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Kinetics of cellular oncogene expression in mouse lymphocytes
I. Expression of c-myc and c-ras^{Ha} in T lymphocytes induced by various mitogens*

Murine splenic T lymphocytes display maximal cellular myc gene (c-myc) expression already 3 h after concanavalin A stimulation and subsequent down-regulation before the onset of DNA synthesis. Stimulation by leucoagglutinin in the presence or absence of interleukin 2 leads to only low initial levels of c-myc-specific RNA which, however, increase later on. A similar pattern of c-myc expression is shown by the Lyt-2$^+$ T cell subpopulation stimulated with either concanavalin A or leucoagglutinin in the presence of interleukin 2. Although [^{3}H]thymidine incorporation was identical, the leucoagglutinin-stimulated Lyt-2$^+$ T cells were void of any demonstrable c-myc-specific RNA at 3 h post-stimulation. Thus, the kinetics of c-myc expression in mouse T lymphocytes are not at all uniform, but depend on the mitogen and the subpopulation. In contrast, levels of c-ras^{Ha}-specific RNA were always low at early times, always increased towards the onset of DNA synthesis and down-regulation was not observed.

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* This work was supported by the Deutsche Forschungsgemeinschaft, SFB 165, Würzburg.

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Abbreviations: Con A: Concanavalin A **c-myc:** Cellular myc (gene) **c-ras^{Ha}:** Cellular ras Harvey (gene) **IL 2:** Interleukin 2 **LA:** Leucoagglutinin

1 Introduction

Among the cell types originally investigated for cellular myc (c-myc) gene expression, cells of the hematopoietic lineage were found to be particularly active [1]. Kelly et al. [2, 3] reported an approximately 20-fold increase of c-myc-specific RNA in unseparated mouse spleen cells shortly after stimulation with lipopolysaccharide or concanavalin A (Con A). The steady-state level of myc-specific RNA then declined rather sharply before the onset of DNA synthesis. Similar results were obtained with mitogen-activated fibroblasts [2, 3] and regenerating rat liver cells [4].

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This led to the suggestion that the c-myc gene product may function during the transition through the G1 phase of the cell cycle. Subsequent reports have shown, however, that, among others, chicken embryo fibroblasts have a constant level of c-myc RNA [5] and that in quail embryo fibroblasts synthesis, half life and modification of c-myc proteins are independent of cell cycle stage [6].

The expression of another c-oncogene, cellular ras Harvey gene (c-ras^{Ha}), has also been reported to be growth cycle dependent [7] and ras-gene products were found in a variety of normal mammalian cells [8].

We here describe the kinetics of expression of c-myc and c-ras^{Ha} genes in mitogen-activated mouse T lymphocytes and T cell subpopulations. The Lyt-2$^+$ T cell subpopulation was chosen because stimulation by Con A does not result in actual growth unless interleukin 2 is added. This system therefore allows the analysis of c-oncogene expression as a result of Con A stimulation with or without subsequent proliferation. We show that levels of c-myc-specific RNA depend on the mitogen used and the subpopulation of T cells investigated. The kinetics of myc gene expression differ considerably in the two subpopulations of T cells in spite of identical proliferative responses of the cells. c-ras^{Ha} gene expression shows a more uniform pattern under the various conditions investigated. It occurred always late after stimulation and seemed to be more closely related to actual cell growth.

2 Materials and methods

2.1 Isolation and activation of T lymphocytes

T cells from mouse spleen cells were purified by passage through a nylon wool column (Leuko-Pak Leukocyte Filter, Fenwal Laboratories, Morton Grove, IL) [9]. To allow the mitogenic lectins to act optimally after the partial depletion of accessory cells, the cells were incubated (10^7 cells/ml balanced salt solution, BSS) with neuraminidase from *V. cholerae* (Test-Neuraminidase, Behringwerke AG., MarburW, FRG) at a dilution of 1/50 for 1 h at 37 °C to remove negatively charged sialic acid from the cell surface [10]. Lyt-2$^+$ T cells were obtained by treatment of purified T lymphocytes (1×10^7/ml BSS) with rat anti-mouse T4 monoclonal antibody at a dilution of 1:100 (H1.29.19) [11] followed by rabbit anti-rat Ig antibody (Dako immunoglobulins, Hamburg, FRG) at a dilution of 1:250 for 30 min at 0 °C and rabbit complement for 45 min at 37 °C. Lyt-2$^+$ cells (5×10^6/ml) were activated with Con A (2 μg/ml) or leucoagglutinin (LA; 0.5 μg/ml) [12] in the presence of IL 2 (30 U/ml; recombinant human IL 2; Dr. Fiers and Devos, Biogen, Gent, Belgium). All cells were cultured in RPMI medium (Gibco, Grand Island, NY) supplemented with nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol and 5% heat-inactivated fetal calf serum (Gibco).

2.2 Determination of proliferative activity

Two $\times 10^4$ cells were pulsed for 24 h with 5 μCi = 185 kBq of [^{3}H]thymidine, spec. act. 2 Ci/mmol. Determination of the incorporated radioactivity was performed as given in [10]. Data are presented as triplicate means of cpm.

2.3 Preparation of RNA

The lymphocytes were pelleted and resuspended in a guanidinium rhodanid buffer (4 M guanidinium rhodanid; 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, pH 7.0). RNA was pelleted through a CsCl cushion (1.7 kg/l) at 80000 × g for 20 h, resolved in TES buffer (10 mM Tris-HCl, pH 7.0, 5 mM EDTA, 1% sodium dodecyl sulfate, SDS) extracted by chloroform/butanol (4:1) and purified twice by ethanol precipitation. Twenty μg of total cellular RNA/slot was electrophoresed on 1.5% agarose gels containing 6.3% formaldehyde. Gels were blotted on nitrocellulose sheets (0.2 μm, Schleicher and Schüll, Dassel, FRG) with 20 × sodium chloride, sodium citrate (SSC) for 36 h and baked for 2 h at 80 °C in vacuum. The hybridization probes of specific DNA fragments were labeled by nick translation with ^{32}P-dATP to a spec. act. of 1×10^8 cpm/μg and hybridized for 36 h at 42 °C in 50% formamide, 6 × SSC, 1 × Denhardt's solution and 100 μ/ml salmon sperm DNA. Filters were washed in 0.2 × SSC, 0.1% SDS for 60 min at 60 °C. Autoradiography was performed with Cronex-2 films (Dupon, Wilmington, DE) at –70 °C.

2.4 Hybridization probes

The 1.5 kb Sst I fragment from the second exon of a human c-myc clone (a gift from Dr. Stehelin, Institut Pasteur de Lille, Lille, France) served as a myc-specific probe. As a ras^{Ha}-specific probe we used the 1.3 kb Kpn I/Pst I fragment of the clone H1 of Ha-MuSV [13] (a gift from J. Doehmer, MPI for Biochemie, Martinsried, FRG). The H-2-specific probe was pH-2^d-1 [14]. The probe for env is a pUC8 subclone containing the 1.0 kb Eco RI/Bgl II fragment of clone 36.1 of a xenotropic viral envelope gene [15].

3 Results and discussion

3.1 c-myc and c-ras^{Ha} expression in T lymphocytes

Mouse T lymphocytes isolated by passage through nylon wool columns contain apart from some residual macrophages and B

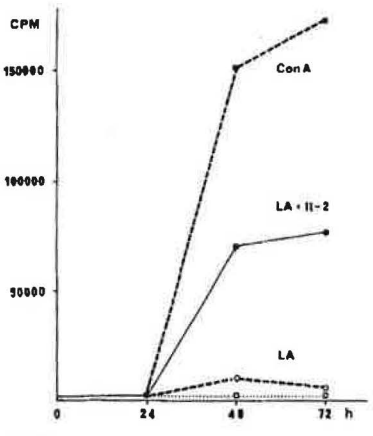

Figure 1. [^{3}H]thymidine incorporation by nylon wool-purified mouse T lymphocytes following activation by Con A, LA and IL2 and LA alone. Experimental conditions as detailed in Sect. 2.2.

lymphocytes (Lyt-2$^-$) helper and (Lyt-2$^+$) cytotoxic or suppressor T lymphocytes [16]. Stimulation by Con A leads to (a) the expression of IL2 receptors; (b) production and secretion of IL2, particularly by helper T cells and (c) proliferation of both helper and cytotoxic T cells [17]. Stimulation by low doses of LA only results in the expression of IL2 receptors but insufficient production of IL2 [12]. These cells therefore proliferate only upon addition of IL2. This is shown in Fig. 1.

Northern blot analyses using c-myc, c-ras^{Ha} and H-2-specific cloned probes were performed with total RNA derived from the variously treated cells. Fig. 2 gives the results obtained with Con A-stimulated T cells. c-myc transcripts were increased drastically within 3 h post-stimulation and down-regulated towards the onset of DNA synthesis. This is in agreement with the findings described by Kelly et al. for unseparated spleen cells [2, 3]. To correct for possibly unequal amounts of total RNA loaded onto the gels, the amounts of c-myc relative to those of H-2-specific RNA were determined by densitometry in each case (see Fig. 6). c-ras^{Ha}-specific RNA increased much slower, but also seemed to be down-regulated somewhat between 20–48 h post stimulation. The hybridizations with the H-2-specific probe are given as controls for intact RNA.

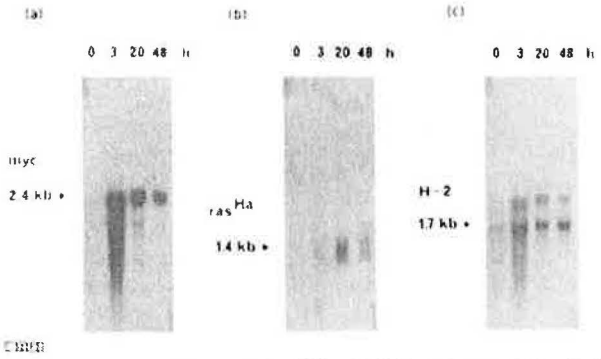

Figure 2. Northern blot analysis of the RNA of nylon wool purified mouse T lymphocytes stimulated by Con A. Hybridization with radiolabeled probes specific for (a) c-myc, (b) c-ras^{Ha} and (c) H-2. Panels a) and b) represent blots derived from parallel gels loaded each with 20 µg of the same RNA preparation. They were developed separately with the respective probes. Panel c) shows the result of a subsequent hybridization with an H-2-specific probe of blot (a). The remaining c-myc-specific probe was not melted-off and c-myc specific bands are therefore still visible above the 1.7 kb H-2-specific band. Preparation of RNA, the probes and the hybridization conditions are described in Sect. 2.4.

In Fig. 3 the results obtained with LA-stimulated T cells are presented. Again, c-myc-specific RNA increased within 3 h post-stimulation, but to a much lower extent than in the Con A-treated cells. Most importantly, the amount of c-myc transcripts further increased at later times even in the absence of IL2 when the cells did not proliferate. The same kinetics were found in the presence of IL2 which allowed cell proliferation, the total amounts of myc-specific RNA being elevated at 20 and 48 h.

These results indicate that (a) early c-myc expression per se does not lead to proliferation and (b) the type of mitogen used to activate mouse T lymphocytes determines the kinetics of c-myc expression in T cells and down-regulation is not concomitant with cell growth. Regarding c-ras^{Ha}-specific RNA, there was a minor increase throughout the post-stimulation period in the presence or absence of IL2. H-2 controls are included in Fig. 3.

3.2 c-myc and c-ras^{Ha} expression in Lyt-2$^+$ T lymphocytes

The above results had been obtained with T cells containing both the Lyt-2$^+$ and Lyt-2$^-$ T cell subpopulations. The differences found with respect to c-myc RNA after Con A and LA stimulation could be due to a preferential stimulation of the two different subpopulations by the two mitogens [12]. Therefore, Lyt-2$^+$ T cells were isolated. Owing to the lack of helper T cells, even Con A at the concentrations used hardly resulted in any cell growth (Fig. 4). Addition of IL2, however, led to an increase of [^{3}H]thymidine incorporation, confirming that IL2 receptors had been induced by Con A. Exactly the same pattern as with Con A was observed after stimulation with LA and these cells, in the presence of IL2, showed identical growth potential. However, as shown in Fig. 5, the amounts of myc-specific RNA found 3 h post-stimulation were quite different (track 5 *vs.* 8) with comparable loads of RNA as shown by H-2 controls (see also Fig. 5). Indeed, LA-stimulated cells in the presence of IL2 early on had barely demonstrable amounts of c-myc-specific RNA which, however, increased significantly later on. It is noteworthy that c-myc-specific transcripts, having already increased demonstrably within 3 h of Con A stimulation, were also increased after

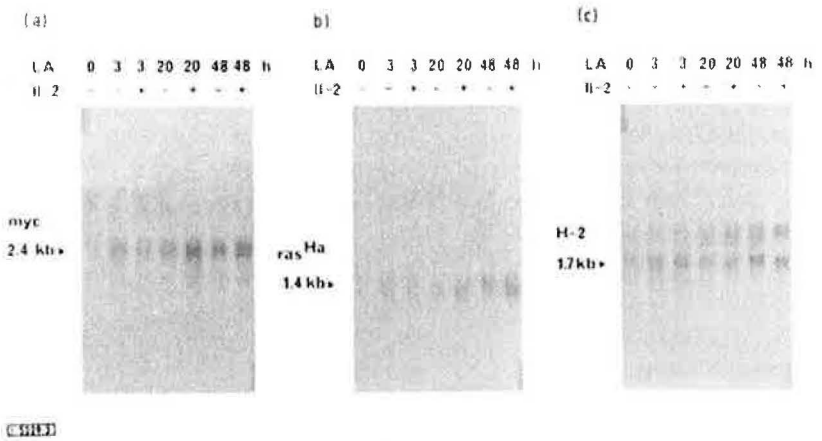

Figure 3. Northern blot analysis of the RNA of nylon wool purified mouse T lymphocytes stimulated by LA in the absence (−) or presence (+) of recombinant IL2. Hybridization probes as described in Fig. 2. (a) c-myc, (b) c-ras^{Ha} and (c) H-2. Other conditions as described for Fig. 2. Note that panel c) (again) shows the result of re-hybridization of blot a) with an H-2-specific probe and the remaining c-myc-specific bands.

20 h with or without added IL2. Note that tracks 1, 7 and 10 contained more intact RNA as revealed by the H-2 controls although, by absorbance determination, 20 μg of total RNA were applied in all cases. The c-myc-specific signals in tracks 7 and 10 can therefore only be compared directly to the time 0 control shown in track 1. However, as shown in Fig. 6, the amounts of c-myc-specific RNA relative to those of H-2 increased up to 20 h in every case and decreased thereafter. Regarding c-ras expression, a comparison of tracks 1, 7 and 10 of Fig. 5 reveals a true increase from 0 h to 48 h post-stimulation.

The differences found in total T cell populations regarding the kinetics of c-myc gene expression after Con A and LA activation can thus in part be explained by the different T cell subpopulations addressed. While Con A stimulates both Lyt-2$^-$ and Lyt-2$^+$ T cells, LA at the concentrations used only activates Lyt-2$^+$ cells [12]. The latter display their peculiar kinetics of c-myc gene expression even after stimulation with Con A.

In contrast to IL2, the platelet-derived fibroblast growth factor as such induces c-myc expression in its target cells and in that respect mimics the action of mitogens on lymphocytes [2, 3, 18]. At the same time, however, it leads to cell proliferation. This is unlike the growth requirements of Lyt-2$^+$ T cells which need both induction by antigens or lectins and the growth factor IL2. Since the latter did not significantly influence the expression of c-myc genes one indeed comes to the conclusion that, if at all, c-myc expression must be correlated with the mitogen-induced commitment of the cell to grow, rather than with actual growth.

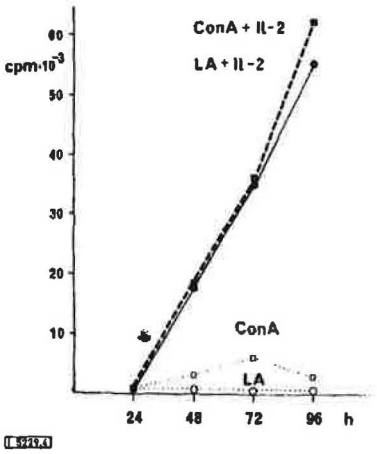

Figure 4. [^{3}H]thymidine incorporation by Lyt-2$^+$ and mouse T lymphocytes stimulated by Con A or LA, both in the presence and absence of recombinant IL2.

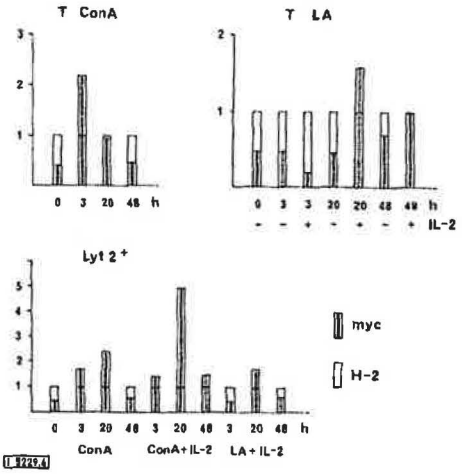

Figure 6. Summary evaluation of c-myc expression in T lymphocytes. Densitometry readings were obtained of each of the c-myc- and H-2-specific bands shown in Figs. 2, 3 and 5. The values obtained for H-2 were normalized to arbitrary units of 1. The values obtained for the corresponding c-myc bands were then computed in the same manner to present the densitometry readings of c-myc relative to H-2. This presentation corrects for possibly unequal amounts of total RNA loads in the various tracks.

However, from our results a strong correlation between onset and strength of c-myc gene expression or steady state level of specific mRNA and commitment of the cells to grow is not obvious either. In the case of Lyt-2$^+$ T cells stimulated by LA, very little of c-myc-specific RNA would suffice early after mitogenic stimulation and down-regulation is not observed at all. Yet these cells, upon addition of the growth factor IL2, proliferate just as well as unseparated Con A-stimulated T

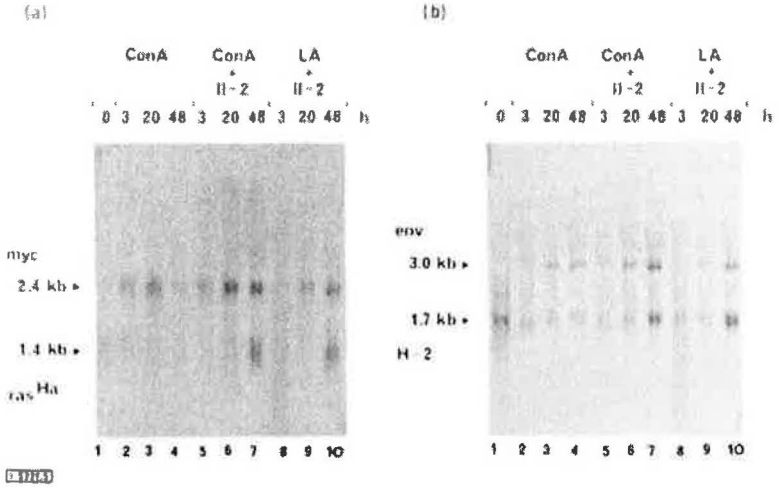

Figure 5. Northern blot analysis of the RNA of Lyt-2$^+$ mouse T lymphocytes stimulated by Con A alone and by Con A or by LA in the presence of recombinant IL2. Panels a) and d) are two parallel blots derived from two parallel gels loaded with 20 μg per slot of the same RNA preparation. Blot a) was hybridized simultaneously with c-myc and c-ras^{Ha}-specific probes. Blot b) was hybridized simultaneously with an H-2-specific probe and, as an additional marker for intact RNA, with a probe specific for the env gene of an endogeneous mouse retrovirus [15]. Note that tracks 1, 7 and 10 contained more intact RNA as revealed by the H-2 controls.

cells in which a very early and very strong optimum of c-myc gene expression occurs.

A much better correlation would seem to exist between c-myc and IL2 gene expression or IL2 production, respectively. Indeed, mitogen-induced Lyt-2$^-$ cells show similar optima in the kinetics of c-myc, as shown here, and IL2-specific mRNA [19]. Also, in both cases the down-regulation appears to be due to similar post-transcriptional mechanisms as indicated by the protective effect of cycloheximide on steady state levels of both types of mRNA [19, 20]. Conversely, Lyt-2$^+$ cells produce, if any, very small amounts of IL2 and their levels of myc-specific RNA are very low.

Thus, the investigation of the two phenotypically distinct T lymphocyte subpopulations revealed novel and interesting phenomena regarding c-oncogene expression and growth regulation. The uniform pattern of c-myc gene expression described so far for mouse lymphocytes [1, 3] does not apply to all T cell subsets in spite of comparable mitogen plus IL2-induced proliferative responses. Therefore, a different type of correlation between c-myc gene expression and growth regulation may have to be considered. We hope to gain further insight into the actual involvement of the c-myc gene by ongoing studies using appropriate and specific anti-sense probes.

We thank Dr. A. Begue, Lille, for the c-myc clone and Dr. E. U. Scolnick, West Point, for the ^{H}ras clone. We are grateful to Drs. Fiers and Devos, Gent, Belgium, for a generous gift of recombinant human interleukin 2. The expert technical assistance of Ms. Renate Schmitt is gratefully acknowledged. We also thank Ms. Helga Kriesinger for the preparation of the manuscript.

Received August 17, 1985; in revised form October 22, 1985.

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