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Immunology

# Ly-1 B-cell clones similar to human chronic lymphocytic leukemias routinely develop in older normal mice and young autoimmune (New Zealand Black-related) animals

(fluorescence-activated cell sorting)

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ABSTRACT **Studies presented here demonstrate that individually expanded clones of murine Ly-1 B cells, perhaps analogous to the expanded neoplastic Leu-1 B-cell clones in human chronic lymphocytic leukemias, are universally detectable in young New Zealand Black (NZB)-related autoimmune mice and in senescent normal mice (>18 months old). These clones are visible as phenotypically homogeneous cell populations in multiparameter fluorescence-activated cell sorter analyses of peritoneal and splenic B cells; they show unique immunoglobulin heavy- and light-chain gene rearrangements in Southern gel analyses of peritoneal and splenic DNA; and, like the self-replenishing Ly-1 B-cell population from which they are drawn, they tend to grow readily in irradiated or unirradiated syngeneic or allotype congenic hosts. Furthermore, they develop and generalize in primary and secondary hosts in a characteristic pattern (peritoneum >> spleen > lymph node > bone marrow) that suggests that their initial growth is controlled by the mechanisms that normally control Ly-1 B-cell distribution in lymphoid organs. The universal emergence of these clones within the Ly-1 B-cell lineage may be explained by the substantially greater opportunity for hyperplastic and neoplastic transformation events in this long-lived self-replenishing Ly-1 B-cell population, which must divide relatively frequently to maintain its normal size throughout adulthood. Repeated exposure to internal or environmental antigens (with which Ly-1 B cells are known to react) may also play a role in driving the development of these clones.**

Recent studies divide murine B lymphocytes into two lineages: the conventional B-cell lineage, which is replenished from surface immunoglobulin-negative progenitors in the bone marrow; and the Ly-1 B lineage (Ly-1 B), which in the adult is a self-renewing population replenished from immunoglobulin-positive progenitors found largely in the peritoneal cavity (1–3). Ly-1 B cells normally comprise only 1–3% of splenic B cells; however, they constitute 40–80% of the B cells in the peritoneum (1–3). They can express two surface antigens not found on conventional murine B cells, CD5 (Ly-1) (1, 4) and CD11 (MAC-1) (5). In addition, recent studies have shown that within the Ly-1 B-cell lineage two populations exist that can be distinguished by their expression of the CD5 (Ly-1) antigen (ref. 1; A.M.S., unpublished observation). The $CD5^+$ Ly-1 lineage B cells and the $CD5^-$ Ly-1 B cells (also referred to as the sister population) are, except for the expression of the CD5 antigen, phenotypically, developmentally, and functionally indistinguishable. Homologous antigens Leu-1 (CD5) (6) and Leu-15 (CD11) (7) are expressed on human Leu-1 B cells.

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At the functional level, Ly-1 lineage B cells produce many of the autoantibodies found in normal and New Zealand Black (NZB)-related autoimmune mice (4); in addition, Ly-1 B-cell frequencies are much higher in autoimmune NZB and (NZB × NZW)$F_1$ (B/W) than in normal mouse strains (8) (NZW, New Zealand White). Similarly, the homologous population in humans (Leu-1 B cells) have been shown to produce certain autoantibodies—e.g., IgM rheumatoid factor (9, 10)—and are increased in frequency in some autoimmune diseases (10, 11). The autoimmune disease in B/W mice has been studied for years as a murine model for human systemic lupus erythematosus (12, 13).

We report here that NZB-related mice have an additional and perhaps related immunological defect: they uniformly develop clonal populations of self-perpetuating Ly-1 B cells that arise in the peritoneal cavity early in life and eventually invade all lymphoid tissues. We also show that similar clonal Ly-1 B-cell populations can be detected in older mice (>15 months old) of all strains. The phenotypic and growth characteristics of these clonal populations suggest that they represent the murine equivalent of human B-cell chronic lymphocytic leukemia (B-CLL).

## MATERIALS AND METHODS

**Animals.** Two-month-old NZB, NZW, and B/W female mice were obtained from The Jackson Laboratories and maintained at Stanford. All other mice were bred in the Stanford Department of Genetics Mouse Facility.

**Fluorescence-Activated Cell Sorter (FACS) Analysis.** Single cell suspensions were prepared from lymphoid organs as described (14) and stained with optimal amounts of monoclonal antibodies: anti-IgM, 331.12/fluorescein isothiocyanate; anti-CD5 (Ly-1), 53.7/allophycocyanin; anti-IgD, AMS 15.1/biotin; anti-B220/6B2, RA3-6B2/biotin. Purification and fluorochrome conjugation of the monoclonal antibodies have been described in detail (5, 15). Biotin-conjugated antibodies were revealed with Texas Red avidin. FACS analyses were conducted as described (5, 16). Dead cells were stained with propidium iodide and excluded from the analyses (16). For each analysis, data from 30,000 viable cells were collected. Data are presented as 5% probability contour maps (17).

**Southern Analysis.** Approximately 10 μg of genomic DNA, extracted from total peritoneal cells, was digested with *Eco*RI, fractionated on 0.7% agarose gels, transferred to nitrocellulose filters (18), and probed with a heavy-chain

Abbreviations: FACS, fluorescence-activated cell sorter; B-CLL, B-cell chronic lymphocytic leukemia; PerC, peritoneal cells; $J_H$, heavy-chain joining region; D, diversity.

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joining region ($J_H$) probe (pJ11) that was radiolabeled by hexamer priming (19). Hybridizations were done in 6× SSPE/5× Denhardt's solution/0.5% $NaDodSO_4$/100 μg of salmon sperm DNA (1 ml) at 65°C, and washed in 2× SSPE/0.1% $NaDodSO_4$ and 0.2× SSPE/0.1% $NaDodSO_4$ at 65°C (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). The probe pJ11 is a 2.0-kilobase *Bam*HI/*Eco*RI fragment that includes JH3 and JH4.

**Adoptive Transfer of Clonal Populations.** Recipient 2-month-old B/W mice were irradiated with 850 rads (1 rad = 0.01 Gy) and injected i.v. the following day with $10^6$ syngeneic bone marrow cells and $5 \times 10^6$ peritoneal cells from a 7- to 9-month-old B/W mouse. The peritoneal cells were analyzed before transfer to confirm the presence of phenotypically homogeneous populations of Ly-1 B cells.

## RESULTS

**FACS Analyses Reveal Phenotypically Homogeneous B-Cell Populations in B/W Mice.** Comparison of data from multiparameter FACS analyses of peritoneal lymphocytes (PerC) from BALB/c and B/W mice clearly demonstrates the presence of phenotypically homogeneous Ly-1 B-cell populations in the B/W animals (Fig. 1). In BALB/c mice, as in all normal adult animals, the peritoneal Ly-1 B-cell population is heterogeneous in expression of surface IgM, which characteristically varies over a 100-fold range (1, 3, 12, 20). In B/W mice, in contrast, the expression of IgM on Ly-1 B cells is highly restricted and varies over only a 10- to 20-fold range. Similarly, Ly-1 expression is more restricted on the B/W Ly-1 B cells. Analyses of PerC from >50 animals show that every B/W mouse over the age of 3 months has one to three such phenotypically homogeneous populations, whose FACS profiles resemble those obtained in the analysis of monoclonal cell lines and tumors.

**B/W Phenotypically Homogeneous B-Cell Populations Each Contain at Least One Ly-1 B-Cell Clone.** The clonality suggested by the FACS phenotypic analyses is confirmed at the molecular level by Southern analysis of PerC DNA for clonal rearrangements using a $J_H$ probe (Fig. 2*B*). Two or more clonal D–J or V–D–J (D, diversity; V, variable) rearrangements were detected in the PerC DNA from each of 14 B/W animals examined at 3 months or older, whereas no clonal rearrangements were detected by this method in PerC DNA from 8 age-matched normal mice (BALB/c and CBA) (data not shown). In general, the number of clones estimated from immunoglobulin rearrangements in individual mice is greater than the number of phenotypically distinct populations, and FACS-sorted populations of phenotypically homogeneous cells often have more than two $J_H$ rearrangements (D.M.T., unpublished data). Thus, FACS analyses provide a minimum estimate of the number of clones present in the peritoneum of a given animal.

The FACS and Southern analyses shown in Fig. 2 illustrate the development of the clonal Ly-1 B populations with time. In 1-month-old mice, the peritoneal Ly-1 B are phenotypically indistinguishable from BALB/c and no clonal bands are visible by Southern analysis. Phenotypically homogeneous populations of peritoneal Ly-1 B cells first become visible in 3- to 4-month-old mice at the time when multiple faint hybridization bands (suggestive of a relatively large number of small clonal populations) become detectable on Southern blots. By 7 months of age, Ly-1 B-cell clones dominate in the peritoneal cavity and a few strong bands are detectable on the blots. During this period—i.e., from 1 to 7 months—the number of peritoneal Ly-1 B cells increases slowly but steadily, from $\approx 3 \times 10^6$ to $>3 \times 10^7$. Virtually all of this increase is due to the expansion of Ly-1 B clonal populations.

**Ly-1 B Clones Follow a Characteristic Pattern When They Generalize from the Peritoneum to Other Lymphoid Sites.** The expansion of the peritoneal Ly-1 B clones in older animals is usually accompanied by the migration (generalization) of one or more clones to peripheral lymphoid tissues (Fig. 3). During this migration, the FACS phenotype of a given clone within a given animal remains the same, regardless of the lymphoid organ in which the clone resides. Furthermore, the $J_H$ rearrangements detected by Southern analysis of individual Ly-1 B clones sorted from spleen or lymph node in a given animal are found amongst the $J_H$ rearrangements detected in PerC taken from the same animal (D.M.T., unpublished

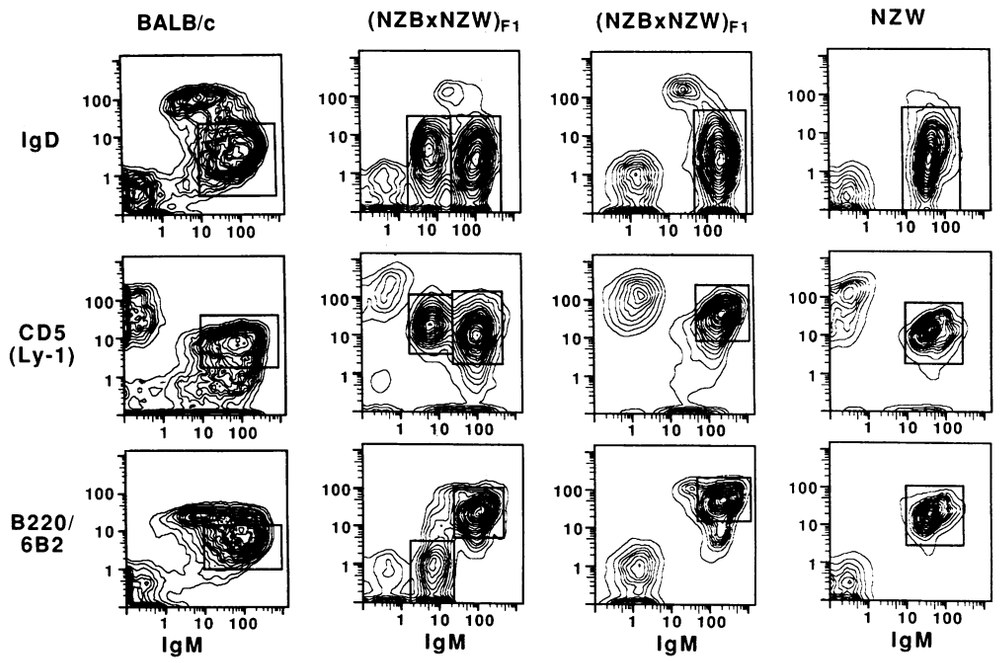


FIG. 1. Phenotypically homogeneous (clonal) populations of Ly-1 B cells in the peritoneum of B/W mice. The Ly-1 B populations are boxed. The ages of the mice are as follows: BALB/c, 8 months; B/W, 10 and 11 months; NZW, 9 months. See text for explanation.

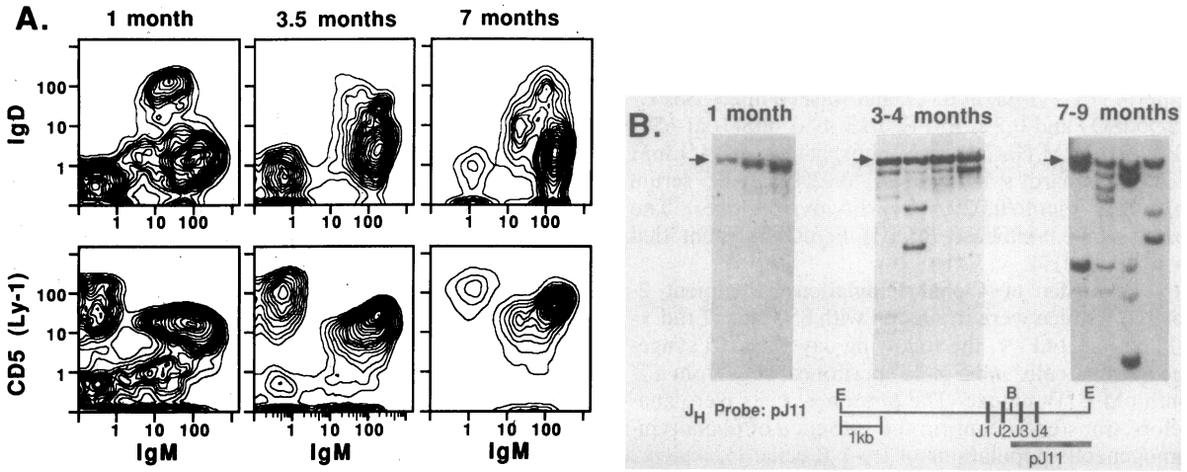


FIG. 2. Clonal populations of Ly-1 B cells develop with age. (*A*) FACS analysis, PerC from 1-, 3.5-, and 7-month-old B/W mice were prepared and characterized by FACS analysis. (*B*) Southern analysis of D–J rearrangements with the pJ11 probe indicated in the schematic diagram at the base of the figure. The positions of the 6.2-kilobase *Eco*RI germ-line fragment are indicated by arrows. We have not observed any polymorphism for this fragment between BALB/c and B/W. E, *Eco*RI; B, *Bam*HI; kb, kilobase.

data). Thus, the clones in the peripheral lymphoid organs derive from clones that originate in the peritoneum in young animals and generalize to the periphery as the animals age.

This generalization from the peritoneum follows a characteristic pattern in that the clonal populations initially maintain Ly-1 B tissue specificity but eventually become invasive and expand into tissues in which Ly-1 B cells are not normally found. Clones first become detectable (by FACS analysis) in spleen and peripheral blood, where Ly-1 B cells are rare but always present. In some animals, clones then emerge 2–3 months later in lymph nodes and bone marrow even though Ly-1 B cells are not normally detectable in these organs (1) (Fig. 3). Clonal expansion outside the spleen and peritoneal cavity, however, appears to proceed relatively slowly. For example, the Ly-1 B clone present in the bone marrow of an 11-month-old mouse represents <1% of nucleated cells in the bone marrow (Fig. 3). Similarly, while Ly-1 B-cell clones are readily apparent in the peripheral blood of 7-month-old mice (Fig. 3) and gradually increase over time, the overall blood lymphocyte counts in B/W mice do not reach leukemic levels at 8–12 months of age when these mice typically die from autoimmune disease. B/W mice that survive longer, however, do become leukemic, since Ly-1 B frequencies in the blood of 2-year-old animals in which the autoimmune disease is reversed by anti-L3T4 treatment increase as much as 40-fold above normal (21).

Splenic or peritoneal Ly-1 B clones can be transferred indefinitely in irradiated or nonirradiated syngeneic or allotype congenic recipients. As shown in Fig. 4, the phenotypes of individual Ly-1 B clones remain relatively stable after transfer, although individual populations may expand at different rates. The donor origin of the Ly-1 B-cell clones was confirmed by Southern analysis. The same clonal $J_H$ rearrangement bands were present in the analysis of pre- and 7-month posttransfer peritoneal samples (data not shown).

**Early Development of Ly-1 B Clonal Populations Is Characteristic of All New Zealand-Related Strains of Mice (NZB, NZW, and B/W).** Ly-1 B-cell clones develop in both B/W parental strains—i.e., NZB (22) and NZW (Fig. 1). The development of the clones (age of appearance, pattern of

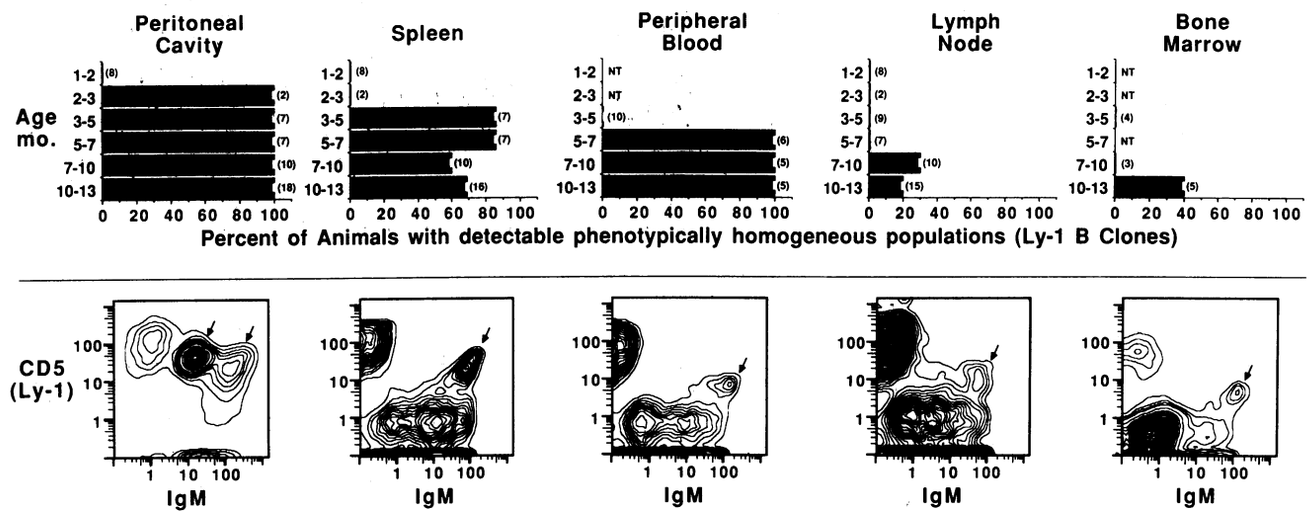


FIG. 3. Clonal Ly-1 B-cell populations have a characteristic order of appearance in peripheral lymphoid tissues. Bar graphs show the percentage of B/W mice analyzed at the indicated ages that have detectable phenotypically homogeneous (clonal) populations (by FACS analysis) in the indicated tissues of B/W mice. Values in parentheses indicate the number of animals at each age tested; NT, none tested. All animals with clonal populations in the lymph nodes or bone marrow also had clones detectable in spleen and peritoneum. Representative FACS profiles of the clonal populations (arrows) found in each of the tissues are shown below each bar graph. For the FACS profiles, the PerC, splenic, and peripheral blood lymphocytes were obtained from 7-month-old mice, lymph node cells were from an 8-month-old mouse, and the bone marrow cells were from an 11-month-old mouse.

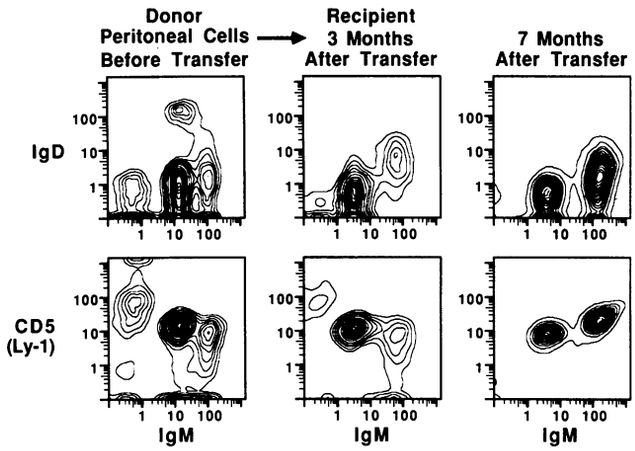


FIG. 4. Ly-1 B clones can be adoptively transferred. PerC ($5 \times 10^6$) from an 8-month-old B/W were transferred into an irradiated 2-month-old syngeneic recipient. PerC obtained from the recipient 3 and 7 months after transfer were analyzed by FACS.

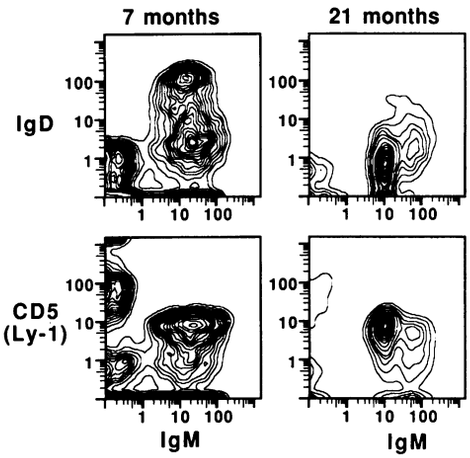


FIG. 5. Clonal populations of Ly-1 B cells develop in older animals in normal strains of mice. PerC were obtained from a 7-month-old and a 21-month-old BAB/25 mouse and analyzed by FACS. The presence of clones in the 21-month-old mouse indicated by FACS analysis was confirmed by Southern blot analysis (data not shown).

generalization to peripheral tissues) appears similar in the NZB-related strains. Clones were detectable in the peritoneum of each of five NZB and five NZW 3.5-month-old mice analyzed (data not shown). Nevertheless, a difference can be seen in the expansion of the clones in individual NZB mice. In two of four 11-month-old NZB mice tested, the Ly-1 B clones comprised >95% of the cells in the spleen and lymph nodes. We have never observed such enormous clonal expansion in B/W or NZW mice.

**Ly-1 B Clones Are Routinely Detectable in Normal Mice Over 15 Months of Age.** The development of Ly-1 B clonal populations is not a unique characteristic of B/W-related mice. The development of clones in these mice is, in fact, an acceleration of a process that occurs in all mice. Typical phenotypically homogeneous populations of peritoneal Ly-1 B cells were found in each of 40 BALB/c, C57BL/6, and CBA mice tested over the age of 15 months (compare Figs. 2 and 5). In addition, Southern analysis of the PerC from 12 older (15–22 months) normal mice reveals the same pattern of clonal $J_H$ rearrangement bands observed in 3- to 4-month-old B/W animals (data not shown). While every B/W, NZW, and NZB mouse has detectable clones by 3–4 months of age, normal mice such as BALB/c and CBA rarely have detectable clones (by FACS or Southern analysis) until 12–15 months of age. Thus, the major difference between normal mice and New Zealand-related mice is the age of onset of clonal development. However, once present, the Ly-1 B clones expand and migrate similarly in all strains.

The clones that develop in a given strain appear to be derived from a random sample of the Ly-1 B-cell lineage cells in that strain. For example, the relative frequencies of the two Ly-1 B-cell lineage subpopulations ($CD5^+$ and $CD5^-$ sister) observed in different strains are reflected in the clonal populations found in these strains—i.e., the $CD5^-$ sister population constitutes <3% of B/W Ly-1 B cells, 20% of BALB/c, and 50% of CBA Ly-1 lineage B cells (A.M.S., unpublished observation). Similarly, we have never observed a $CD5^-$ sister clone in B/W mice, only 1 in 15 1- to 2-year-old BALB/c mice, while 5 of 14 1- to 2-year-old CBA mice had $CD5^-$ sister clones (Fig. 6).

**Ly-1 B Clones Do Not Secrete Significant Levels of Immunoglobulin.** Even though the Ly-1 B clones become a significant proportion of the B lymphocytes, in general, they do not appear to produce significant levels of immunoglobulin. No monoclonal immunoglobulin spikes were detected (by serum electrophoresis) in the serum of 18 7- to 11-month-old B/W mice with demonstrable clonal populations (data not shown). Furthermore, transfer studies of BCL-85, a BALB/c ($Igh^a$) Ly-1 B clone, into congenic BAB/25 ($Igh^b$) mice revealed no immunoglobulin production by the clone (P.A.L., unpublished data).

## DISCUSSION

Studies presented here demonstrate the routine expansion of hyperplastic or neoplastic Ly-1 B-cell clones whose phenotypic and developmental characteristics strongly suggest that they represent the murine equivalent of human B-CLL tumors. That is, like human B-CLLs, these Ly-1 B clones express CD-5 (Leu-1/Ly-1) and CD-11 (Leu-15/MAC-1) cell-surface antigens (3, 6, 7, 23) and rarely secrete large amounts of antibody (24). Furthermore, they are usually found in older individuals (23) and/or in association with

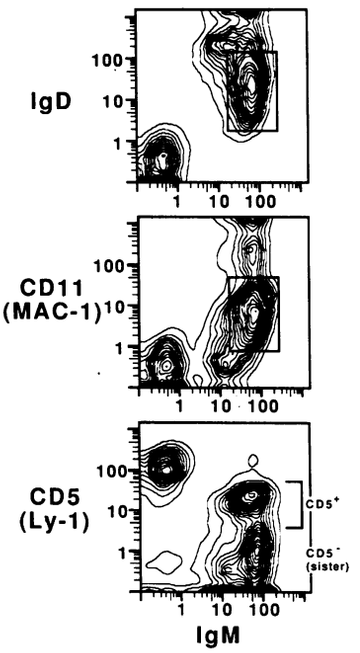


FIG. 6. Clones develop from both $CD5^+$ and $CD5^-$ sister Ly-1 B cells. PerC from a 24-month-old CBA/Ca were analyzed by FACS. The $CD5^+$ and $CD5^-$ sister clones were indistinguishable for the expression of the IgM, IgD, and Ly-1 antigens. See text for explanation.

autoimmune dysfunction (24–26); they are relatively benign in that they usually grow slowly and tend not to be the primary cause of death in the affected individual (24, 27).

A variety of other murine B-cell tumors have recently been shown to be Ly-1 B neoplasms—e.g., spontaneous CH tumors in C57BL/10.H-2a,H-4b (28); spontaneous leukemias in NZB (21); spontaneous hyperdiploid tumors in NZB (E. S. Raveché, P.A.L., A.M.S., and J. Conroy, unpublished data); tumors in CBA animals given large amounts of congenic bone marrow (J. Ansel, personal communication); and a series of Ly-1 B clones that grow *in vitro* and *in vivo* (ref. 29; J. Braun, personal communication). In addition, BCL-1, 70Z, WEHI-231, and several other well-studied murine B-cell tumors have been shown to express Ly-1 (30). Thus, Ly-1 B clones and tumors offer an attractive model for investigating mechanisms underlying the origin and progress of CLL-like disease and the relationship(s) between autoimmunity and CLL.

The emergence of Ly-1 B clonal populations in mice and humans may be the natural result of the unique ontogeny of the murine Ly-1 B-cell lineage and its putative human (Leu-1 B) counterpart. That is, conventional B cells are replenished from immunoglobulin-negative precursors throughout the life of the animal. Ly-1 B cells, in contrast, are primarily generated from immunoglobulin-negative progenitors during the first few weeks of life and become a self-replenishing population that maintains itself by division of mature, immunoglobulin-positive Ly-1 B cells thereafter (1–3). Thus, inherent or induced differences in the division rates of individual Ly-1 B clones apparently translate over time into the development of dominant clones within the Ly-1 B-cell population. These clones then apparently undergo further growth deregulation, finally generalizing to peripheral lymphoid sites, where they behave like neoplastic cell populations—i.e., they grow unrestrictedly and can be readily passaged.

The detection of hyperplastic/neoplastic Ly-1 B clones in NZB-related mice raises questions about the relationship between increased Ly-1 B-cell frequencies and autoantibody production. In previous studies, we demonstrated increased splenic Ly-1 B-cell frequencies (16) and spontaneous IgM secretion and autoantibody production by splenic Ly-1 B cells in NZB mice (4). We interpreted these findings as reflecting the polyclonal expansion and activation of a population of autoantibody-producing Ly-1 B cells. Data presented here, however, indicate that the increased Ly-1 B-cell frequencies that were observed could also be due to the expansion of individual Ly-1 B clones. Since these clones generally do not secrete large amounts of immunoglobulin, this would mean that the increased splenic Ly-1 B-cell frequencies are probably not responsible for the increased IgM and autoantibody production in these strains. Consequently, the autoantibodies and the IgM are probably produced by what may be a normal number of polyclonal Ly-1 B cells.

The demonstration of Ly-1 B clones in apparently healthy mice (young NZW; older BALB/c, CBA, etc.) further dissociates the Ly-1 B-cell tumors from a causative role in autoimmune disease. Although Ly-1 B (Leu-1) B-cell tumors tend to occur more frequently in autoimmune individuals, this tendency could be explained by an overall increase in the stimulation of the Ly-1 (Leu-1) B-cell population in these individuals. Alternatively, the tumor could arise in response to the autoimmune disease and perhaps even be an effort to regulate the disease process. Finally, these clones might represent an independent but correlated genetic predisposition toward Ly-1 B-cell tumor development and the development of autoimmune disease. Further studies are required to resolve these alternatives.

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