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Acknowledgement——We thank members of the EORTC Screening and Pharmacology Group for helpful discussions during the performance of this work.

Eur J Cancer, Vol. 28A, No. 10, pp. 1622–1627, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Downmodulation of c-*myc* Expression by Interferon γ and Tumour Necrosis Factor α Precedes Growth Arrest in Human Melanoma Cells

Susanne Osanto, Rumo Jansen and Monica Vloemans

After *in vitro* incubation of melanoma tumour cells Cmel453A with either recombinant interferon gamma (rIFN-γ) or tumour necrosis factor alpha (rTNF-α) a dose-dependent inhibition of cell growth occurred; when both cytokines were added, a synergistic action was observed. Inhibition of DNA synthesis, as measured by [^{3}H] thymidine incorporation, occurred after 6 h of incubation with rIFN-γ or rTNF-α, and this action was potentiated when the two cytokines were applied simultaneously. Within 1 h, the level of c-*myc* mRNA in tumour cells had already decreased by, respectively, 60% (S.D. 7) and 25% (S.D. 7); the combined addition of the cytokines resulted in a greater reduction of c-*myc* mRNA than by each cytokine alone. Downregulation of c-*myc* expression is an early event, occurring hours before the actual inhibition of outgrowth. Thus, in melanoma cells like Cmel with a high constitutive expression of the c-*myc* oncogene, the antiproliferative action of rIFN-γ and rTNF-α may be mediated by an inhibition of the expression of c-*myc*.
Eur J Cancer, Vol. 28A, No. 10, pp. 1622–1627, 1992.

INTRODUCTION

INTERFERON GAMMA (IFN-γ) and tumor necrosis factor alpha (TNF-α) are regulatory cytokines with pleiotropic biological activities in addition to antitumour actions. Both cytokines exert a potent antiproliferative action, *in vitro* as well as *in vivo*, on the growth of various tumour cells [1, 2].

The mechanism of the antiproliferative action of these cytokines is uncertain. Recent evidence suggests that the growth-inhibiting action of cytokines in tumour cells can be mediated by a suppression of the expression of oncogenes [3, 4]. In this context, attention is focused on the c-*myc* oncogene that is thought to play a key role in the control of cell proliferation [5,

6]. Expression of c-*myc* is tightly linked to the cell cycle: c-*myc* mRNA levels change dramatically during the transition from the quiescent to the proliferating state induced by growth or mitogenic factors [5], whereas c-*myc* expression is invariant throughout the cell cycle [7]. Attenuation of the c-*myc* oncogene has been shown to prevent cellular DNA replication [8,9]. The exact function of the nuclear protein product of the c-*myc* oncogene is still uncertain but the *myc* protein may serve to induce expression of G0/G1 transition genes [10]. Overexpression of c-*myc* may contribute to tumorigenesis [11, 12]; the findings that elevated c-*myc* expression is associated with many naturally occurring tumours [13, 14], supports a role for c-*myc* in the control of cell proliferation. Also the melanoma cells (Cmel453A) used in this study have a high constitutive expression of c-*myc* [15].

In addition to their antiproliferative action, both cytokines are capable of modulating the expression of cell surface molecules, e.g. HLA class I antigens and intercellular adhesion molecules, with a pivotal role in the interaction of tumour cells with the host's immune system. These effects of IFN-γ and TNF-α seem most important in the case of melanoma: firstly, because melanoma lesions are often infiltrated by immune cells, e.g. cytotoxic T lymphocytes, IFN-γ and TNF-α are likely to be produced *in vivo* at the site of the tumour cells. Secondly, melanoma patients are often treated with interleukin 2 based regimens and during immunotherapy, increased serum levels of both IFN-γ and TNF-α are found ([16] and R. van Oosterom, personal communication). Thus, cytokine-induced changes in the expression of both HLA class I molecules as well as adhesion molecules may determine the efficiency of eliciting a specific immune response.

To investigate the antiproliferative effect and its mechanism of action of recombinant IFN-γ and TNF-α on melanoma tumour cells, we determined the outgrowth, [^{3}H] thymidine incorporation and changes in expression of the c-*myc* oncogene of the human melanoma cell line Cmel453A, after addition of these cytokines.

In addition, changes in the melanoma cell surface expression of HLA class I antigens, i.e. HLA-ABC, -A and -B, and of adhesion molecules ICAM-1 and LFA-3, were investigated following incubation with these two cytokines.

MATERIALS AND METHODS

Cell culture

Cmel453A is a human melanoma cell line (Cmel) in low passage number, with a high constitutive level of c-*myc* expression [15]. Cells were grown in monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 100 IE/ml penicillin plus 0.05 mg/ml streptomycin. Monolayers were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagents

Recombinant human interferon gamma, rIFN-γ (specific activity 2×10^7 U/mg protein), and recombinant human tumour necrosis factor alpha, rTNF-α (specific activity 6×10^7 U/mg protein) were kindly provided by O. Damsma and G.A. Adolf, Boehringer Ingelheim BV (Alkmaar; Vienna). Cycloheximide was obtained from Sigma.

Assessment of cell growth rate

After trypsinisation, 2×10^5 cells were seeded in a volume of 6 ml in 6-cm dishes and allowed to attach during a period of more than 24 h. Under these conditions control cells grow exponentially for 6 days, before cell growth ceases as a result of contact-inhibition. To study the effects of rIFN-γ and rTNF-α on cell number, exponentially growing cells were incubated with 10, 100, or 1000 U/ml of rIFN-γ, rTNF-α or the various combinations. Cell numbers were determined 2 and 5 days after administration of the cytokines. To this purpose, cells were washed, trypsinised and cell numbers determined using a Coulter Counter as well as by counting viable cells by trypan-blue dye exclusion using a haemocytometer. Each group of cell cultures consisted of six replicates.

Assay of DNA synthesis

The inhibitory effect of rIFN-γ and rTNF-α on DNA synthesis of exponentially growing melanoma cells was assessed *in vitro* after exposure to 200 U/ml of each cytokine, either alone or in combination, in three independent experiments, each in triplicate. [^{3}H]thymidine incorporation into DNA was determined by pulsing the cells with 74 KBq [^{3}H]thymidine/dish for 1 h. At 1, 3, 6, 24 and 30 h after administration of the cytokines, the medium was aspirated, cells washed, the cells were collected by trypsinisation and cold trichloroacetic acid-precipitable radioactive material was collected on glass-fibre discs. The amount of radioactivity incorporated into DNA was determined by liquid scintillation spectrometry. Control cultures consisted of cells incubated without rIFN-γ or rTNF-α.

The results are expressed as:

$$\% \text{ incorporation} = \frac{\text{cpm (of) experimental culture}}{\text{cpm (of) control culture}} \times 100.$$

RNA isolation and northern blotting

At time points indicated in the text, RNA isolation was performed using the LiCl/ureum method, as described elsewhere [17]. Total RNA (15 ug per lane) was size-fractionated on a 1% agarose–formaldehyde gel and transferred to nitrocellulose. Hybridisations were performed at 42°C for 24 h with probes radiolabelled by the random primer method [18]. The following probes were used: c-*myc*: a ClaI-EcoRI fragment of a human genomic clone, spanning most of exon 3 [19]; class I HLA: a B7 cDNA clone [20] and γ-actin (provided by R. Bernards). Accurate determination and application of identical RNA amounts was checked by staining the gels with ethidiumbromide and by rehybridisation of the filters with γ-actin and HLA-B7. Autoradiographs were scanned by densitometric tracing (Desaga Densitometric Scanner), intensities of hybridisation signals quantitated and c-*myc* mRNA levels expressed as the ratio of c-*myc*/γ-actin and/or c-*myc*/HLA signals.

Serological reagents

The following monoclonal antibodies (Mabs) were used: W6/32 (Sera Laboratories), reacting with monomorphic HLA class I (HLA-ABC), Mab 4E, recognising all HLA-B and HLA-A29 up to -A33, and Mab 4B, recognising HLA-A2 and -A28 alleles [21], F-10 (a generous gift from A.C. Bloem, Department of Immunology, Utrecht), reacting with adhesion molecule

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Revised 24 Mar. 1992; accepted 31 Mar. 1992.

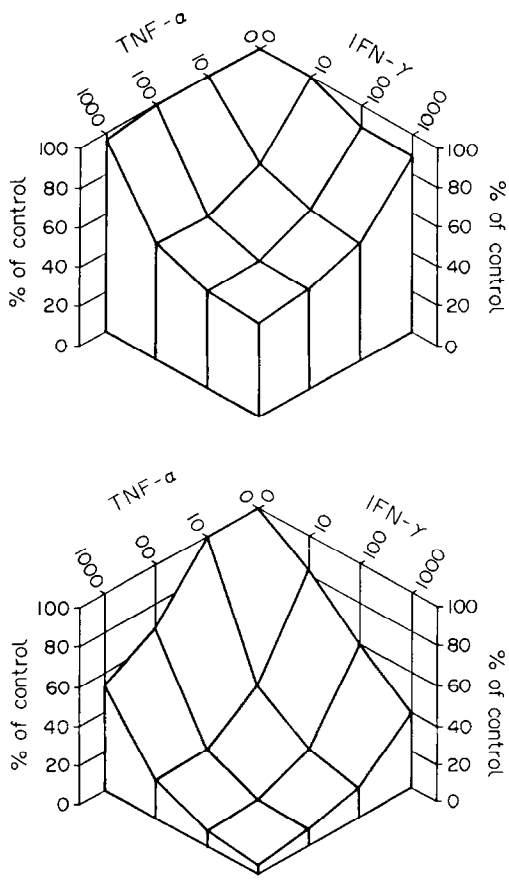

Fig. 1. Effect of various concentrations of rIFN-γ alone, rTNF-α alone or the combination of rIFN-γ and rTNF-α on the growth of melanoma cells. Exponentially growing melanoma cells were continuously exposed to rIFN-γ (10, 100 or 1000 U/ml), rTNF-α (10, 100 or 1000 U/ml) or the various combinations of these concentrations of rIFN-γ and rTNF-α, indicated in the figure. Viable cell numbers were counted after 2 (upper panel) and 5 (lower panel) days. Values are expressed as percentage of control.

ICAM-1, TS2/9, reacting with adhesion molecule LFA-3 [22], and goat anti-mouse IgG2a antibody directly labelled with fluorescein isothiocyanate (GAM/FITC, green) obtained from Nordic Immunological Laboratories, Tilburg.

FACS analysis

For FACS analysis, 0.25×10^6 cells were incubated with Mabs in appropriate dilutions in ice-cold phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.01 N sodium azide. After three washes, GAM/FITC diluted 1:30 was added. Background fluorescence was determined by incubating the cells with a non-reactive IgG2a antibody and GAM/FITC. All incubations were performed for 30 min at 4°C. After one more wash the samples were analysed in list mode on a FACSTAR flowcytometer (Becton Dickinson). The results were expressed as net fluorescence units (Fl-U).

RESULTS

Effect of rIFN-γ and TNF-α on cell growth of melanoma tumour cells

In vitro incubation of Cmel with 10–1000 U/ml of rIFN-γ or rTNF-α led to a dose-dependent inhibition of tumour cell growth indicated by a decrease in cell number relative to control cells after 5 days (Fig. 1, lower panel). In this respect, rIFN-γ, in equivalent units, was more active than rTNF-α, whereas the combined action proved synergistic. The differences in cell number between cells exposed to rIFN-γ, rTNF-α, or the combination and control cells were greater after 5 (Fig. 1, lower panel) than after 2 days (Fig. 1, upper panel) following exposure to the cytokines. Treated and untreated cells showed more than 95% viability, as indicated by trypan-blue dye exclusion tests, after 2 and 5 days of cell growth.

*Table 1. Effect of rIFN-γ, rTNF-α or both on DNA synthesis as measured by [³H]thymidine incorporation in melanoma cells**

Exposure time (h)	Control medium†	rTNF-α (200 U/ml)	rIFN-γ (200 U/ml)	rTNF-α + rIFN-γ (200 U/ml)
1	100.0	100.0 (2.8)	98.1 (3.1)	91.3 (1.0)
3	100.0	99.9 (3.4)	99.7 (1.6)	86.0 (4.3)
6	100.0	93.0 (0.8)	83.8 (3.9)	65.4 (3.3)
24	100.0	50.6 (5.2)	24.1 (1.8)	2.6 (0.2)
30	100.0	44.6 (1.7)	19.4 (1.8)	1.5 (0.04)

*Values are expressed as a percentage of [³H]thymidine incorporated into DNA compared to untreated controls (see Materials and Methods).
†The coefficient of variance of increase in cell number in control dishes was less than 4%.

Effect of rIFN-γ and TNF-α on [³H] *thymidine incorporation by melanoma tumour cells*

In the previous experiments it was demonstrated that rIFN-γ and rTNF-α alone inhibited the growth of Cmel cells already in concentrations between 100 and 1000 U/ml. On the basis of these experiments, it was decided to study effects of cytokines on [³H]thymidine incorporation up to 30 h, at a fixed dose within this concentration range, i.e. a dose of 200 U/ml of rIFN-γ or rTNF-α, in order to quantitate cellular effects occurring within hours after exposure to the cytokines. *In vitro* incubation of melanoma cells with rIFN-γ or rTNF-α led to a reduction of [³H]thymidine incorporation by tumour cells that started after about 6 h and continued up to at least 30 h (Table 1). The decrease in DNA synthesis as reflected by a reduction of thymidine incorporation, was greater upon incubation with equivalent units of rIFN-γ than rTNF-α, and was greatly enhanced when tumour cells were exposed to both cytokines (Table 1).

*Effect of rIFN-γ and TNF-α on expression of the c-*myc *oncogene by melanoma tumour cells*

After 2 days of continuous exposure to 200 U/ml of rTNF-α alone the number of Cmel cells did not differ significantly from untreated control cells, but DNA synthesis was already significantly inhibited after 24 h, i.e. [mean (S.D.)] 51 (5)% of that in untreated control cells (Table 1). Following continuous exposure to 200 U/ml of rIFN-γ alone, DNA synthesis was reduced to 24 (2)% of that in untreated cells. This large reduction in DNA synthesis became evident in a decrease in cell number by two days of exposure to IFN-γ. To determine whether this growth-inhibition by rIFN-γ and rTNF-α correlated with quantitative changes in c-*myc* mRNA steady state levels, we determined the levels of c-*myc* expression at time points between 10 min and 24 h after administration of 200 U/ml of each cytokine alone, or in combination, in various experiments. *In vitro* incubation of tumour cells with rIFN-γ or rTNF-α led to a

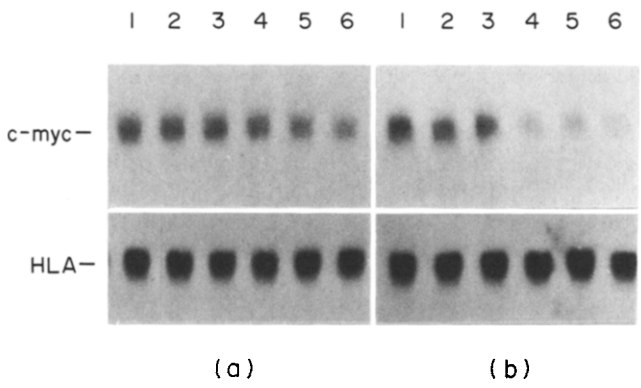

Fig. 2. Time course of c-*myc* mRNA after 10–50 min exposure to rIFN-γ or rTNF-α alone. Subconfluent monolayers of melanoma cells were continuously exposed to (a) rTNF-α (200 U/ml) or (b) rIFN-γ (200 U/ml) until RNA was isolated at various times thereafter and analysed on northern blots. Lanes 1, untreated controls (no rTNF-α or rIFN-γ); lanes 2, 10 min exposure; lanes 3, 20 min exposure; lanes 4, 30 min exposure; lanes 5, 40 min exposure; lanes 6, 50 min exposure. The northern blots were probed for expression of c-*myc*. The same filters were rehybridised with a class I HLA gene probe as described in Materials and Methods.

rapid reduction of the level of c-*myc* mRNA, that started within 10, respectively 30–40 min after exposure to rIFN-γ or rTNF-α (Fig. 2) and reached a maximum reduction after 8 h (Fig. 3). The decrease in c-*myc* mRNA occurred more rapidly and was greater upon incubation with equivalent units of rIFN-γ than rTNF-α. Incubation of tumour cells with both rIFN-γ and rTNF-α resulted in a significantly greater reduction in c-*myc* mRNA compared to either cytokine alone (Figs. 3 and 4), and resulted in an almost complete shut-off of c-*myc*. In contrast, already 4 h after addition of rIFN-γ combined with rTNF-α maximal inhibition of c-*myc* occurred, reaching a plateau; the expression level of c-*myc* mRNA had decreased more than 89% (S.D. 3%).

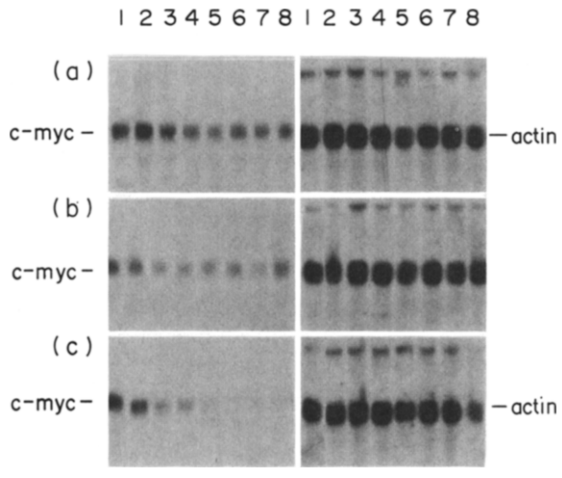

Fig. 3. Time course of c-*myc* mRNA of melanoma cells exposed for 0.5, 1, 2, 4, 6, 8 and 24 h to (a) rTNF-α (200 U/ml), (b) rIFN-γ (200 U/ml), or (c) the combination. Total RNA was extracted at the indicated times. Lanes 1, untreated controls; lanes 2, 30 min exposure; lanes 3, 1 h exposure; lanes 4, 2 h exposure; lanes 5, 4 h exposure; lanes 6, 6 h exposure; lanes 7, 8 h exposure; lanes 8, 24 h exposure. The northern blot was probed for expression of c-*myc* and γ-actin as described in Materials and Methods.

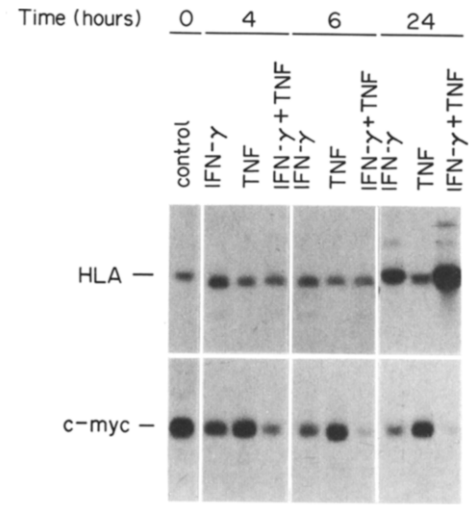

Fig. 4. Time course of class I mRNA of melanoma cells exposed for 4, 6 and 24 h to rIFN-γ (200 U/ml), rTNF-α (200 U/ml) or the combination. Total RNA was extracted at the indicated times. The northern blot was probed for expression of class I HLA and c-*myc* as described in Materials and Methods. Following exposure to rTNF-α for 4, 6 and 24 h, the c-*myc* mRNA level was reduced to 76, 58 and 60%, respectively, and following exposure to rIFN-γ for the same period of time, the c-*myc* mRNA level was reduced to 45, 28 and 22%, respectively, of that in the control, as evidenced by scanning of autoradiograms of different exposure times of the same northern blot.

Cycloheximide experiments

To determine whether synthesis of new proteins is required for the reduction in c-*myc* expression, the effect of cycloheximide on rIFN-γ- and rTNF-α-mediated c-*myc* inhibition was studied. C-*myc* mRNA is superinduced by cyclohexamide in the presence of serum factors, indicating that a short-lived protein is involved in the negative regulation of c-*myc*. The inhibitory effect of rIFN-γ and rTNF-α on c-*myc* mRNA was not abrogated in the absence of new protein synthesis. Furthermore, the inhibition of c-*myc* mRNA by IFN-γ, TNF-α or the combination after 0.5 (results not shown) and 2 h exposure in the presence of cycloheximide, was proportional to the inhibition of c-*myc* at the same time points in the absence of cycloheximide (Fig. 5).

Effect of IFN-γ and TNF-α on HLA class I expression

As internal control, the same filters of the time course experiments were hybridised to the HLA-B7 cDNA probe. In contrast to the reduction in c-*myc* mRNA, the levels of the 1.7-kb class I HLA mRNA were not changed up to 6 h after treatment with rIFN-γ or rTNF-α alone, and subsequently increased. 24 h following exposure to the cytokines, rTNF-α had enhanced class I mRNA 1.6-fold (S.D. 0.28), whereas rIFN-γ had increased class I mRNA 3.8-fold (S.D. 1.09); the combination had enhanced class I HLA expression 8.4-fold (S.D. 4.19). Upregulation of HLA-class I by the combination of rIFN-γ and rTNF-α was already evident after 4 h, before enhancement of class I by rIFN-γ or rTNF-α alone occurred (Fig. 4).

Effect of IFN-γ and TNF-α on cell surface expression of HLA class I antigens and adhesion molecules ICAM-1 and LFA-3

IFN-γ, and to a lesser extent TNF-α, enhanced HLA class I cell surface expression, as indicated by the increase in the binding of Mab W6/32 (Table 2). Class I expression was synergistically enhanced when the cytokines were applied together. Interest-

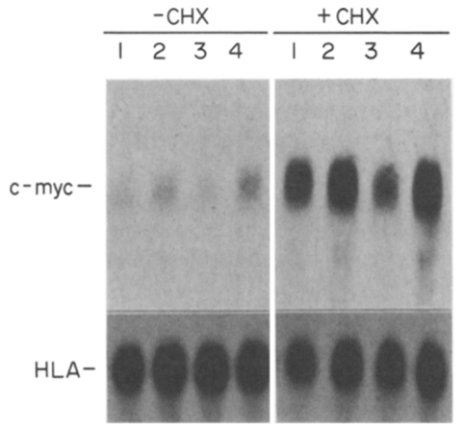

Fig. 5. Effect of cylcoheximide (CHX) on the reduction of c-*myc* by rIFN-γ and rTNF-α. Subconfluent monolayers of melanoma cells were incubated for 15 min in the absence (−CHX) or presence (+CHX) of CHX (25 ug/ml). Next, rIFN-γ (200 U/ml) or rTNF-α (200 U/ml) was added alone or in combination for 2 h, and RNA extracted. Lanes 1, exposure to rIFN-γ; lanes 2, exposure to rTNF-α; lanes 3, exposure to the combination of rIFN-γ and rTNF-α; and lanes 4, untreated controls. The northern blot was probed for expression of c-*myc* and class I HLA as described in Materials and Methods.

ingly, HLA-B expression was increased to a much greater extent than HLA-A, as indicated by the increase in binding of Mab 4E and 4B, by IFN-γ, whereas both HLA-A and HLA-B were only slightly increased by TNF-α.

Following exposure to IFN-γ, TNF-α and the combination of IFN-γ and TNF-α, expression of adhesion molecule ICAM-1, as indicated by the binding of Mab F-10, was increased by a factor 7.1, 4.3 and 12.8, respectively (Table 2). In contrast, the expression of LFA-3, as measured by the binding of Mab TS2/9, was not affected by incubation with either of the cytokines (Table 2).

DISCUSSION

The findings of the present study demonstrate that rIFN-γ and rTNF-α exert a potent dose-dependent antiproliferative activity on human melanoma tumour cells, grown *in vitro*, while the combination of the two cytokines results in an enhanced antiproliferative effect. The decline in cell proliferation, as reflected in a reduction of [^{3}H]thymidine incorporation, is long preceded by a rapid fall in expression of the c-*myc* oncogene. Downregulation of c-*myc* requires no new protein synthesis. The antiproliferative activity and the downregulation of the c-*myc* oncogene can be strikingly potentiated when both cytokines are applied together. HLA class I (in particular HLA-B) and ICAM-1 expression was markedly enhanced by IFN-γ. This effect was markedly potentiated when IFN-γ and TNF-α were applied together.

*Table 2. Surface markers on cytokine-treated Cmel453A melanoma cells**

Treatment	HLA class I			Adhesion molecules	
	W/6/32	4E	4B	F-10	TS2/9
	Fl-U				
No	36.1	6.0	12.8	13.4	15.1
IFN-γ	85.7	37.8	17.4	95.6	14.4
TNF-α	45.2	10.9	17.2	58.8	17.1
IFN-γ+TNF-α	124.6	89.5	24.7	172.5	15.7

*Surface marker analysis of cultured Cmel453A melanoma cells with W6/32, 4E, 4B, F-10 and TS2/9. IFN-γ and TNF-α were both used at 200 U/ml for 48 h of incubation. Fl-U is determined as described in Materials and Methods. The background Fl-U with non-reactive Mab and GAM-FITC stained cells is 3.5 and identical for treated and untreated melanoma cells.

The present findings suggest that the downregulation of the c-*myc* oncogene is causally related to the observed arrest in tumour cell growth. The extent of suppression of c-*myc* in the tumour cells may determine whether complete cessation of cell growth occurs. In agreement herewith, experiments with Balb/c 3T3 fibroblasts, transfected with c-*myc*, and expressing high levels of the exogenous c-*myc*, indicate that reduction of c-*myc* expression is a prerequisite for getting an efficient growth arrest of the cells following administration of interferon type I (IFN-α and -β) [23]. The downmodulation of c-*myc* by IFN-γ and TNF-α in these melanoma cells with high constitutive expression of c-*myc* parallels the findings with IFN-α or -β in Daudi cells and with IFN-γ and TNF-α in HeLa cells [3, 24, 25]. However, because the regulation of cell growth is multifactorial, changes in expression of c-*myc* may not be the only cause of the inhibition of growth following exposure to rIFN-γ and rTNF-α.

Our study demonstrates that the downregulation of c-myc by rIFN-γ and rTNF-α occurs within 10, respectively 30 min after administration of the cytokines. These findings are in accordance with earlier reports, which indicate that rIFN-γ can affect the transcription of various genes within minutes of binding to its cell surface receptor [26]. In some tumour cell lines, rIFN-γ apparently increases the number or affinity of TNF receptors on tumour cells, thus rendering these cells more susceptible to TNF action [27]. In the Cmel cells used in this study, neither the number nor the affinity of TNF receptors changed after incubation of the cells with rIFN-γ (results not shown), indicating that another mechanism is responsible for the synergistic action of the combination of the cytokines. Such a mechanism probably was reflected, however, in the strikingly potentiated downregulation of the c-*myc* oncogene that occurs when both cytokines are applied together.

In contrast to c-*myc*, class I mRNA as well as cell surface expression of HLA class I is increased by rIFN-γ and rTNF-α in Cmel cells, the two cytokines interacting in a synergistic way. The enhancement of class I HLA mRNA is inversely proportional to the reduction of expression level of c-*myc*. Both IFN-γ and TNF-α have been shown to increase the expression of MHC class I genes in a variety of cell types [28, 29]. In human melanoma cells, including Cmel453A cells, and in rat neuroblastoma cells, overexpression of c-*myc* and of the related N-*myc* has been shown to cause a dramatic reduction in the expression of class I HLA mRNA [15, 30]. Thus, in the melanoma cells under study, abolition of the suppressive effect on class I gene expression by c-*myc* could contribute to the enhancement of class I expression. However, at least for IFN-γ, this mechanism seems less likely, because IFN-γ has been shown to be able to reverse N-*myc* mediated downmodulation of class I in N-*myc* transfected cells, without affecting steady state levels of N-*myc* [30].

In the Cmel453A cells, the surface expression of HLA molecules, in particular HLA-B, and of ICAM-1 is markedly increased by IFN-γ and synergistically by the combination of

IFN-γ and TNF-α. These molecules play an important role in presentation of (tumour) peptides, respectively intercellular adhesion of (tumour) cells and lymphocytes. Thus, besides exerting direct antiproliferative effects on melanoma tumour cells, IFN-γ and TNF-α may also modulate the cellular immune response of the host via enhancement of cell surface expression of HLA class I and adhesion molecules, resulting in a more efficient eradication of the tumour cells *in vivo*.

At present, a large number of immunomodulators including rIFN-γ and rTNF-α are under investigation as anticancer agents in patients with metastatic melanoma. However, immunotherapy with these agents has been successful in only a limited number of patients. More precise identification of prognostic factors in the patient and the tumours should lead to a more successful, individualised approach. In this respect, the level of c-*myc* expression in melanoma tumour cells may represent an important factor determining the response to immunotherapy with rIFN-γ and rTNF. Furthermore, cytokine-induced enhancement of class I expression and ICAM-1 on melanoma tumour cells could lead to a more effective elimination of tumour cells by the host via facilitated T-cell mediated defence.

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Acknowledgements—The authors would like to thank Dr P.I. Schrier and Dr J.T. van Dissel for reading the manuscript. This study was supported by a grant (IKW 87-14) of The Netherlands Cancer Foundation (KWF) and by the Maurits en Anna de Kock Foundation.