ELEVATED *myc* EXPRESSION AND c-*myc* AMPLIFICATION IN SPONTANEOUSLY OCCURRING B LYMPHOID CELL LINES

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Recently, a minor subpopulation of murine B lymphocytes has been distinguished by its unique role in immune physiology and disease. These cells, initially characterized by their novel surface elevation of the Ly-1 antigen, are most prominent early in ontogeny, and have a restricted tissue distribution and precursor origin (1). They are the predominant source of autoantibody production in normal and autoimmune mouse strains (2, 3), and provide a novel stimulatory influence to the production of antiallotypic and antiidiotypic antibodies (4, 5). The Ly-1$^+$ B cell phenotype accounts for most spontaneous murine B cell lymphomas and leukemias (6, 7). In man, B lymphocytes expressing CD5, the human Ly-1 homologue, are the predominant phenotype in chronic lymphocytic leukemia (8), and are elevated early in bone marrow transplantation (9), and possibly in certain autoimmune diseases (10, 11).

We have reported a simple cell culture strategy that allows the exclusive outgrowth of long-term Ly-1$^+$ B cell lines (12, 13). T cell–depleted splenic lymphocyte cultures, maintained at high cell density for 2 wk in the absence of nominal lymphocytotrophic hormones or stimuli, enter a period of crisis. However, by 3–4 wk, Ly-1$^+$ B lymphoblasts are detectable; within an additional 2–3 wk, stable, robustly proliferating cell lines may be established. These cell lines, termed B Ly-1 cells, have been serially passaged for >3 yr, and hence have an immortal growth phenotype. In addition, they are clonable in soft agar, and at limiting dilution at high cloning efficiency (>50%). However, they are not tumorigenic after inoculation into syngeneic animals.

The spontaneous occurrence and sustained in vitro growth of B Ly-1 cells led us to assess their expression of c-*myc*, a protooncogene with elevated transcription during the initial phase of B cell mitogenesis (14, 15) and deregulated expression in many B cell neoplasms (16, 17, reviewed in 18). Here we report high steady-state levels of c-*myc* mRNA in B Ly-1 cells, which correlates with amplification of the c-*myc* locus.

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Materials and Methods

Cell Lines. B Ly-1 cell lines were established according to the previously established method (13) from C3H/HeJ, MRL/++, and A/J mice, and cloned by two or more serial limiting-dilution cultures in the absence of feeder cells. 70Z/3 is a pre-B cell line established from a methyl nitrosourea–induced tumor in thymectomized (DBA × C57BC/6)F_1 mouse (19). WEHI 231, WEHI 279, and 74.10 (a tissue culture–adapted subclone of CH1) are surface IgM^+ B lymphocyte cell lines described previously (6, 11). All cell lines were cultured in RPMI 1640, supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 50 μM 2-ME, and penicillin/streptomycin.

Purification and Analysis of RNA and DNA. Cytoplasmic RNA was prepared from the postnuclear fraction of NP-40 cell lysates (20). RNA (15 μg/lane) was electrophoresed on 1.2% formaldehyde agarose gels (21), and stained with ethidium bromide to visualize ribosomal RNA bands for use as internal molecular mass standards. Gels were blotted by capillary transfer to Zetabind nylon filters (AMF-Cuno, Meriden, CT) following the commercial protocol.

Genomic DNA was prepared from the nuclear fraction of NP-40 lysates by standard methods (21). After exhaustive restriction enzyme digestion, DNA (12 μg/lane) was electrophoresed on 0.8% agarose gels in TBE buffer (0.089 M Tris, 0.89 M boric acid, 0.002 M EDTA), including 0.5 μg/ml ethidium bromide. Eco RI/Hind III–digested λ DNA (Boehringer-Mannheim Biochemicals, Indianapolis, IN) served as molecular mass standard. Gels were photographed and blotted to Zetabind nylon filters using the commercial modification of the method of Southern.

Blots were hybridized with nick-translated plasmid fragments in 5× SSCPE (0.75 M NaCl, 0.075 M sodium citrate, 0.0375 M sodium phosphate, 0.005 M EDTA, pH 7.4), 1× Denhardt's solution, 50% formamide, 0.1 mg/ml herring sperm DNA, for 36 h at 42°C. Blots were washed at 65°C in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS and autoradiographed using Kodak XAR film at −70°C with an intensifying screen. In some cases, autoradiograms were analyzed by densitometry using a LKB Ultrascan with a 2220 recording integrator (LKB-Produktor AB, Bromma, Sweden); for this purpose, exposure times were chosen giving a linear range of film development (0.2–0.6 OD).

Recombinant DNA. pmyc-26, a murine c-*myc* genomic clone (30) was used to prepare a 2.0 kb Xba I–Hind III fragment for use as a *myc* probe. pβ_2m, a gift from J. Seidman, consists of a 4 kb Hind III genomic segment (including second and third exons) of the murine β_2 microglobulin gene subcloned into pBR322 (31); a Sac I/Kpn I subfragment was used as specific β_2 microglobulin probe. Probes were nick-translated with α-[^{32}P]ATP (New England Nuclear, Boston, MA) for blot hybridization. All restriction enzymes were purchased from New England Biolabs, Beverley, MA.

Results

The expression of *myc* transcripts was analyzed by Northern blots of cytoplasmic RNA. 750-13, a cloned B Ly-1 cell line, derived from C3H/HeJ mice, expressed a 2.3 kb *myc* RNA corresponding in size to the conventional murine c-*myc* transcript. The level of expression, however, when compared with the murine pre-B cell line 70Z/3, was strikingly elevated (Fig. 1 *a*). Similar results were also observed in independent clones of B Ly-1 cell lines derived from two other mouse strains (AJ and MRL; Fig. 1 *b*).

To assess the degree of *myc* overexpression, Northern blots were prepared using RNA from a number of B Ly-1 clones and other B lymphocyte cell lines. Integrative densitometry was then carried out on blots which were sequentially hybridized with probes for *myc* and β_2 microglobulin (chosen as an independent, constitutively expressed RNA). The resulting values of *myc* transcript expression

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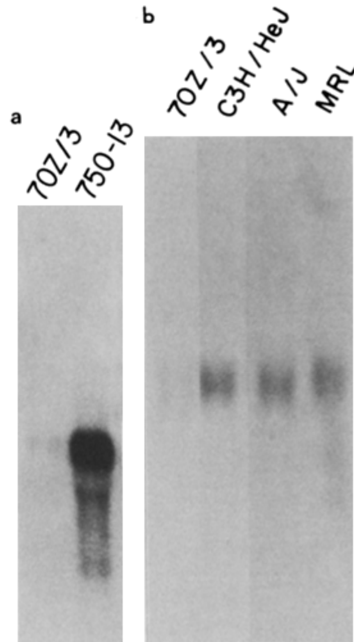

FIGURE 1. Elevated c-*myc* RNA transcripts in B Ly-1 cells. Formaldehyde-denatured cytoplasmic RNA was electrophoresed, Northern transferred to nitrocellulose, and hybridized with a nick-translated *myc* probe. (*a*) 70Z/3, 750-13; (*b*) 70Z/3, C3H/HeJ (670-6), A/J (AJ 12), MRL (MRL 37).

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in B Ly-1 cells, as normalized to the levels of the β_2 microglobulin transcript, ranged up to 45-fold above the level observed in 70Z/3 RNA (Table I).

The genomic c-*myc* configuration was evaluated by Southern blot analysis of DNA from 750-13 as compared with syngeneic C3H/HeJ liver DNA (Fig. 2). Digestion of genomic DNA from 750-13 with four restriction endonucleases revealed a pattern of *myc* hybridization indistinguishable from that of the liver DNA, indicating no DNA rearrangement within the c-*myc* transcription unit. The intensity of the hybridization signal, however, (using β_2 microglobulin as a single copy gene internal standard) revealed that the c-*myc* band in the 750-13 was amplified 5–10-fold (Fig. 2). Independently derived cell lines from another two mouse strains also exhibited amplification of the c-*myc* locus (Fig. 3). Prolonged tissue culture passage (6–9 mo) correlated, in the one C3H/HeJ cell line examined, with increased c-*myc* amplification (data not shown).

Discussion

Quantitative analysis of cytoplasmic RNA from B Ly-1 cells has revealed a substantially elevated steady-state level of c-*myc* RNA. Genomic rearrangements of the c-*myc* locus were not evident within the transcription unit, but a 2–10-fold c-*myc* amplification was observed.

Constitutively elevated *myc* RNA expression is a characteristic feature in lymphoid neoplasia. The mechanisms for this altered regulation include c-*myc* translocation to the immunoglobulin locus (reviewed in 22–24), and proviral insertion near the *myc* locus (25–28). The absence of short-range c-*myc* rearrangements (Fig. 1) argues against the role of such events in B Ly-1 cells. However B Ly-1 cytogenetic abnormalities, including lack of a normal chromosome 6 (κ locus) and 15 (*myc* locus), leave open the possibility that a more distant rearrange-

TABLE I
Levels of myc RNA in B Ly-1 Compared with Other Lymphoid Cell Lines

Strain	B cell line	Band intensity
	70Z/3	1.0
	WEHI 279	1.5
	CH1	2.2
	WEHI 231	5.5
	B Ly-1 cell lines	
A/J	AJ7	45.0
	AJ9	18.0
	AJ12	20.0
	AJ13	15.0
MRL/++	MRL17	26.2
	MRL37	18.8
C3H/HeJ	670-6	13.8
	750-13	31.2

Cytoplasmic RNA from cloned cell lines derived from the three mouse strains denoted, and from four other B cell lines were electrophoresed in agarose, Northern transferred, and hybridized sequentially with probes for *myc* and β_2 microglobulin. Band intensities were quantitated by integrative densitometry, and the level of *myc* expression was calculated by normalizing *myc* integration values with the corresponding β_2 microglobulin value. Normalized results are tabulated in arbitrary integration units relative to 70Z/3.

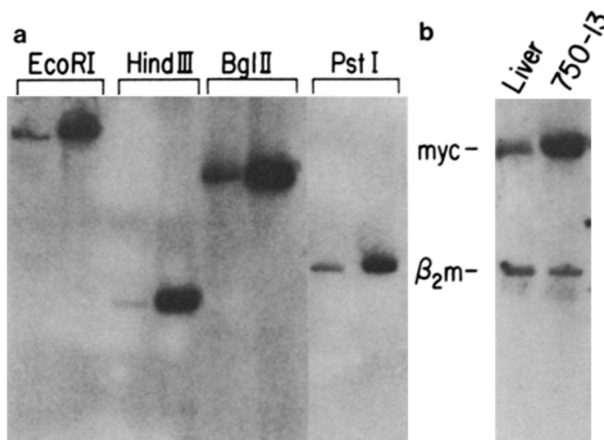

FIGURE 2. Amplification of the c-*myc* DNA segment. (*a*) Liver (*left*) and 750-13 (*right*) DNA were digested with the indicated restriction enzymes, then electrophoresed, Southern transferred and hybridized with a nick-translated *myc* probe. (*b*) High-molecular-mass DNA was digested with Eco RI, electrophoresed, Southern transferred to nitrocellulose, and hybridized with nick-translated probes for *myc* and β_2 microglobulin (β_2m). *Left,*C3H/HeJ liver; *right,* 750-13.

ment may have occurred, with long-range effects on *myc* transcription (cytogenetic data not shown). Such events may also account for the lack of a linear correlation between the moderate degree of *myc* amplification and the remarkable elevation of c-*myc* expression in the B Ly-1 cells.

It is striking that all of the spontaneously immortalized lymphocytes that are obtained in these spleen cultures are both Ly-1^+ (1, 13) and have an amplified

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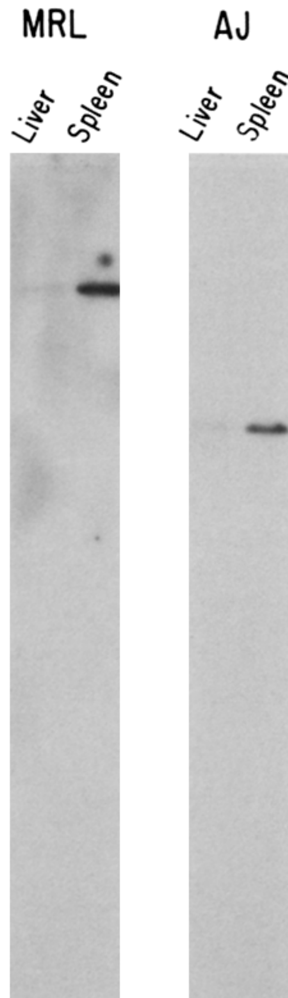

FIGURE 3. Amplification of the c-*myc* in cells derived from MRL and A/J mouse strains. Liver (*left*) and cloned B Ly-1 cell lines (*right*) derived from MRL/++ and A/J were analyzed for the level of c-*myc* amplification as described in Fig. 2*a*.

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and highly expressed c-*myc* locus. It is possible thus that the Ly-1$^+$ cells have c-*myc* loci that are in a state that may be readily amplified. Another possibility is that the physiological levels of c-*myc* expression in these cells is high so that even moderate amplification of the locus contributes the extra expression needed to pass the threshold of immortalization. Finally, it is possible that the Ly-1$^+$ B lymphocytes may be the target of a yet-unidentified transforming agent, analogous to the unique sensitivity of CR2$^+$ human B lymphocytes to EBV transformation. Analysis of these possibilities may shed light on the peculiar malignant potential of Ly-1$^+$ B cells in the mouse and on the prevalence of CD-5$^+$ cells in human chronic lymphocytic leukemia.

Summary

Recently, a minor subpopulation of murine B lymphocytes, Ly-1$^+$ B cells, has been distinguished by its unique ontogeny, tissue distribution, and prominence

in certain autoimmune and neoplastic B cell diseases. We have previously described a simple murine spleen culture system that results in the spontaneous and exclusive outgrowth of long-term Ly-1^+ B cell lines (B Ly-1 cells). Here, we report that the immortal growth property of B Ly-1 cells correlates with a 10–45-fold elevation of steady-state *myc* RNA and 2–10-fold amplification of the c-*myc* locus. While c-*myc* amplification has been observed in malignant cell lines derived from several tissues of origin, its occurrence in lymphoid cells has not been previously reported. The consistent c-*myc* amplification in B Ly-1 cells may reflect a unique state of this locus in the Ly-1^+ B lymphocyte lineage, and contribute to the spontaneous immortalization of this B cell population in vitro, and its apparent predilection for malignant transformation in vivo.

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