 protein, serially diluted in 10% fetal calf serum (FCS), as a competitive inhibitor in the same assay. This assay detects amounts greater than 0.3 μg IgM/ml in culture supernatants.

Results

Cells Bearing Ly-1, IgM, and Other Classic B Cell Markers Are Present in Mouse Spleen. A small subpopulation of spleen cells in all mouse strains carries both IgM and Ly-1 (Figs. 1 and 2). These Ly-1 B cells, revealed by two-color FACS analysis of cells stained with fluorescent monoclonal antibodies, have more surface IgM than most B cells and considerably less Ly-1 than most T cells (i.e., they cluster in the high IgM, low Ly-1 region of the contour plot as shown in Fig. 1). They can be demonstrated equally well by staining with fluorescein-anti-IgM and biotin-anti-Ly-1 plus TR-avidin (as shown in Fig. 1) or with the converse pair of staining reagents (Fl-anti-Ly-1 and Bi-anti-IgM plus TR-avidin) (data not shown), and their frequency is well above the consistent "background" staining frequency (0.5–1% of spleen cells) (Fig. 2 and Table II).

Ly-1 B carry all differentiation markers found on B cells and none of the differentiation markers found on T cells, except Ly-1. Thus, as Table II shows, monoclonal antibodies to IgD, Ia, ThB, and some less well-known B cell markers, E2 (19), Ly-19, and Ly-20 (24, 25) stain the same number of Ly-1-bearing cells as the anti-IgM reagent. Monoclonal antibodies to Thy-1 and Lyt-2, in contrast, do not stain IgM-bearing cells above the typical background frequency of 0.5–1%. Preliminary data from staining experiments with FACS-isolated Ly-1 B cells (IgM^+, $Ly\text{-}1^+$) are consistent with these findings.

Most mice, including nude (*nu/nu*) and CBA/N (X-id), have about the same numbers of Ly-1 B in spleen (~2% of total spleen cells); however, the Ly-1 B frequency in NZB mice is substantially higher (usually >5% of spleen cells) and is even greater in the BW hybrid, i.e., (NZB × NZW)F_1 (Fig. 2). NZB X-id congenic mice, which fail to develop the typical NZB autoimmunity, and MRL mice, which develop a different type of autoimmune disease than NZB (32), apparently have normal numbers of Ly-1 B (Fig. 2 and preliminary observations, A. D. Steinberg and the authors).

In all mice tested (including X-id, nude, NZB, and BW hybrids), Ly-1 B are clearly detectable in spleen but essentially undetectable in lymph nodes, bone marrow, and thymus, i.e., the Ly-1 B frequency in the latter organs is equal to or barely above background staining frequency (Table III). The contrast between Ly-1 B representation in spleen and lymph node is particularly dramatic in NZB and BW mice, where there are 5 to 10 times as many Ly-1 B detectable in spleen as in lymph node. In addition, Ly-1 B can sometimes be found in peripheral blood, particularly in NZB and BW mice.

Ly-1 B Appear Early during Ontogeny. The appearance of Ly-1 B coincides with the appearance of the first IgD-bearing B cells detectable in neonatal spleen; however, unlike other B (or T) lymphocyte subpopulations, the frequency of the Ly-1 B subpopulation rapidly reaches adult levels. Thus, the proportion of Ly-1 B cells relative to other lymphocyte populations in the spleen decreases markedly with age (as these other populations reach mature levels), and Ly-1 B become a minor subpopulation in the adult. This ontogenic pattern is similar in BALB/c and NZB mice, even though NZB have many more Ly-1 B (Table IV).

Ly-1 B in the neonate have the same properties as Ly-1 B in the adult with regard to expression of surface markers, light scatter, and organ location (data not shown).

Ly-1 B Are in B Lymphocyte Subpopulation III. We recently resolved splenic B cells into three subpopulations (I–III) on the basis of the correlated expression of IgM and

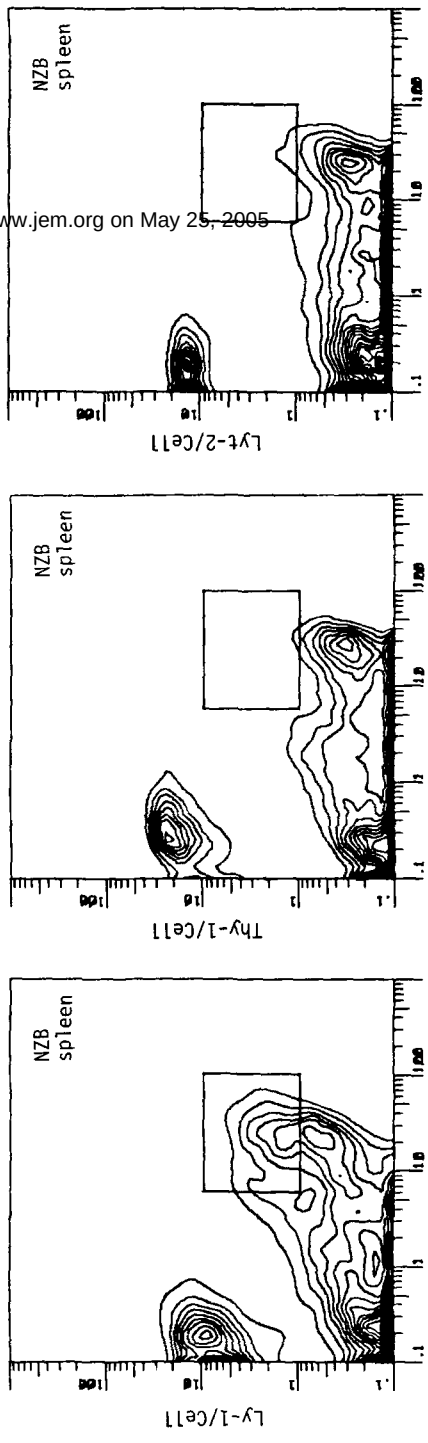

FIG. 1. Resolution of a population of Ly-1-positive B cells (IgM bright, Ly-1 dull) by two-color immunofluorescence. Spleen cells from a 2-mo-old NZB mouse were costained with Fl-anti IgM and either Bi-anti-Ly-1, Bi-anti-Thy-1 or Bi-anti-Lyt-2 + TR-avidin. The integration boundaries used to determine Ly-1 B frequencies reported in this publication are drawn onto the contour plot. In each experiment, frequency of Ly-1 B report is corrected by subtracting the "background" staining frequency (within the integration boundaries shown) of an aliquot stained with anti-IgM and anti-Thy-1 (see *Materials and Methods*).

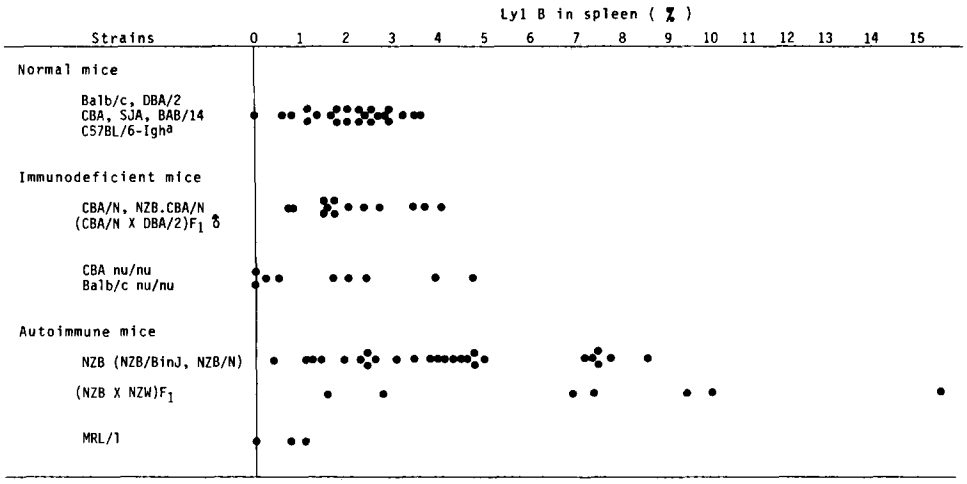

FIG. 2. Ly 1 B are present in all strains of mice tested and elevated in NZB and related strains. Spleen cells from 2- to 3-mo-old mice were stained and frequencies of Ly-1 B determined as described in Fig. 1

TABLE II

Ly-1 B Express B Cell Markers

Determinants detected by staining reagents		Positive cells in spleen		
		NZB	BALB/c	SJA
			%	
Experiment 1	IgM	49	59	43
	Ly-19 (polymorphic)	41	54	0
	Ly-20 (polymorphic)	0	54	0
	Ly-1 and IgM	8	3	4
	Ly-1 and Ly-19	7	3	0
	Ly-1 and Ly-20	0	2	0
Experiment 2	Ly-1 and IgM	4	ND‡	ND
	Ly-1 and Ia^d	5	ND	ND
	Ly-1 and IgD^a	4	ND	ND
	Ly-1 and E2	4	ND	ND
	Ly-1 and ThB	6	ND	ND
	Ly-2 and IgM	0.5*		
	Thy-1 and IgM	1*		

* Staining with these reagents is defined as background and subtracted from all other frequencies reported (see Materials and Methods). Background values for reagents detecting polymorphic determinants were obtained by staining the genetically negative controls (~2%).

‡ Not determined.

IgD (10, 17). Subpopulation III, like Ly-1 B, has high levels of surface IgM and low to intermediate levels of surface IgD. Its levels of other B cell markers match the levels found on Ly-1 B and it, too, is found almost exclusively in spleen (Fig. 3).

Ly-1 B frequencies approximate B cell subpopulation III frequencies in all mouse

TABLE III
Ly-1 B Reside Primarily in Spleen

	NZB cells* carrying		
	IgM	Ly-1	IgM and Ly-1 (Ly-1 B)
		%	
Spleen	55	48	5
Peripheral blood (lymphocyte)	23	58	0‡
	23	79	0
Lymph node	13	1	0
Bone marrow	2	98	0
Thymus			

* Average frequencies shown here for 2-mo-old NZB mice are representative of frequencies measured for adult mice in this strain. Ly-1 B frequencies in other mouse strains are roughly half as high.
‡ 1–2% Ly-1 B occasionally detectable.

TABLE IV
Ly-1 B Appear Early in Ontogeny

Surface marker(s) on cells	Spleen cells at various ages							
	NZB mice				BALB/c mice			
	5 d	2 wk	4 wk	8 wk	5 d	2 wk	4 wk	8 wk
				%				
IgM	10	30	56	53	15	17	48	52
Ly-1	9	16	43	57	8	13	34	41
IgM and Ly-1*	3	4	7	7	2	1	1	1
	(30)	(13)	(13)	(13)	(13)	(6)	(2)	(2)

* Ly-1 B in spleen (%) and, shown in parentheses, Ly-1 B in splenic IgM-bearing population (%).

strains except those with the X-linked immunodeficiency (X-id) defect (33) (Table V). In NZB, the increase in Ly-1 B frequency translates to a very high frequency of subpopulation III, making this normally small subpopulation easily visible in FACS analysis (Fig. 3). X-id mice show a similar elevation in subpopulation III; however, this elevation is not accompanied by an increase in Ly-1 B frequency. Thus, X-id mice have relatively large numbers of Ly-1-negative cells in subpopulation III, suggesting that, although subpopulation III in normal animals consists largely of Ly-1 B, it may also contain some cells that do not have surface Ly-1.

FACS Isolation of Ly-1 B. Classic methods that enrich for Ig-positive (B) cells (killing or panning with anti-Thy-1, isolating nylon-adherent cells), also enrich for Ly-1 B. None of these T-depletion methods, however, substantially alters the frequency of Ly-1 B relative to the frequency of other B cell subpopulations (data not shown). Thus, they offer little advantage for functional studies aimed at identifying unique role(s) played by Ly-1 B.

Using the FACS to sort spleen cells that costain with anti-IgM and anti-Ly-1 yields suspensions in which Ly-1 B represent 80–90% of the sorted cells. Ly-1-negative B cells constitute the major contamination; however, a small number of T cells (1–2%) are often present. Histological staining (Wright-Giemsa) of FACS-isolated Ly-1 B

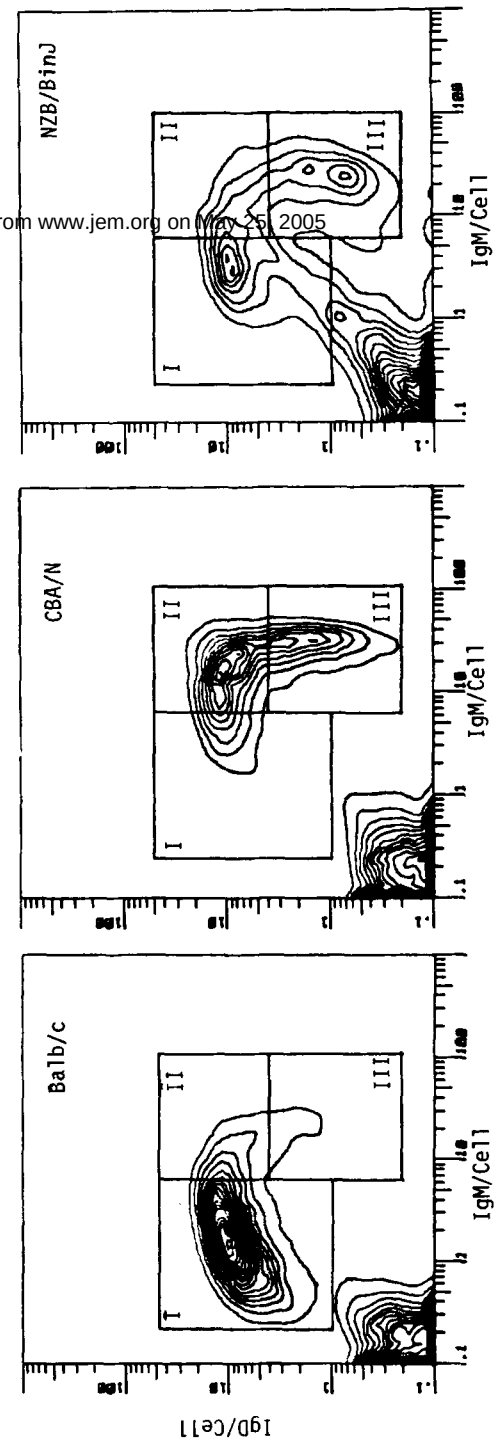

FIG. 3. B cell subpopulation III is elevated both in CBA/N and NZB mice (relative to all normal strains tested). Spleen cells were stained with Fl-anti-IgM together with Bi-anti-IgD + TR-avidin. The boundaries of the three B cell populations (10, 11) are shown for all three strains. In NZB mice the increase of population III is accounted for by the Ly-1 B cells, whereas the excess population III cells in CBA/N are Ly-1-negative (see Table V). All normal strains are similar in relative population frequencies to BALB/c.

TABLE V

B Cell Subpopulation III and Ly-1 B Frequencies Coincide Except in CBA/N (X-id) Mice

Mouse strain	Spleen cells	
	III*	Ly-1 B
	%	
Balb/c	3	3
DBA/2	4	3
C57BL/6.Igh^a	4	5
CBA	2	2
SJA	2	3
NZB	8	10
(NZB × NZW)F1	11	15
CBA/N	12	2

* Subpopulation III, defined by large amounts of surface IgM and relatively small amounts of surface IgD (see Fig. 3).

from either BALB/c or NZB mice reveals a population consisting mainly of "lymphoblastoid" cells whose appearance contrasts sharply with that of the typical small "resting" lymphocytes that constitute the majority of the B cells in BALB/c spleen (Fig. 4).

Ly-1 B from NZB Mice Spontaneously Secrete IgM In Vitro and Are Distinct from Antigen-stimulated IgM-secreting Cells. FACS-sorted Ly-1 B from NZB mice secrete ~40 times as much IgM as sorted Ly-1 B from BALB/c mice when cultured in the absence of added antigens or mitogens (Table VI). Ly-1-negative B cells, in contrast, do not secrete detectable levels of IgM, except when sorted from recently immunized animals (Tables VI and VII). Thus, the spontaneous IgM secretion by spleen cells cultured from nonimmunized NZB mice is due entirely to the activity of the Ly-1 B cells in these mice.

4 d after immunization with sheep erythrocytes (SRBC), in contrast, sorted Ly-1-negative cells from NZB spleen contain "direct" plaque-forming cells (PFC) that secrete IgM antibody (Table VII). The small number of PFC recovered in the sorted Ly-1 B population is proportional to the contamination of this sorted population by Ly-1-negative B cells. Thus, although Ly-1 B from NZB mice secrete IgM, they do not include the IgM-secreting cells (PFC) generated in the response to SRBC.

The IgM-secreting PFC in the Ly-1-negative population increase the amount of IgM produced by this population in comparison with the sorted Ly-1-negative population from nonimmunized animals. Ly-1 B IgM secretion, however, does not change after immunization (Table VII). Thus, IgM-secreting Ly-1 B are completely distinct from the PFC generated by immunization with SRBC.

Ly-1 Is More Firmly Bound to Ly-1 B Than IgM. Most of the IgM on Ly-1 B can be readily removed ("stripped") after staining with anti-IgM by culturing the stained cells for several hours. Ly-1, however, strips off only minimally under the same conditions. Thus, when FACS-isolated Ly-1 B that were stained before sorting with Fl-anti-IgM and Bi-anti-Ly-1 plus TR-avidin are cultured overnight, virtually all of the IgM-associated (green) fluorescence disappears from the cells, while most of the Ly-1 associated (red) fluorescence remains (Fig. 5). Ly-1 B stained with the converse pair of reagents (green Ly-1 and red IgM) yield the same results. Furthermore,

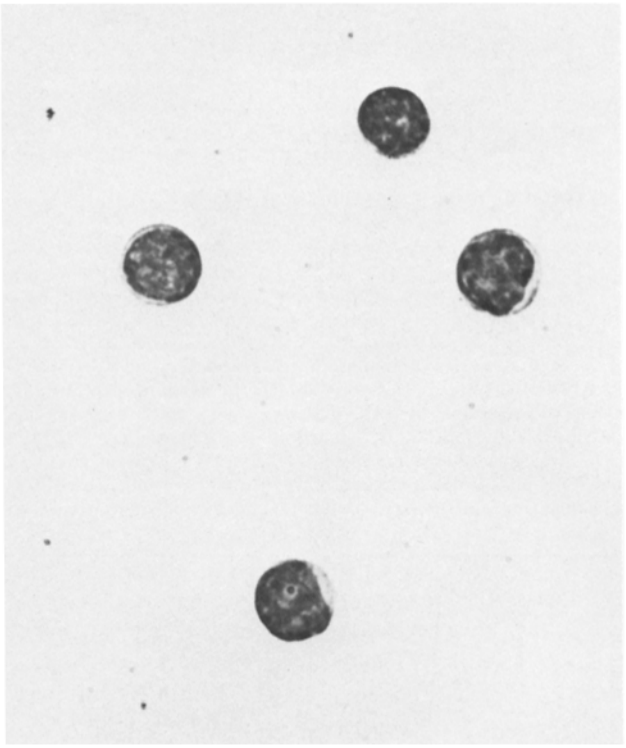

FIG. 4. Ly 1 B appear to be "lymphoblastoid" cells. Ly-1 B and other (Ly-1-negative) B cells sorted from a Thy-1-depleted NZB spleen cell suspension stained with Fl-anti-IgM and Bi-anti-Ly-1 + TR-avidin. Sorted cells were smeared onto glass slides, fixed, and processed by the standard Wright-Giemsa staining procedure.

TABLE VI

NZB Mice Have Ly-1 B That Secrete IgM

Mouse strain	Cultured spleen cell population	IgM secreted in vitro*			
		4 h		6 d	
		per well	per 10^6	per well	per 10^6
			μg		
NZB	Unseparated spleen	0.3	0.8	3.1	7.8
	FACS-sorted cells				
	Ly-1 B	0.3	0.8	15.0	37.5
	Other IgM^+ B	<0.03	<0.08	0.4	1.0
Balb/c	Unseparated spleen	<0.03	<0.08	<0.1	<0.3
	FACS-isolated cells				
	Ly-1 B	<0.03	<0.08	0.4	1.0
	Other IgM^+ B	<0.03	<0.08	<0.1	<0.3

* IgM secreted during the indicated culture period. 4×10^5 cells per well cultured for the indicated time in 0.1 ml RPMI 1640 + 10% FCS. No exogeneous antigens or mitogens were added. IgM in culture supernatant was measured by RIA. Sorted fractions represent 8, 17, 4, and 33% of spleen, respectively.

TABLE VII

Antigen-stimulated IgM-secreting Cells (PFC) Do Not Carry Ly-1

Spleen cell populations from SRBC-primed NZB‡	PFC*		Secreted IgM* per 10^6	Ly-1 B frequency*
	per chamber	per 10^6		
	number		*μg*	%
Stained unseparated spleen	83	553	12.7	5
Sorted cells				
All IgM^+	138	920	14.0	10
IgM^+, Ly-1^+ (Ly-1 B)	19	127	30.7	85
IgM^+, Ly-1^-	103	687	12.0	<1
IgM^-, Ly-1^+ (T cells)	0	<7	<0.2	<1

* Half of the cells in the sorted population were cultured at 1.5×10^5 cells per well in 0.04 ml RPMI 1640 + 10% FCS for 6 d, and the amount of secreted IgM in the supernatant was measured by RIA. The remaining cells were used immediately to estimate direct PFC (anti-SRBC) in the Cunningham assay (1.5×10^5 cells/chamber). Frequency of Ly-1 B was determined by reanalysis of sorted cells.

‡ 2-mo-old NZB mice immunized with 2×10^8 SRBC intraperitoneally 4 d before sorting. Sorted fractions represent 51, 11, 14, and 26% of spleen, respectively.

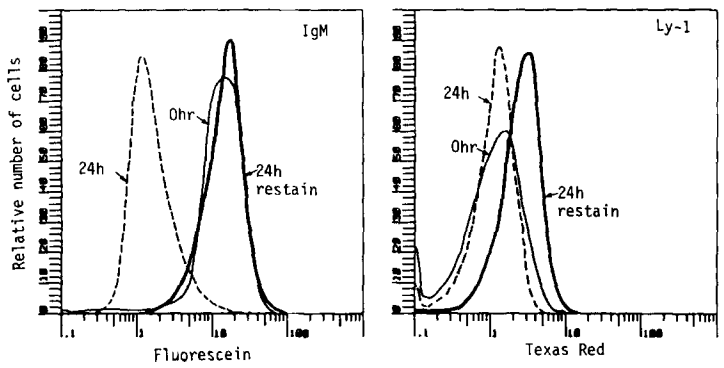

FIG. 5. Ly-1 remains firmly bound to Ly-1 B cells after 24 h in culture. An aliquot of FACS-isolated Ly-1 B (costained with Fl-anti-IgM and Bi-anti-Ly-1 + TR-avidin) from NZB spleen was analyzed immediately after sorting. The remaining Ly-1 B were cultured in RPMI 1640 + 10% FCS for 24 h. An aliquot of these cells was then incubated with TR-avidin alone (control), and another aliquot was restained with Fl-anti-IgM and Bi-anti-Ly-1 + TR-avidin. Staining histograms show the immediate reanalysis of the sorted population (0 h), the residual staining after 24 h in culture (24 h), and the amount of reexpression found by restaining this 24-h culture (24 h restain).

incubating stained and sorted Ly-1 B (before culture) with "second-step" anti-rat-Ig antibodies, which bind to the monoclonal anti-Ly-1 reagent and should thus facilitate stripping, fails to remove Ly-1. Thus, Ly-1 specifically remains on the cells, despite the removal of most of the IgM under the same conditions.

The difficulty in stripping Ly-1 poses a problem for attempts to directly demonstrate that Ly-1 B express, rather than acquire, Ly-1. Regrowth experiments in which sorted Ly-1 B are cultured after stripping show that surface IgM reappears and reaches its original level within 24 h (Fig. 5); however, because the original Ly-1-staining reagent remains associated with the cells, the increase in Ly-1 observed after culture and restaining is difficult to interpret. This increase could be due (*a*) to newly synthesized Ly-1; (*b*) to unmasking of previously present Ly-1 molecules; or (*c*) to a lack of saturation of all of the Ly-1 on the cells during the initial staining. Thus, although we

can show that Ly-1 B are clearly B cells in that they resynthesize their IgM receptors, we cannot state that these cells have synthesized the Ly-1 they carry.

These findings with splenic Ly-1 B recall evidence from similar studies with a B cell lymphoma that carries both Ly-1 and IgM (7). The Ly-1 on this lymphoma was shown to be a normal membrane component, internally synthesized and indistinguishable biochemically from the Ly-1 on T cells; however, it clearly remains associated with the cell membrane under conditions that readily remove IgM. Thus, our failure to strip Ly-1 from Ly-1 B appears to reflect the normal behavior of the Ly-1 molecule when expressed on B cell membranes.

Discussion

Ly-1 B first attracted attention (10, 11) because they carry surface molecules that were believed to be present only on T cells. However, as we have now shown, the presence of surface Ly-1 represents only one of a set of equally surprising characteristics that distinguish these cells from the typical B cells present in peripheral lymphoid organs. In essence, Ly-1 B constitute a B cell subpopulation different enough to raise the possibility that it might represent a distinct lymphocyte lineage separated quite early in evolutionary time.

We mainly used normal mice to define the characteristics of this subpopulation, since it constitutes a virtually invariant component of the overall B cell population in such mice. We completed this characterization, however, with data derived from studies with three types of immunodefective animals: nude (*nu/nu*) mice that have few (if any) mature T cells; X-linked immunodeficiency (X-id) mice such as CBA/N that have only two of the three B cell subpopulations found in normal mice (17) (and which show other B cell developmental abnormalities mentioned here); and NZB-related autoimmune mice that produce large amounts of IgM autoantibodies as part of a characteristic disease pattern (34–37).

Thus we show (*a*) that Ly-1 B carry all of the classic B cell differentiation markers (IgM, IgD, Ia, and ThB) and none of the classic T cell markers, except Ly-1; (*b*) that these cells nevertheless have more IgM, less IgD, more ThB, and somewhat less Ia than most B cells; (*c*) that they are present almost exclusively in spleen; and (*d*) that they are well represented in neonatal animals but constitute only a very small proportion of the overall splenic B cell population in adults. In normal mice, this subpopulation essentially coincides with B cell subpopulation III (the least frequent of three subpopulations that we recently defined according to correlations in the quantitative expression of IgM and IgD) (10, 17).

Studies with nude mice add two key points. First, the presence of normal numbers of Ly-1 B in these genetically T-deficient animals severs Ly-1 B from the T cell lineage (which requires a functional thymus for development). Thus, Ly-1 B emerge as a true B cell subpopulation rather than a subpopulation, that is, in some way "part T and part B." Second, the presence of normal amounts of Ly-1 on the Ly-1 B subpopulation in nude mice strongly suggests that Ly-1 B produce rather than acquire their surface Ly-1, since there are very few T cells to serve as Ly-1 "donors" in nude animals.

Gene(s) controlling the X-linked immunodeficiency in CBA/N (X-id) mice (33, 36), which deplete the normally predominant B cell subpopulation I and increase subpopulations II and III (10, 17), leave the Ly-1 B population at its normal

frequency. Since Ly-1 B normally comprise all or nearly all of the cells in subpopulation III, these studies also show that the X-id defect causes a selective expansion of a Ly-1-negative component of B cell subpopulation III that is either minimally represented or completely missing in normal animals.

Gene(s) in the autoimmune NZB mouse strain (38), in contrast, selectively increase Ly-1 B frequencies. From neonatal life onward, NZB mice have abnormally large numbers of Ly-1 B (and correspondingly increased numbers of subpopulation III cells). The presence of these cells, which have large amounts of surface IgM and relatively small amounts of surface IgD, accounts for previous reports indicating that NZB B cells (studied as a bulk population) have abnormally high IgM/IgD ratios (39).

The enlarged Ly-1 B population in NZB mice appears homogeneous and cannot be distinguished from normal Ly-1 B by the analytic criteria used here (surface marker distribution, light scatter, organ location); however, functional studies show that it contains unique cells missing or minimally represented in normal mice. Unlike normal Ly-1 B, these cells (isolated from unimmunized animals) spontaneously secrete large amounts of IgM when cultured in the absence of added stimulants (mitogens or antigens).

Antigenic stimulation generates antibody-producing cells that also secrete IgM in vitro, e.g., large numbers of IgM-secreting ("direct") PFC appear in the spleen after immunization with SRBC. These normal IgM-secreting cells, however, are clearly Ly-1 negative and thus distinct from the atypical IgM-secreting Ly-1 B population in NZB mice. This distinction is further demonstrated by the fact that Ly-1 B frequencies in NZB (and normal) mice remain constant after SRBC immunization, whereas PFC frequencies rise dramatically; in addition, sorted Ly-1 B secrete equivalent amounts of IgM whether taken from SRBC-immunized or nonimmunized NZB animals, whereas Ly-1-negative populations secrete detectable IgM only if isolated from recently immunized donors.

Since SRBC immunization may not stimulate all B cell subpopulations capable of responding to other kinds of antigens (40, 41), the Ly-1 B population could contain a special subset of normal, antigen-stimulated IgM-secreting cells. But even if this were the case, our data still clearly demonstrate that the IgM secretion obtained from NZB spleen derives from a unique population of (Ly-1 B) cells rather than, as previously suggested (42), from excessive numbers of normal IgM-secreting cells generated by the "hyperactivity" of the predominant B cell populations in NZB. Earlier studies (35) also attributed spontaneous NZB IgM secretion to atypical cells; however, this conclusion was based on kinetic evidence indicating that the IgM-secreting cells in NZB spleen differ from NZB splenic precursor B cells that respond normally to in vitro LPS stimulation (by differentiating to IgM-secreting cells after several days in culture).

The elevated Ly-1 B frequencies in NZB mice reported here have been demonstrated previously (10, 11). Similarly, as indicated above, the spontaneous IgM secretion by cultured NZB spleen cells was described years ago as one of the complex phenomena that occur in these autoimmune mice (34–36). Our studies confirm and extend these findings. In addition, however, we make a key connection by demonstrating that the IgM secretion is due to an aberrant, IgM-secreting B cell population that exists within the enlarged Ly-1 B subpopulation in these mice.

The NZB gene(s) that cause the enlargement of this population does not necessarily influence the representation of normal Ly-1 B, either in NZB or in other mouse strains. The proportion of atypical cells in the NZB Ly-1 B population and the relationship of these cells to normal Ly-1 B has yet to be determined. Thus, Ly-1 B cells such as those in BALB/c mice could be present at the normal frequency in NZB mice and in fact, could be completely unrelated to the atypical IgM-secreting Ly-1 B in these mice.

Normal Ly-1 B appear to have evolved in response to strong selective pressures that permit little variation from animal to animal. All mouse strains (except perhaps NZB) have comparable numbers of these cells, which characteristically reside almost exclusively in the spleen and emerge early in ontogeny as a major lymphocyte population that decreases in relative frequency as other lymphocyte populations approach their adult levels. The constancy of these properties suggests that Ly-1 B play one or more key functional roles (as yet undefined) in the immune system.

A murine population of normal antigen-specific (B′) cells lacking Thy-1 and (apparently) carrying both Ly-1 and IgM has been shown to augment in vitro IgG antibody responses in the presence of carrier-specific help (16). Our data demonstrating Ia on Ly-1 B would appear to distinguish Ly-1 B from B′ cells (which are not killed by anti-Ia plus complement); however, preliminary observations indicate that Ly-1 B are not easily depleted by cytotoxic treatment with anti-Ia. Thus, the studies with B′ cells suggest that the normal Ly-1 B subpopulation may contain cells active in the regulation of antibody responses mounted by other B cells.

Summary

A small subpopulation of normal murine splenic B cells carrying all of the classic B cells markers (IgM, IgD, Ia, and ThB) also carries Ly-1, one of the major T cell surface molecules. This "Ly-1 B" subpopulation (identified and characterized by multiparameter FACS analyses) consists of relatively large, high-IgM/low-IgD/low-Ly-1 lymphocytes that represent ~2% of the spleen cells in normal animals and, generally, 5–10% of spleen cells in NZB mice. Ly-1 B are clearly detectable in all normal mouse strains tested as well as NZB, CBA/N, other X-id mice and nude (nu/nu) mice. They are found primarily in the spleen; are either absent or very poorly represented in lymph node, bone marrow, and thymus; appear early during ontogeny; and comprise about a third of the small number of lymphocytes present in 5-d-old mice.

NZB and (NZB × NZW)F1 mice have more Ly-1 B than all other strains and, furthermore, have a unique Ly-1 B population that secretes IgM when cultured under usual conditions in the absence of added antigen. The IgM secretion by these Ly-1 B cells accounts for the previously reported "spontaneous" IgM secretion by NZB spleen cells in culture. Studies with FACS-sorted cells show that the presence of Ly-1 on these IgM-secreting cells distinguishes them from the (Ly-1 negative) IgM-secreting "direct" plaque-forming cells generated in NZB mice after stimulation with sheep erythrocytes.

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