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Differential c-*myc* Protein Expression in Burkitt's Lymphomas and EBV-transformed Lymphoblastoid Lines

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The levels of c-*myc* protein expression in three types of Epstein–Barr virus (EBV) transformed human B-cell derived lines were examined with an ELISA assay. Six independently maintained sublines of the same EBV-transformed pro-B-cell line (FLEB-14), six B-cell lines (LCL) and six Burkitt's lymphoma lines (BL) were compared. The average amount of c-*myc* protein, calculated from at least three independent tests on each line, differed between the three groups. Expressed in relative units, the ratio of the means was 1:2:5 for the LCL:FLEB:BL lines. The differences were statistically significant at $P < 0.01$.
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INTRODUCTION

DEREGULATION OF c-*myc* expression plays an essential role in the pathogenesis of B-lymphocyte-derived neoplasia in several species. Constitutive activation of c-*myc* by retroviral insertion [1] or chromosomal translocation to an immunoglobulin locus [2] is apparently a rate-limiting step in the genesis of avian leukosis, mouse and rat plasmacytomas and human Burkitt's lymphoma.

It is generally believed that the tumorigenic contribution of the pathologically activated c-*myc* gene stems from its unresponsiveness to normal regulation. All proliferating cells express c-*myc*, whereas resting cells, including small B-lymphocytes, have low or undetectable levels. When proliferating cells leave the cycling compartment, as part of their spontaneous life cycle or differentiation program, they downregulate c-*myc*. Due to its subordination to a retroviral enhancer or a juxtaposed immunoglobulin gene, the pathologically activated c-*myc* may resist this normal downregulation, thereby preventing the cell from leaving the cycling compartment. The validity of this hypothesis has been proven by facsimile experiments. Introduction into B-cells of constructs containing a c-*myc* gene linked to a retroviral LTR or an immunoglobulin enhancer had a tumourigenic effect in transfected cells and in transgenic mice, respectively [3, 4]. The presence of such constructs obviated the need for the *myc*/Ig translocation, observed in the corresponding unmanipulated tumour induction system.

The question whether the Ig/*myc* translocation contributes to the genesis of Burkitt's lymphoma (BL) by altering the regulability of c-*myc*, or also by increasing the constitutive steady-state level of expression, has been controversial [5]. Semiquantitative comparisons have been performed at the RNA [6] or at the protein level [7–9]. We have approached this question by a quantitative ELISA technique for the c-*myc* protein [10]. In order to equalise trivial fluctuations in expression, known to occur even under standardised culture conditions, we have assayed the level of the c-*myc* protein on a statistical basis. Each line has been tested three to five times and the mean levels and their standard errors compared. The material consisted of six BL lines, six regular Epstein–Barr virus (EBV) transformed lymphoblastoid cell lines (LCLs) and six different sublines of an EBV-transformed human pro-B-cell line.

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MATERIALS AND METHODS

Cell lines

An EBV-transformed pro-B cell line that maintained its immunoglobulin genes in the germ line configuration, designated FLEB-14, was derived from fetal liver [11, 12]. A series of separate clonal sublines was propagated independently. None of the six sublines tested has undergone any physiological IgH rearrangement. Sublines 14-3, 14-4, 14-7 and 14-14 carried 14q+ markers that arose from illegitimate recombination between the S_μ locus and another chromosome [13]. Sublines 14-6 and 14-8 carried no such translocation. None of the translocations affected chromosome 8, the site of the c-*myc* locus.

All Burkitt's lymphoma-derived lines carried the reciprocal 8;14 (IgH/c-*myc*) translocation. All except Ramos were EBV-positive. Their origins have been described: Daudi [14], Jijoye M13 [15], Raji [16], Ramos [17] and Rael [18]. P3HR-1 was a variant subline, originally a clonal derivative of Jijoye.

The EBV-transformed LCLs carried normal, non-translocated c-*myc* genes. They were phenotypically different from the BL lines with regard to morphological characteristics, growth patterns and B-cell markers [19]. CBMl-RALSTO was derived from cord blood cells transformed by EBV rescued from the BL line Rael after induction with sodium n-butyrate [20]. CBC-KINA-9 (unpublished) is a cord blood line transformed with virus from the saliva of a healthy donor. WWl-LCL [21] originated from *in vitro* infected normal B-lymphocytes of a patient with an EBV-positive Burkitt's lymphoma. NAD20, GSBl and RSBl (unpublished) were established from the

peripheral blood of healthy adult donors by the exposure to EBV of the B95-8 strain.

The cells were maintained in RPMI 1640 supplemented with 8% fetal calf serum (FCS) and antibiotics. The number of viable cells per ml were obtained by trypan blue exclusion.

c-myc protein ELISA

Samples from 10×10^6 cells were extracted and assayed as described [3]. The extraction buffer contained 1.9 μg/ml aprotinin (Sigma) and 5 mmol/l PMSF (Sigma), the latter added freshly from a stock solution in butanol. The samples were stored frozen between the extraction and the ELISA. The absorbance at 490 nm of duplicate 100 μl sample aliquots was compared to a standard prepared from bacterially expressed c-*myc* protein [22].

Lowry total protein assay

With the standard Lowry reagents [23], a precipitate was formed with the extraction buffer. A modified protocol [24], designed to minimise detergent interference, was therefore employed, where 100 μl sample aliquots were TCA-precipitated and made 1% SDS in addition to the standard Lowry procedure. A final sample volume of 600 μl was distributed in three 190 μl aliquots on a 96-well microtitre plate, and the absorbance at 700 nm measured. Bovine serum albumin (Sigma) was used as standard reference, assuming a linear relationship of absorbance vs. protein content in a log–log graph.

RESULTS

Considerable variations in the level of c-*myc* expression have been noticed during normal *in vitro* growth of established cell lines by us (unpublished) and others [25]. Apart from random errors in the assay methods [3], these fluctuations are most likely due to uncontrollable variables of cell culturing, which affects growth rate, cell density, metabolic adjustments to medium changes, etc. In order to measure the intrinsic, constitutional level of c-*myc* expression, we took three to five samples during different passages of six cell lines representing each cell type. Computation of the mean levels was expected to reduce the influence of random fluctuations and provide more representative values.

The mean values of each of the 18 lines are shown in Fig. 1.Figure 2 shows the distribution of the mean values for the

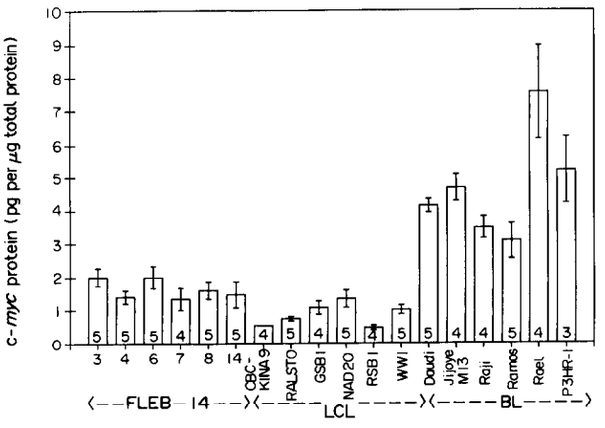

Fig. 1. Mean (S.E.) c-*myc* protein per total protein in individual cell lines. The number of samples (3–5) is indicated by the figure in the lower part of each bar.

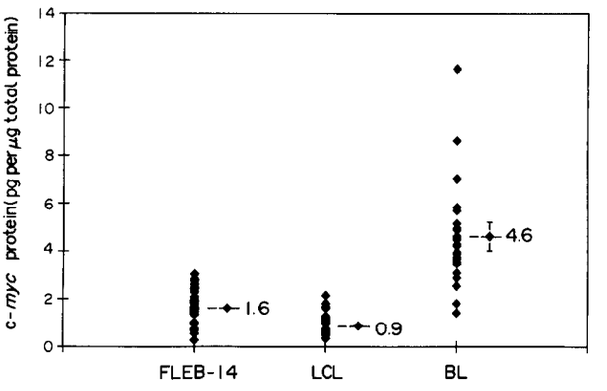

Fig. 2. Average level of c-*myc* expression in FLEB-14s, LCLs and BLs. Pooled data from Fig. 1, where all data points are plotted for the three cell line groups. Mean (S.E.): FLEB-14, 1.6 (0.14), $n = 29$; LCL, 0.9 (0.09), $n = 27$; BL, 4.6 (0.43), $n = 25$.

three groups. Means were 0.9 vs. 1.6 vs. 4.6 for LCL vs. FLEB-14 vs. BL, with <10% S.E. and all pairwise comparisons showing statistically significant differences with Student's *t* test.

DISCUSSION

In the present study, three types of human B-cell lines were compared. The LCLs had normal c-*myc* and immunoglobulin loci, the FLEB-14s had normal c-*myc* and either normal or, in the case of sublines 14-3, 14-4, 14-7 and 14-4, non-functionally rearranged IgH loci due to chromosomal translocations that did not involve c-*myc* [13]. The BLs carried typical 8;14, c-*myc*/IgH translocations. The average relative c-*myc* expression of LCL:FLEB-14:BL was found to be approximately 1:2:5.

As seen in Fig. 2, there was a considerable overlap between the c-*myc* expression of the FLEB-14 and the ordinary LCL lines, although the average differed by a factor of two ($P < 0.01$). FLEB-14 and LCL lines were phenotypically indistinguishable [13]. Both carried the surface markers and adhesion molecules characteristic of activated immunoblasts, notwithstanding the lack of any physiological Ig rearrangement in the FLEB-14 lines. The biological meaning of the 2-fold difference in the overall c-*myc* protein levels between the FLEB-14 and LCL lines is doubtful. It may simply reflect the fact that all six FLEB-14 sublines are the derivatives of a single original EBV-transformed pro-B cell clone.

The more than 5-fold increase of the average *myc* level in BL compared to the LCLs appears more convincing, particularly since none of the mean values for any of the six lines overlapped with the mean values for either the LCLs or the FLEB-14s (Fig. 1). This finding is at variance with the earlier papers of Ramsay *et al.* [9] and of Hann *et al.* [7, 8]. Hann *et al.* compared the *myc* protein expression in 17 BL and six LCL by immunoprecipitation [8]. This study had a qualitative rather than quantitative focus, and only one reported experiment directly compared three LCL and six BL samples. Visual inspection of electrophoretic bands led to the conclusion that no significant differences existed between BL and LCL lines. This method and evaluation cannot be regarded as quantitative, however; nor is the need for repeated analysis of each line taken into consideration. The same is true for the studies of Ramsay *et al.* [9] and Hann *et al.* [7], where similar comparisons were made of one LCL vs. two BL and three LCL vs. three BL samples, respectively.

Using the same quantitative ELISA technique but a different total protein assay than used here, Sullivan *et al.* [26] observed a more variable expression of c-*myc* in LCL lines. Specifically, the c-*myc* level in five LCLs from patients with Bloom's syndrome was higher than the level in two LCLs derived from normal donors, and only slightly lower than in three BL lines. The results presented here include LCL lines from cord blood, healthy donors as well as a Burkitt's lymphoma patient. Despite their diverse origin, no individual LCL has a mean c-*myc* level similar to that of any BL, lending support to the interpretation that the increased c-*myc* expression in the LCLs of Bloom's syndrome patients may be related to that disease and not due to natural variability among LCLs.

Our results thus suggest that the translocated c-*myc* gene expresses more *myc* protein in BL, on average, than the corresponding non-translocated gene in the EBV-transformed LCL. This is not surprising in itself, since the displaced gene is under the control of immunoglobulin sequences and may therefore not be subject to the same rules of synthesis and processing as its normal counterpart. The molecular basis of this abnormal constitutive activation differs among different BL lines. It consists of various defects in several regulatory systems that operate alone or in combination, depending on the fine anatomy of the translocation. They include increased transcription, loss of block to transcriptional elongation and increased mRNA half-life [27].

It does not necessarily follow that the elevated *myc* protein level is directly responsible for the tumorigenicity and high clonability of the BL cells. The normal counterpart of the BL cell probably has a silent c-*myc* gene and the translocation may act by the constitutive activation of c-*myc* expression at a critical point where sustained c-*myc* levels may prevent the cells from leaving the cycling compartment as programmed. A high constitutive *myc* protein level during *in vitro* proliferation, as found here for the BLs, may be seen as reflecting this relaxed cellular control, but need not indicate the critical pathogenic threshold *in vivo*. In view of the fact that even small quantitative changes in key regulatory proteins can lead to profound phenotypic changes in cells that are poised to respond to them, the settlement of this question will have to await more precise quantitative studies with inducible and finely tuned expression systems.

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