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Relationship of Cellular Oncogene Expression to Inhibition of Growth and Induction of Differentiation of Daudi Cells by Interferons or TPA

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Human α or β interferons inhibit the proliferation of Daudi Burkitt lymphoma cells and induce the differentiation of these cells towards a mature plasma cell phenotype. Similar responses are seen when Daudi cells are treated with the phorbol ester, TPA. Both interferons and TPA down-regulate expression of the c-myc oncogene in these cells. Although TPA can mimic the effect of interferon on cell differentiation, it does not induce 2′5′ oligoadenylate synthetase or the interferon-sensitive mRNAs, 6–16 or 9–27. Thus chronic stimulation of protein kinase C by TPA cannot mimic all of the effects of interferon treatment on gene expression. Inhibition of ADP-ribosyl transferase activity by 3-methoxybenzamide impairs interferon- or TPA-induced differentiation of Daudi cells. This agent induces a higher level of c-myc mRNA in the cells and stimulates the incorporation of [^{3}H]thymidine into DNA; although these effects are partially counteracted by interferon or TPA treatment, the elevated expression of the c-myc gene may be sufficient to prevent terminal differentiation and allow cell proliferation to continue.

Key words: ADP-ribosyl transferase, cell proliferation, gene expression, protein kinase C, c-myc expression

Treatment of a number of immortalized or transformed cell lines with homologous interferons has been shown to inhibit cell proliferation [1-3]. In many cases this effect is accompanied by the differentiation of the cells towards a more mature phenotype [3,4]. This suggests the possibility that the loss of proliferative ability may be related to a change in the pattern of gene expression in these cells that leads to terminal differentiation [5]. We have shown recently that the Burkitt lymphoma-derived Daudi cell line, which is particularly susceptible to growth inhibition by human α or β interferons, is induced to express surface antigens characteristic of antibody-secreting plasma cells when treated with highly purified Namalwa cell

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interferons or cloned IFN-α_2 [6,7]. This response was inhibited if the cells were pretreated with the differentiation inhibitors 3-methoxybenzamide (3-MB) or 3-aminobenzamide (3-AB), which block ADP-ribosyl transferase activity [7]. These agents had a protective effect on the cells against the growth inhibitory action of the interferons; such a result is consistent with an obligatory link between cell differentiation and growth inhibition in this system. The expression of plasma cell antigens by Daudi cells also can be stimulated by treatment with the phorbol ester, 12-O-tetradecanoyl phorbol 13-acetate (TPA), and this effect too is inhibited by 3-MB or 3-AB [7].

Insight into a possible molecular basis for the antiproliferative effects of interferons has come from the demonstration that the expression of a number of cellular oncogenes associated with cell growth and/or maintenance of a transformed state is down-regulated by interferon treatment [8-10]. In Daudi cells the expression of the c-myc gene is inhibited by interferons at both the RNA and protein levels [8,9].

Although the function of the DNA binding protein encoded by c-myc is not known, unregulated expression of the gene is believed to be a cause of the tumourigenic properties of Burkitt lymphomas. In a large proportion of these tumours transcriptional activation of c-myc has occurred as a result of a reciprocal translocation of the gene to an immunoglobulin locus [11]. Having identified conditions under which the antiproliferative effect of interferons can be inhibited in Daudi cells, we took interest in investigating the relative levels of expression of c-myc under these conditions. The effects of the ADP-ribosyl transferase inhibitor 3-MB on c-myc and on other genes that are regulated by interferons has been examined. At the same time we have analysed the effect of TPA treatment on cell proliferation and on expression of these genes in order to determine the extent to which chronic exposure to phorbol esters can mimic the effects of interferons.

MATERIALS AND METHODS

Materials

Highly purified human lymphoblastoid (Namalwa) cell interferons (7.1×10^7 units/mg protein) were kindly provided by Wellcome Biotechnology (Beckenham, England). TPA was from Sigma and 3-MB was from Aldrich. DNA probes specific for human c-myc [12], 2′5′ oligoadenylate synthetase [12], 6–16 and 9–27 mRNAs [12–14] were as described. The monoclonal antibodies against surface antigens characteristic of plasma cells also have been previously described [15,16].

Cell Culture

Culture of Daudi cells; treatment with 3-MB, interferons, or TPA; determination of cell number; and measurement of incorporation of [^{3}H]thymidine into DNA were all carried out as in earlier reports [7,17,18].

RNA Extraction and Analysis

Total cell RNA was extracted with the LiCl/urea procedure of Auffray and Rougeon [19]. For analysis of the content of individual mRNA sequences, the RNA was glyoxylated and subjected to electrophoresis on 1.4% agarose gels in 10 mM sodium phosphate buffer (pH 7.0) and then blotted on to NNG nylon filters (Pall Corp.), as previously described [20]. These blots were hybridized with ^{32}P-labelled

DNA probes specific for c-myc, 2′5′ oligoadenylate synthetase, 6–16 or 9–27 mRNA sequences, as described previously [12–14]. After stringent washes (final wash in 0.1 × SSC, 0.1% SDS at 60°C), the blots were subjected to autoradiography and the exposed x-ray film was scanned with an LKB 2202 laser densitometer. Relative densities of the bands were quantified with the LKB 2190 GELSCAN program.

Cell Differentiation

The extent of differentiation of Daudi cells towards the plasma cell phenotype was determined by flow cytometry, with monoclonal antibodies against four different cell surface markers (BU5, 7, 9, and 11) [15,16]. The conditions of antibody binding have been described previously [6,7].

The results are expressed as the percentage of positively staining cells obtained, after subtraction of background values for nonspecific binding of antibody against human immunoglobulin λ light chain. The latter is not synthesized by Daudi cells.

RESULTS

The ability of human α and β interferons to inhibit Daudi cell proliferation is well documented [6–9]. Table I shows that treatment of these cells with TPA at 10^{-9} M or greater also inhibits proliferation within 24 h. In order to examine the effects of 3-MB on the expression of various genes that may be associated with inhibition of growth or induction of differentiation by interferon or TPA treatment, cells were

TABLE I. Effects of TPA Treatment on Cell Proliferation and Thymidine Incorporation Into DNA*

TPA concentration (M)	Time (days)	Cell number ($\times 10^{-5}$/ml)	Cell doublings	Inhibition by TPA (%)	^{3}H-TdR incorporation (cpm/10^5 cells)	Inhibition by TPA (%)
0	0	2.24			8510	
	1	4.32	0.95		4920	
	2	7.88	1.81		6190	
	3	11.60	2.37		5570	
10^{-9}	0	2.04			7340	13.7
	1	3.48	0.77	18.9	8490	Stimulation
	2	6.07	1.57	13.3	11500	Stimulation
	3	9.80	2.25	4.6	10090	Stimulation
10^{-8}	0	2.12			9260	Stimulation
	1	2.46	0.21	77.9	4150	15.6
	2	2.45	0.21	88.4	6560	Stimulation
	3	4.19	0.98	58.6	3430	38.3
10^{-7}	0	2.35			8280	2.7
	1	2.28	0	100	3180	35.4
	2	2.42	0.04	97.8	3290	46.8
	3	3.80	0.69	70.9	1860	66.5

*Exponentially growing Daudi cells were diluted to the cell densities indicated at time 0 and incubated with the concentrations of TPA shown. Cell numbers were determined at daily intervals; the incorporation of ^{3}H-thymidine into DNA was measured on a small sample over a 1-h incubation period. The percentage inhibition of cell growth rate and thymidine incorporation are calculated relative to the corresponding values for the control cells on each day of the experiment.

preincubated with or without 3-MB and then exposed to either agent for 24 h. RNA was extracted from the cells and subjected to Northern blotting analysis with a human c-myc cDNA probe. Figure 1 shows the relative levels of c-myc mRNA in total cell RNA after the various cell treatments. In cells not exposed to 3-MB, both interferon and TPA lowered the level of c-myc RNA (by 66% and 48%, respectively). In cells preincubated with 3-MB for 24 h, the level of c-myc sequences was dramatically elevated (by a factor of 4.4-fold). Again, both interferon and TPA treatment resulted in a reduction in c-myc mRNA, but the concentration of this RNA remained substantially higher than in the control cells. Thus, while 3-MB does not prevent an inhibitory effect of interferon or TPA on c-myc expression, this agent has the effect of raising the absolute level of mRNA for this oncogene in the cells under all these conditions.

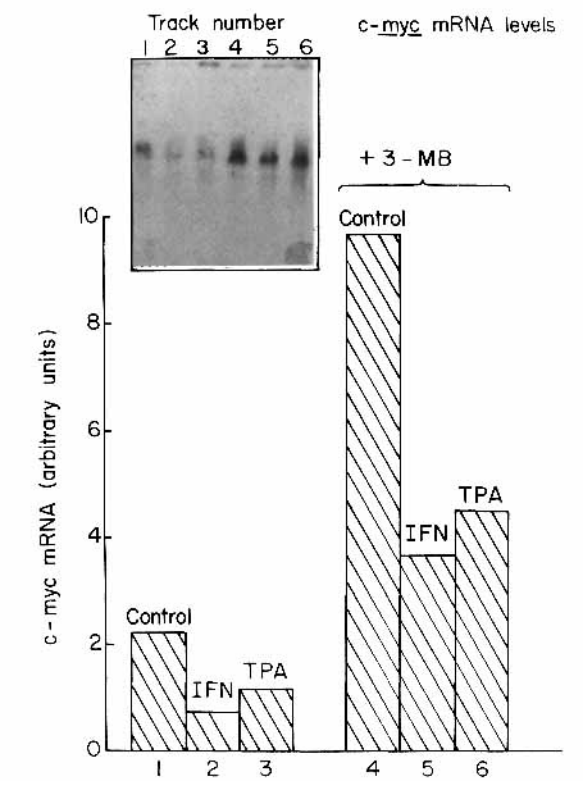

Fig. 1. Effects of preincubation with 3-methoxybenzamide on c-myc mRNA levels in control, interferon-treated and TPA-treated Daudi cells. Exponentially growing Daudi cells were diluted to approximately 1.8×10^5 cells/ml and incubated with or without 3-MB (3 mM) for 24 h. Each batch of cells was then divided into three aliquots of 250 ml, which were incubated with or without interferons (200 units/ml) or TPA (1.6×10^{-8} M) for an additional 24 h. Cell numbers were then determined and total cell RNA was extracted for analysis by Northern blotting. Equal quantities per track of each RNA preparation were loaded and fractionated on a 1.4% agarose gel. The RNA was transferred to nylon paper and hybridized with a probe for the human c-myc sequence (ca. 10^9 cpm/μg DNA). After washing, the blot was subjected to autoradiography and the relative intensities of the bands (inset) were quantified by laser densitometry. The values obtained are plotted in arbitrary units.

The ability of TPA to mimic the effect of interferon treatment on differentiation of Daudi cells [7] and on the down-regulation of c-myc mRNA (Fig. 1) suggests that these two agents may exert their actions by a common biochemical mechanism. In particular, because TPA is a potent activator of protein kinase C, the results implicate involvement of this enzyme in the growth-regulatory effects of interferons. We therefore examined the extent to which TPA could mimic the ability of interferons to induce other changes in gene expression. As shown in Figure 2, the levels of three interferon-inducible mRNAs, viz. 2′5′ oligoadenylate synthetase, 6–16 RNA, and 9–27 RNA [13,14], were not increased in TPA-treated cells. We also failed to detect any increase in 2′5′ oligoadenylate synthetase enzyme activity in Daudi cells after phorbol ester treatment (data not shown). These results indicate that only part of the cellular response to interferons can be elicited by chronic stimulation of protein kinase C by TPA. Pretreatment of the cells with 3-MB partially inhibited induction of the 2′5′ oligoadenylate synthetase and 9–27 mRNAs by interferon but had no effect on expression of the 6–16 mRNA.

The effects of interferon, TPA, and 3-MB on thymidine incorporation into DNA, in relation to the relative levels of c-myc expression, are shown in Figure 3A. As reported previously [17,18], interferon strongly inhibited thymidine incorporation into DNA. Curiously, although TPA is very potent in blocking cell growth (Table I) [21] it has a less marked effect than interferon on thymidine incorporation. Preincu-

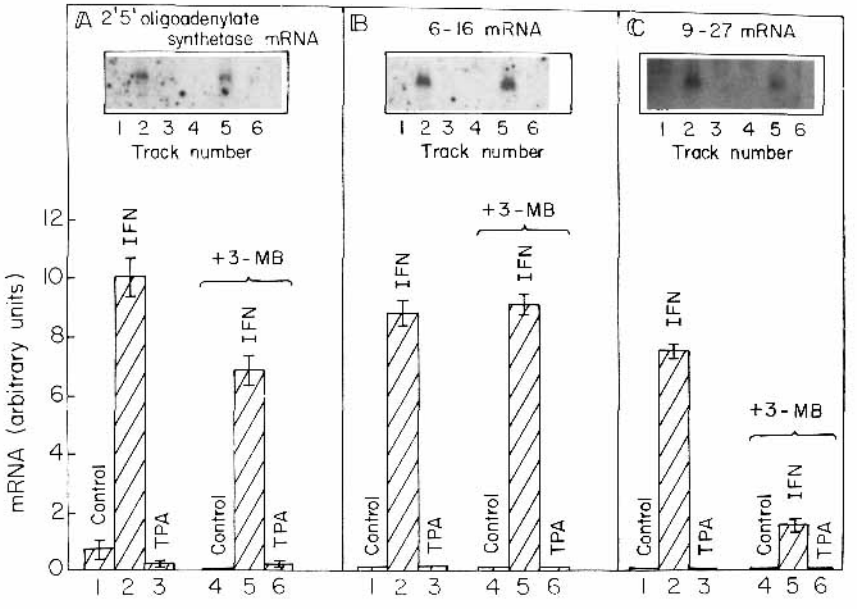

Fig. 2. Interferon-inducible mRNA levels in Daudi cells treated with interferon, TPA, and 3-methoxybenzamide. The procedures were exactly as described in Figure 1, except that the DNA probes used for hybridization analysis of the Northern blots were (**A**) 2′5′ oligoadenylate synthetase cDNA; (**B**) 6–16 cDNA; (**C**) 9–27 cDNA. All probes had specific activities of 5×10^8–1×10^9 cpm/μg DNA. The relative intensities of the bands (inset) were quantified by laser densitometry (4 scans per track) and the mean values ± standard errors of the mean are shown in arbitrary units.

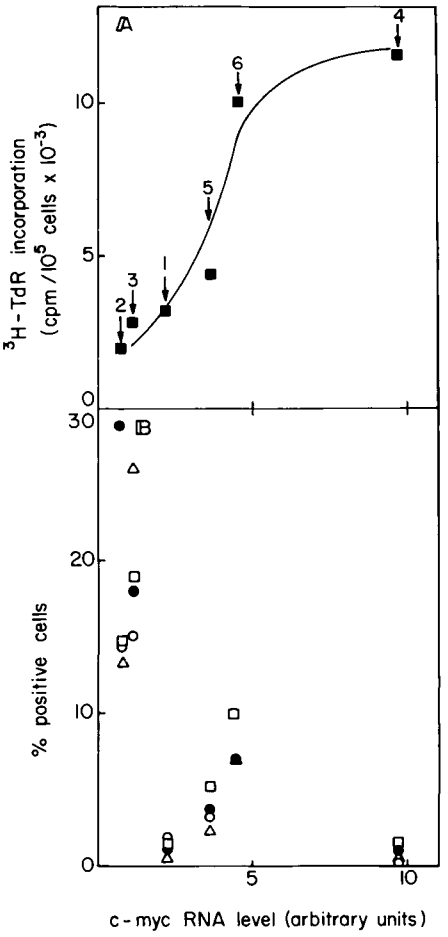

Fig. 3. Relationship of the level of c-myc mRNA to thymidine incorporation into DNA and the expression of differentiation antigens in Daudi cells. **Panel A** shows the relationship between DNA labelling with thymidine and relative levels of c-myc mRNA for the six conditions described in Figure 1: **1,** controls; **2,** interferon-treated; **3,** TPA-treated; **4,** 3-MB-treated; **5,** interferon- and 3-MB-treated; **6,** TPA- and 3-MB-treated. **Panel B** shows a correlation between cell differentiation (expressed as percentage of cells stained positively by monoclonal antibodies against plasma cell surface markers [7]) and levels of c-myc mRNA. For each of the six conditions staining with four different antibodies is shown: ○, BU5; △, BU7; □, BU9; ●, BU11.

bation of the cells with 3-MB stimulated thymidine incorporation into DNA substantially. Nevertheless, as shown previously [7], exposure to 3-MB did not prevent inhibition of thymidine incorporation by interferon.

Figure 3B illustrates the relationship in Daudi cells under these various conditions between c-myc mRNA level and expression of cell-surface antigens characteristic of plasma cells (a measure of differentiation). It can be seen that conditions that favour c-myc expression are associated with low levels of plasma cell antigens, and vice versa. Recent literature has strongly suggested a role for c-myc in the regulation of both cell proliferation and differentiation in a variety of systems; although the evidence is so far only circumstantial, our data support this concept of a direct

relationship between c-myc expression and parameters associated with cell growth and DNA synthesis, on the one hand, and an inverse relationship with cell differentiation on the other.

DISCUSSION

We have shown here that the level of c-myc oncogene mRNA can be regulated by three distinct agents: interferons, a phorbol ester (TPA), and an inhibitor of ADP-ribosyl transferase (3-MB). The first two down-regulate while the latter enhances expression of the c-myc gene at the RNA level; the interactions between these opposing effects may account for some of the antagonistic actions of 3-MB on the biological effects of interferons and TPA in Daudi cells. The data presented here do not distinguish between possible effects of the different agents at the level of transcription of the c-myc gene and effects on the stability of c-myc mRNA in the cytoplasm. Further experiments are necessary to examine these potential mechanisms of action. In the case of interferon action on Daudi cells, reports suggest control of c-myc expression at both the transcriptional and post-transcriptional levels [22,23]. Whichever is the case, the unusually short half-life of both c-myc mRNA and the c-myc protein [11] would allow rapid changes in the concentration of the gene product to occur in the nucleus in response to interferon, TPA, or 3-MB treatment.

Perhaps the most surprising finding from this work is the large increase in c-myc mRNA that occurs within 24 h of exposure of cells to 3-MB (Fig. 1). This is not the first instance of such an observation; McNerney et al. [24] have reported an increase in c-myc mRNA levels of about ninefold in serum-stimulated 3T3 cells after incubation with 3-MB. The physiological significance of such a response is not yet clear. Inhibitors of ADP-ribosyl transferase have been shown to inhibit the repair of DNA strand breaks [25], and it has been suggested that poly(ADP-ribose) is an activator of DNA ligase [26]. There is no evidence connecting the accumulation of DNA strand breaks with activation of c-myc expression, but the ability of the c-myc protein to bind DNA and its possible role in DNA replication [27] could provide the basis of a feedback relationship between certain steps in DNA synthesis and c-myc gene activity. A role for c-myc in promoting DNA ligation would be consistent with earlier data from our laboratory indicating impairment of this process in interferon-treated Daudi cells [18].

The ability of the phorbol ester TPA to mimic the effects of interferon treatment on growth and differentiation of Daudi cells [7] raises the possibility that the protein kinase C pathway may be involved in the mechanism of interferon action. This possibility is supported by the observation that interferon treatment transiently raises the intracellular concentration of diacylglycerols in these cells [21]. It is clear from our results, however, that chronic treatment with TPA cannot reproduce all the effects of interferons in this cell line. The phorbol ester does not induce 2′5′ oligoadenylate synthetase at either the RNA or protein level and also has no effect on expression of the 6–16 or 9–27 genes (Fig. 2). The 6–16 and 9–27 sequences, the functions of which are unknown, are strongly induced by interferons in a variety of cell types [14]. We cannot exclude the possibility that long-term exposure to TPA, which leads to down-regulation of protein kinase C, is not an appropriate model for the effects of interferon treatment on the activity of this enzyme, which may only be transient [21]. Nevertheless the present data suggest that the second messenger involved in transduc-

ing a signal from the interferon receptor on the cell surface to the nucleus is likely to be distinct from (or at least additional to) diacylglycerols or other components of the protein kinase C pathway. Our data also indicate that induction of 2′5′ oligoadenylate synthetase or the 6–16 or 9–27 genes is not obligatory for inhibition of proliferation and stimulation of differentiation of Daudi cells towards a plasma cell phenotype by TPA. Induction of 6–16 expression by interferon is also not sufficient alone to bring about such effects because this gene is expressed equally well in the presence and absence of 3-MB.

The ability of 3-MB to stimulate incorporation of thymidine into DNA deserves some comment. The biochemical basis for this effect remains to be elucidated, but it seems unlikely that it reflects a true increase in DNA synthesis, since cell proliferation is not correspondingly stimulated. We have repeatedly drawn attention to the poor correlation between thymidine incorporation and DNA synthesis rates in the case of interferon-treated cells [7,17]; the effect of 3-MB may be another example. There is also a lack of correlation between the effects of TPA on thymidine incorporation and cell proliferation, with strong inhibition of the latter and a much smaller effect on the former (Table I). Measurements of thymidine transport, thymidine kinase activity, and the specific activity of the intranuclear TTP pool are necessary in order to define the nature of these apparent anomalies.

We have previously suggested, on the basis of the inhibitory effects of 3-MB and 3-aminobenzamide, that ADP-ribosyl transferase activity is required for the growth-regulatory and differentiation-inducing actions of interferons [7]. Although there is no evidence against this requirement, the results presented here suggest an alternative interpretation of the data. In the light of the reported ability of the c-myc gene, when overexpressed, to block the differentiation of such diverse cell types as murine erythroleukaemia cells and myoblasts [28,29], it is possible that the increase in c-myc mRNA in cells exposed to 3-MB may account for the protection of Daudi cells against the actions of the interferons. This could explain the antagonistic effect of the ADP-ribosyl transferase inhibitor without the need to invoke a specific requirement for this enzyme in the molecular mechanism of action of interferons. The induction of c-myc expression by 3-MB also suggests a possible basis for the inhibition of cell differentiation by this compound in a wide range of other cell systems [30]. It will be of interest to determine whether c-myc expression is generally stimulated by ADP-ribosyl transferase inhibitors and, if so, how the product of this cellular oncogene is able to prevent terminal differentiation.

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