Brief Definitive Report

SEQUENTIAL EXPRESSION OF GENES INVOLVED IN HUMAN T LYMPHOCYTE GROWTH AND DIFFERENTIATION

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The complex series of cellular and molecular interactions that comprise T cell activation can be initiated by diverse agents including antigen, lectins, tumor promoters, and monoclonal antibodies such as OKT3 and OKT11 (1, 2). Each of these activation signals ultimately influence T cell growth and differentiation either by altering the properties or rates of synthesis of existing proteins or by initiating the synthesis of new ones. In this regard, the early phase of T cell activation is characterized by an increase of cellular RNA content, synthesis of new proteins, and the development of receptiveness to further activation signals. Included among this newly induced network of cellular proteins are the interleukin 2 (IL-2) receptor, transferrin (Tf) receptor, and the lymphokines, IL-2 and γ interferon (IFN-γ) (3–6). Through interactions with its cellular receptor, IL-2 is capable of maintaining the long-term growth of normal T cells in vitro (7, 8). Tf receptor expression enables T cells to import Tf, the predominant serum iron–binding glycoprotein. Tf-mediated delivery of iron is absolutely required for eukaryotic cell growth. Although a role for IFN-γ in T cell growth has not yet been elucidated, IFN-γ does promote the differentiation of cytotoxic T cells and natural killer cells, in addition to its antiviral activity (9). T cell activation also results in early expression of the proto-oncogene c-*myc* (10). c-*myc* encodes a DNA-binding protein that may control the transcription of some cellular genes (11). Thus far, little is known about how these genes that control T cell growth and differentiation relate to each other. It has been demonstrated (4) that an interaction of IL-2 with its receptor is required for the expression of Tf receptors. It also has been suggested that IL-2 is involved in the regulation of expression of its own receptor (12–14) and of IFN-γ (12, 15).

As a further step towards understanding the regulation of the genes encoding c-*myc*, IL-2, IFN-γ, and receptors for IL-2 and Tf, we have studied the kinetics of induction of these genes during T lymphocyte activation. Using nuclear transcription assays, we present data demonstrating that expression of each of

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these genes is regulated at the level of transcription, and, with the exception of the Tf receptor, each is induced independently of de novo protein synthesis.

Material and Methods

Preparation of Cells. Peripheral blood lymphocytes (PBL) from normal donors were isolated by Ficoll-Hypaque gradient density centrifugation. T cells were prepared by rosetting PBL with sheep red blood cells as previously described (16). T cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, and stimulated with phytohemagglutinin (PHA) (1 μg/ml) and phorbol myristate acetate (PMA) (20 ng/ml).

Immobilization of cDNA Probes on Nitrocellulose Filters. Linearized cDNAs specific for IL-2, IL-2 receptor (17), IFN-γ, Tf receptor, c-*myc*, HLA, and pBR322 were denatured by boiling for 10 min and incubation in 1 N NaOH for 20 min at room temperature. cDNAs were then neutralized in a buffer containing 1 M NaCl, 0.3 M NaAc, 0.5 M Tris (pH 7.5), 1 M HCl and transferred to nitrocellulose filters using a vacuum-manifold apparatus (Schleicher & Schuell, Inc., Keene, NH). 1 μg of cDNA was applied to each filter, followed by rinsing in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), air drying, and baking in vacuo at 80°C for 2 h.

In Vitro Transcription With Isolated Nuclei. Nuclear "run off" experiments were performed as previously described (18). Briefly, nuclei from induced and uninduced T cells were isolated by cell lysis in a buffer containing 0.05% Nonidet P-40 and subsequent centrifugation through 2 M sucrose gradients. For in vitro transcription, nascent RNA chains were allowed to elongate in the presence of [α^{32}P]UTP. Labeled nuclear RNA was then purified by DNase treatment, proteinase K digestion, phenol extraction, and ethanol precipitation. The labeled nuclear RNA was then hybridized to excess specific cDNA immobilized on nitrocellulose filters. After 2 d, filters were washed and autoradiographed. The radioactivity bound was determined by liquid scintillation counting. The transcriptional activity of a specific gene is expressed as parts per million (ppm). To determine specific ppm, background hybridization to pBR322 DNA was substracted from the actual radioactivity measured. This specific value was then divided by the total amount of input counts per minute of radioactive nuclear RNA and multiplied by 10^6.

Results

Using nuclear transcription assays, we determined the time course of expression of various genes in PHA- and PMA-stimulated T cells. The transcriptional kinetics of genes encoding HLA, c-*myc*, IL-2, and IFN-γ are presented in Fig. 1, *A* and *B*. As shown, HLA is constitutively transcribed, but a modest increase in transcriptional activity occurred after PHA and PMA activation. In contrast, c-*myc* is not transcribed in resting T cells. However, after stimulation, this gene was rapidly induced with peak transcription at 6–9 h. Transcriptional activity of IFN-γ and IL-2 lagged behind c-*myc*, peaking at 9 and 24 h, respectively. Although not returning to basal levels during this assay, transcription of each of these genes declined after peaking, reflecting the generally transient nature of expression of the genes involved in early T cell activation.

Also shown (Fig. 1) are the kinetics of transcription for these genes in the presence of cycloheximide. At 2×10^{-5} M, cycloheximide inhibited >98% of protein synthesis as judged by [^{3}H]leucine incorporation, yet revealed a cell viability of >85% at 48 h. Cycloheximide was added 30 min before stimulation with PHA and PMA to ensure inhibition of protein synthesis at early time points. As shown, cycloheximide did not significantly alter the induction of c-*myc*, IL-2, or IFN-γ gene transcription. Effects of cycloheximide on transcription could not

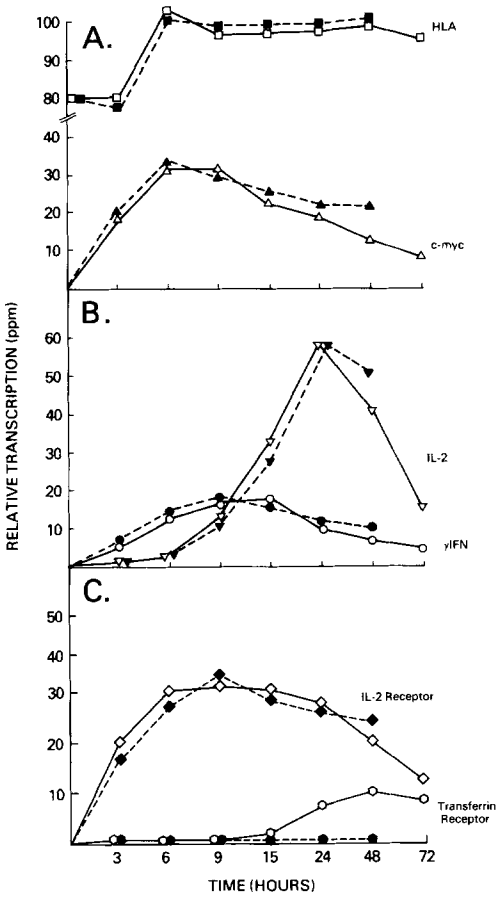

FIGURE 1. Kinetic analysis of gene transcription during human T cell activation. After stimulation with PHA and PMA, nuclei were isolated at indicated times and assayed for transcriptional activity of genes encoding (*A*) HLA (□), *c-myc* (△); (*B*) IL-2 (▽), IFN-γ (○); and (*C*) IL-2 receptor (◇) and Tf receptor (⬡). Relative transcription of these genes is expressed in parts per million and determined as described in Material and Methods. Shown in broken lines and closed symbols are the respective transcriptional activities in the presence of 2×10^{-5} M cycloheximide. Essentially identical results were obtained in two additional experiments using either the same or a different normal lymphocyte donor.

be confidently measured after 48 h due to decreases in cell viability and diminished recovery of ^{32}P-labeled nuclear RNA.

As shown in Fig. 1*C*, neither IL-2 receptor nor Tf receptor genes are transcribed in uninduced T cells. However, after stimulation with PHA and PMA, the IL-2 receptor gene expression was rapidly induced, with peak transcriptional activity from 6 to 24 h. Induction of transcription of the Tf receptor occurred much more slowly, with initiation between 15 and 24 h and peak expression at 48 h. The addition of cycloheximide did not alter IL-2 receptor gene expression. However, Tf receptor gene transcription did not occur in the presence of this agent. Thus, among the genes investigated, only Tf receptor gene expression appears to be dependent upon de novo protein synthesis during T cell activation.

Discussion

Using an in vitro nuclear transcription assay, we have studied the kinetics of activation of select genes involved in T cell growth and differentiation. We demonstrate that c-*myc*, IL-2, IL-2 receptor, IFN-γ, and Tf receptor genes are transcriptionally silent in resting T cells, or at least below the level of detection of our assay system. After T cell activation with PHA and PMA, each of these genes was induced, with different kinetics. PHA- and PMA-mediated activation of c-*myc*, IL-2, IL-2 receptor, and IFN-γ genes occurred in the presence of cycloheximide, indicating that protein synthesis was not required for expression. In contrast, Tf receptor gene expression was blocked by cycloheximide.

Analysis of the time courses of induction indicates that the genes encoding c-*myc* and IL-2 receptor precede the transcription of each of the other genes studied. This finding is consistent with previous studies by Kelly et al. (10), who detected c-*myc* mRNA accumulation in murine T cells as early as 1 h after stimulation with concanavalin A. The c-*myc* gene product is a nuclear protein, whose deregulated expression secondary to chromosomal translocation has been associated with Burkitt's lymphomas (11, 19). This protein has been implicated in transcriptional modulation and appears to be involved in the transit of cells from the G_0 to G_1 phase of the cell cycle. However, as shown in this study, c-*myc* protein production is not required for IL-2, IL-2 receptor, or IFN-γ gene expression, since the induction of these genes is independent of protein synthesis.

IL-2 has been implicated in the regulation of expression of its own receptor and IFN-γ (12–15). However, the results of the present study suggest that the presence of IL-2 protein is not absolutely required for transcriptional activation of either the c-*myc*, IL-2 receptor, or IFN-γ gene. First, c-*myc*, IL-2 receptor, and IFN-γ genes are transcribed before transcription of the IL-2 gene. Second, inhibition of protein synthesis with cycloheximide did not alter transcription of these genes. Reem and Yeh (12) and Welte et al. (13) have demonstrated that recombinant IL-2 can up-regulate IL-2 receptor expression in suboptimally stimulated T cells. These studies involved activation of T cells in the presence of dexamethasone or with submitogenic amounts of OKT3 monoclonal antibody. Similarly, in a different system, we have observed (14) that IL-2 alone can augment IL-2 receptor gene reexpression in activated T cells that have lost >80% of their receptors during a 12 d culture period. In the present study we have activated T cells with optimal concentrations of PHA and PMA. Thus, an amplifying effect of IL-2 on IL-2 receptor gene expression may have been circumvented. We conclude that IL-2 receptor and IFN-γ gene expression is susceptible to IL-2-mediated augmentation in suboptimally stimulated T cells. However, in fully stimulated T cells, IL-2 receptor and IFN-γ gene transcription can clearly occur independent of IL-2.

Fox et al. (20) have suggested that IL-2 receptor expression occurs before IL-2 gene activation during the maturation of thymic T lymphocytes. These findings mirror the kinetics of IL-2 receptor and IL-2 gene expression that occur during T cell activation. Thus, the sequence of IL-2 and IL-2 receptor gene expression in activated T lymphocytes established in this study appears to recapitulate events in early T cell ontogeny.

In contrast to the genes discussed above, Tf receptor gene transcription is

initiated late in the course of T cell activation and its expression required de novo protein synthesis. This is consistent with the studies of Neckers and Cossman (4), who demonstrated that Tf receptor expression requires an interaction of IL-2 with its cellular receptor. In conclusion, we suggest that the genes encoding c-*myc*, IL-2, IL-2 receptor, and IFN-γ are members of a family of genes that are rapidly, independently, and transiently induced during T cell activation. Expression of these gene products may subsequently activate other genes, i.e., the Tf receptor gene, which are required for later steps in T cell growth and differentiation.

Summary

Nuclear transcription assays were performed with isolated nuclei from human peripheral blood T lymphocytes stimulated with phytohemagglutinin and phorbol myristate acetate to determine the kinetics of transcriptional activity of various genes occurring in T cell activation. Although silent in resting T cells, the genes encoding c-*myc* and the interleukin 2 (IL-2) receptor were induced early, preceding γ interferon (IFN-γ), IL-2, and transferrin receptor gene transcription. Transcriptional activity of these genes fell after their respective peaks, indicating that the expression of these genes is a transient event during T cell activation. With the exception of the transferrin receptor gene, the kinetics of induction of these genes were not altered by concentrations of cycloheximide that inhibited protein synthesis. These data indicate that the induction of genes encoding c-*myc*, IL-2, IL-2 receptor, and IFN-γ occur independently of the sequential production of the proteins they encode.

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