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IL-2 Modulation of Murine T-Cell Oncogene Expression (42465)

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Abstract. C-*myb*, a cellular oncogene associated with normal thymic development, was found to be highly expressed in four interleukin 2 (IL-2)-independent T-cell lines, but not in two of three IL-2-dependent T-cell lines. The IL-2-dependent lines, HT2 and CTLL-2, were found to have low levels of c-*myb* mRNA in the presence of IL-2. However, short-term IL-2 depletion resulted in at least fivefold increases in c-*myb* message. Add-back of IL-2 after 30 hr IL-2 depletion of CTLL-2 cells resulted in return to baseline low-level c-*myb* mRNA. Expression of the oncogenes *myc, bas, raf,* and *abl* as well as the T-cell genes Thy-1 and $C_T\beta$ did not parallel that of c-*myb*. These studies indicate that removal of a growth factor can result in increased levels of a specific cellular oncogene and that two nuclear protooncogenes (c-*myb* and c-*myc*) are expressed differentially during cell growth. These results may help to explain aspects of intrathymic T-cell differentiation where there is very high c-*myb* expression in the face of limiting amounts of growth factors such as IL-2.

The nuclear proto-oncogene c-*myc* has been postulated to regulate cell growth. Growth factor stimulation of c-*myc* has been reported in a number of different cell lines (1, 2). Less is known about c-*myb*, another nuclear oncogene, which is of particular interest because of the high level of expression found in thymocytes (3). In addition an unusual subset of T cells associated with lymphadenopathy and autoimmunity in *lpr/lpr* mice has dramatically increased expression of *myb* mRNA (4), as well as impaired production of and response to IL-2 (5, 6).

IL-2 is critical to the growth of many antigen-specific T-cell lines *in vitro*. In order to clarify the relationship between c-*myb* expression and IL-2 effects, we examined the effects of IL-2 administration or depletion on cellular oncogene expression in several T-cell lines. We found a relationship in two IL-2-dependent lines between IL-2 depletion and increased c-*myb* expression which may mimic intrathymic events.

Material and Methods. *Cell lines.* BW 5147 and EL-4 are IL-2-independent T-cell lines derived from C57BL/6 and AKR thymomas, respectively. Although EL-4 can itself produce IL-2, BW 5147 is incapable of IL-2 production (E. Shevach, personal communication). 2B4 is an IL-2-independent T-cell hybridoma which is a cytochrome *c* specific helper-T-cell line derived from a BW 5147 fusion; 8.1 is an IL-2-independent T-cell clone derived from the spleen of an MRL-*lpr/lpr* mouse with many characteristics of the abnormal T cells in *lpr/lpr* mice. HT2 is an IL-2-dependent SRBC-specific helper-T-cell line from BALB/c. CTLL-2 and CT6 are IL-2-dependent C57BL/6 cytotoxic T-cell lines.

Tissue culture. In all experiments, the T-cell lines used were greater than 90% viable and, except where indicated, were manipulated in the same way in an attempt to minimize artifactual changes in RNA expression. IL-2-dependent cells were grown in optimal concentrations (7–10%) of highly purified human IL-2 (Electro-Nucleonics, Inc., Silver Spring, MD) and were subcultured every 48 hr at densities of 5×10^4 cells/ml. Depletion of IL-2 was accomplished by gently harvesting, centrifuging, and then resuspending the cells in media without IL-2 supplementation. Cells were not washed to minimize manipulation artifacts; thus cells were depleted, but not totally deprived, of IL-2 at initial time points. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 m*M* L-glutamine, pen-

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icillin (100 U/ml), and streptomycin (100 μg/ml). 2-Mercaptoethanol (5×10^{-5} *M*) was utilized in all cultures except EL-4.

Molecular techniques. RNA isolation, blotting, and hybridization were performed as previously described (4). Poly(A)$^+$ RNA (10 μg) or 20 μg of total RNA (as indicated) was denatured in 14 m*M* methylmercury hydroxide and subjected to electrophoresis in 1.3% agarose/5 m*M* methylmercury hydroxide gels. Ethidium bromide staining of the gel before and after transfer confirmed that nearly equal amounts of RNA were blotted onto the paper. After washing under stringent conditions, the hybridized blots were exposed to film and the autoradiographs were scanned with a GS 300 scanning densitometer (Hoefer Scientific, San Francisco, CA). Relevant comparisons were made using RNA run in the same gel and transferred to the same blot. The DNA probes c-*myc,* v-*abl,* v-*raf,* and v-*bas,* were as previously described (4). The c-*myb* probe was a 0.54-kb *Eco*RI fragment subcloned from a mouse genomic bacteriophage library (7). The Thy-1 probe was the *Pst*I insert from c-DNA clone pT64 (8). The $C_T\beta$ probe was a 0.5-kb *Eco*RI fragment cleaved from the c-DNA 86T5 (9).

Flow cytometry. For cell cycle analysis, 1×10^6 cells from each culture were stained with 5 mg/100 ml propidium iodide (Calbiochem, La Jolla, CA) in 0.1% sodium citrate. Red fluorescence was measured on a FACS analyzer (Becton–Dickinson, Mountain View, CA) and the percentage of cells in S + G_2 + M was determined using a Hewlett–Packard 9920 computer (Hewlett–Packard, Fort Collins, CO).

Results and Discussion. Expression of the protooncogene c-*myb* in six different T-cell lines was examined by Northern blot analysis of poly(A)$^+$ RNA (Fig. 1). The rapidly growing IL-2-independent T-cell lines, EL-4, BW 5147, and 2B4, expressed high levels of c-*myb* RNA. Previous studies from our laboratory also have shown substantial c-*myb* expression in IL-2-independent T-cell clones derived from MRL-*lpr/lpr* spleen cells. In Fig. 1, 8.1, an MRL-*lpr/lpr* T-cell clone, showed low c-*myb* expression. In contrast, the IL-2-dependent helper-T-cell line HT2 and the IL-2-dependent cytotoxic cell line CTLL-2 both showed very low c-*myb* RNA when grown in the presence

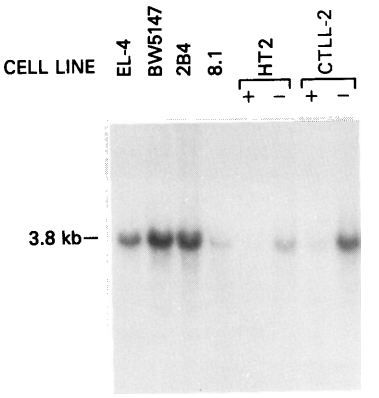

FIG. 1. Analysis of c-*myb* expression in T-cell lines. Northern blots of RNA from indicated cell lines were probed with the 0.5-kb c-*myb* probe which hybridizes to a 3.8-kb transcript. All lanes contained 10 μg of poly(A)$^+$ RNA. HT2 and CTLL-2 cells were pelleted and resuspended with IL-2 (+) or without IL-2 (−) and harvested for RNA 36 hr later.

of IL-2 despite relatively rapid doubling times (approximately 10 hr). Surprisingly, when these latter two cell lines were depleted of IL-2 for 30 hr, a 5- to 20-fold increase in c-*myb* expression resulted. Thus, removal of a required growth factor induced an increase in the amounts of a cellular oncogene transcript.

To examine more carefully the increase in c-*myb* RNA caused by removal of IL-2 from culture media, a kinetic experiment utilizing CTLL-2 cells was performed (Fig. 2). Again, depletion of IL-2 for ≥24 hr resulted in increases in c-*myb* expression. In addition, after add-back of IL-2 to IL-2-depleted cultures, c-*myb* expression decreased back to baseline low levels. The percentage of dividing cells (indicated at the bottom of Fig. 2 by % S + G_2 + M) falls, progressively after IL-2 withdrawal, while *myb* RNA levels rise. Both changes are reversed when IL-2 is added back at 30 hr and analyzed at 36 hr. Levels of Thy-1 RNA were similar at all time points (Fig. 2). Furthermore, the expression of other cellular oncogenes, *myc, bas, raf,* and *abl,* as well as the β chain of the T-cell receptor for antigen ($C_T\beta$), showed different patterns of expression with IL-2 depletion and add-back (Fig. 2). Thus, the increase in c-*myb* with IL-2 depletion appeared to be specific, and not related to a generalized increase in gene expression.

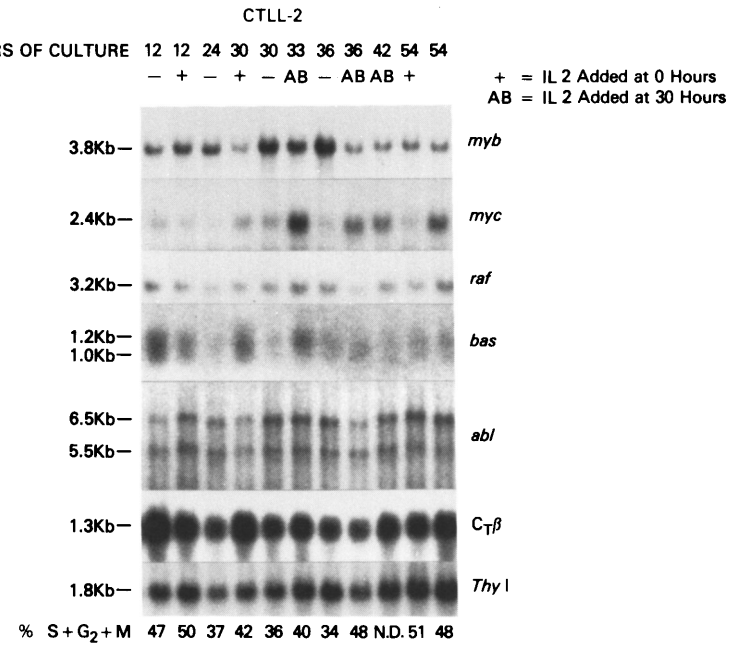

FIG. 2. Effect of IL-2 depletion and add-back on gene expression in IL-2-dependent CTLL-2 cells. Poly(A)$^+$ RNA (10 μg) obtained from each indicated culture were electrophoresed, blotted, and hybridized with probes which detect c-*myb*, c-*myc*, c-*raf*, c-*bas*, c-*abl*, Thy-1, and $C_T\beta$ (indicated to right of the picture). The size of the predominant hybridization transcript detected by each probe is indicated (kb) at the left. Cultures were grown continuously with (+) or without (−) IL-2 as indicated; IL-2 was added back (AB) at 30 hr in some cultures as noted. Cell cycle analysis was performed by flow cytometry on an aliquot of cells from individual cultures; these data are shown at the bottom of the figure. There was a significant trend of reduced percentages of cells in S + G + M with increasing time after IL-2 removal. In addition, there was a significant difference at 36 hr between (−) and AB groups.

In contrast to HT2 or CTLL-2, CT6, another IL-2-dependent T-cell line, showed higher expression of c-*myb* when grown in the presence of IL-2 (Fig. 3). In this cell line, add-back of IL-2 to IL-2-depleted cultures resulted in an increase in both c-*myc* and c-*myb* RNA (Fig. 3). These changes also corresponded to the expression of IL-2 receptors (data not shown). Although IL-2 induced cell cycle activation of CTLL-2 and CT6 cells, opposite effects on the c-*myb* expression were seen in these IL-2-dependent cell lines (compare Figs. 2 and 3). Thus, in different T-cell lines, the expression of c-*myb* is not simply related to IL-2 independence or activation with IL-2.

Although changes in c-*myb* expression varied wtih the T-cell line examined, the kinetics of change in c-*myc* expression were similar. In every case the addition of IL-2 induced a rise in *myc* RNA levels (Figs. 2 and 3). This correlated with the level of the IL-2 receptor message which was relatively constant except after addition of IL-2 when levels increased three- to five-fold in both CTLL-2 and CT6 cell lines (data not shown). Comparison of cultures with similar numbers of cycling cells, with or without depletion and addition of IL-2 indicates that the increase in c-*myc* RNA was not solely related to the percentage of cycling cells. C-*myc* expression at 3 hr (33AB), 6 hr (36AB), 12 hr (42AB), and 24 hr (54AB) after IL-2 add-back was considerably greater than in cultures with a higher percentage of cycling cells in which IL-2 was present continuously (Fig. 2).

C-*myc* remained elevated from 3 to 24 hr after IL-2 add-back to IL-2-depleted cells. C-*myc* elevation at a single time point could be due to cell cycle synchronization; however, this is unlikely to account for the prolonged c-*myc*

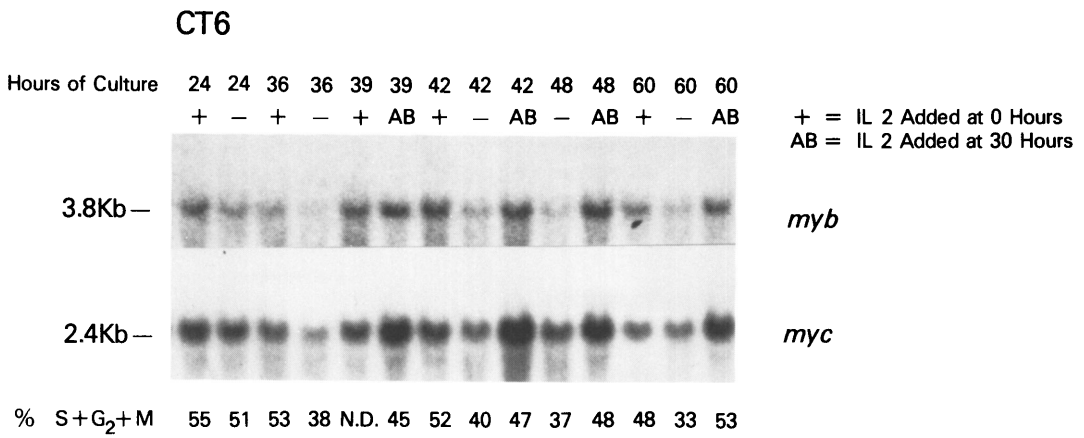

FIG. 3. Expression of c-*myb* and c-*myc* in IL-2-dependent CT6 cells. Total RNA (20 μg) was obtained from each indicated culture, electrophoresed, blotted, and hybridized with c-*myb* and c-*myc* probes. Cell cycle analyses for cultures also are indicated.

elevations seen here. These results agree with recent reports that c-*myc* mRNA and c-*myc* protein dramatically increase when quiescent cells undergo an activational event that allows cells to be competent to enter the cell cycle (10). Subsequently c-*myc* mRNA was shown to be constant throughout the cell cycle and not increased in the G_1 phase in continuously cycling cells.

In the current study c-*myb* and c-*myc* showed opposite patterns of expression in CTLL-2 cells with IL-2 depletion and add-back. These two nuclear oncogenes share structural sequence homologies at both the nucleic acid and the protein levels (11). The nuclear phosphoproteins encoded by v-*myb,* v-*myc,* and c-*myc* all appear to bind within the nuclear matrix; however, differences in the biological effects of these oncogenes have been described. V-*myc* can transform both avian fibroblasts and macrophages, whereas v-*myb* can transform avian macrophages but not fibroblasts. Since the CTLL-2 and CT6 cell lines are closely related IL-2-dependent T-cell clones with differing nuclear oncogene expression, they may prove useful in further dissecting differential and coordinate expression of c-*myc* and c-*myb.*

We do not know yet whether the changes in cellular oncogene mRNA reported in the current study result from altered RNA synthesis, differences in RNA degradation, or both. Recent evidence suggests that changes in the cellular content of both c-*myb* and c-*myc* RNA are frequently modulated by alteration in mRNA stability (12, 13). In contrast, high levels of c-*myb* RNA in both quiescent and proliferating immature thymocytes reflect increased c-*myb* transcription (13).

Although substantial advances have been made with regard to our understanding of intrathymic T-cell maturation, certain details remain to be elucidated. One characteristic of thymocytes is a much higher expression of certain genes relative to peripheral T cells; these include Thy-1, T-cell receptor genes, and c-*myb.* Moreover, reduced IL-2 concentrations in parts of the thymus might, directly or indirectly, led to high c-*myb* expression. Therefore, the present finding of increased c-*myb* expression in certain IL-2-dependent cell lines with IL-2 deprivation may help to explain the high c-*myb* expression in the thymus. It is true that mitogenic stimulation of T cells induces c-*myb* expression (13, 14) and that proliferation signals could be responsible for the high c-*myb* expression in the thymus. However, the levels of c-*myb* RNA in the thymus are substantially higher than those in the mitogenically stimulated peripheral T cells. Furthermore, the current study suggests that in certain cells there can be dissociation of c-*myb* expression and the induction of proliferation. This view is also supported by the findings of high c-*myb* expression in some thymocytes which appear not to be activated or prolifer-

ating (13, plus our own unpublished observations).

Support for the suggestion that the present finding may relate to T-cell maturation comes from studies of certain strains of mice which spontaneously develop massive expansion of a phenotypically unusual T cell. These *lpr/lpr* and *gld/gld* mice have in their lymph nodes cells which fail to produce IL-2 in response to standard signals and which express very high levels of c-*myb* RNA (4–6). Like thymocytes, these unusual lymph nodes cells have very high expression of $C_T\beta$, but unlike thymocytes they do not manifest very high Thy-1 expression (manuscript in preparation). Thus, although high c-*myb*, high $C_T\beta$, and high Thy-1 are all characteristic of thymus gene expression, this high expression need not be coordinate. The one relationship which appears to characterize thymocytes, *lpr/lpr* lymph node cells, and IL-2-depleted CTLL-2 and HT2 cells is a very high expression of c-*myb* in association with reduced amounts of IL-2.

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