

Comparative Studies on the Effects of Doxorubicin and Differentiation Inducing Agents on B16 Melanoma Cells

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The differentiation-inducing activity of doxorubicin on B16 melanoma cells grown *in vitro* was compared with that of other known differentiation inducers, such as theophylline, retinoic acid, and melanocyte-stimulating hormone (MSH). At drug concentrations resulting in cytostatic effects, doxorubicin and theophylline induced morphological changes (dendritic-like structures with a terminal melanin granule) with an enhancement of total melanin content and tyrosinase activity. Retinoic acid did not alter melanin content and cell morphology, although it affected cell growth. MSH enhanced total melanin content and tyrosinase activity, with no significant morphological changes. Flow cytometric analysis showed that MSH led to an accumulation of cells in G1 phase whereas doxorubicin induced an accumulation of cells in G2 + M. Studies on DNA content in doxorubicin-treated cells, selected on the basis of a morphologically differentiated pattern, showed a clustering of these cells in G2 + M, probably due to a cytokinesis block. Thus doxorubicin can induce cell differentiation comparable with other differentiation inducers.

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INTRODUCTION

MALIGNANT MELANOMA cell lines are useful for studies of neoplastic cell differentiation [1, 2]. Indeed, such cells offer at least two different markers of differentiation: a morphological one, i.e. dendrite-like structures from the cell body content [3, 4], and a biochemical, easily quantifiable one, i.e. melanin content [1, 3, 5]. Differentiation of the cells can be revealed also by the production of a bicatalytic enzyme, tyrosinase, which may be present even in non-pigmented cells [6-9] and is a marker of differentiated function in relation to cell potential in producing melanin. Moreover, following differentiation, tumour cells show decreased proliferation, delayed tumour appearance and altered metastatic potential in mice. In melanoma cells such features do not appear simultaneously, thus suggesting a lack of close association between such phenomena [10].

In *in vitro* melanoma cells, differentiation can be induced by extracellular stimuli including α -melanocyte-stimulating hormone (MSH) [3, 11, 12], forbol ester tumour promoters [1], dimethylsulphoxide [10], butyric acid [10], and agents like theophylline [12] and prostaglandins [9] which increase the intracellular level of cyclic adenosine monophosphate able to induce differentiation by itself [13]; moreover contrasting results have been reported on differentiating activity of retinoic acid [14-16].

A differentiation inducing activity of anthracyclines on myeloid and erythroid leukaemias [17-19], rhabdomyoblastoma [20] and neuroblastoma [21] cells has been already described,

but the results cannot be generalised in all the cell systems [22]. Raz [4] reported that daunorubicin induces morphological alterations in B16 melanoma cells toward a differentiation process.

We have previously reported that B16 melanoma cells, with an induced resistance to doxorubicin, are less proliferative [23] and acquire a significantly higher latency and reduced metastatic potential when grown *in vivo* [24], which indicates a reduced tumorigenic behaviour.

Microscopic observations of doxorubicin-treated cells have indicated a differentiated morphology [25]. In an attempt to clarify whether such a phenomenon was due to a differentiating process or to a selection of already differentiated cells, a number of cell differentiation parameters like intracellular and extracellular melanin levels, tyrosinase activity and cell cycle alterations have been quantified in B16 melanoma cells following drug treatment. The results were compared with those obtained with theophylline, retinoic acid and MSH, which have already been reported to be differentiation inducers.

MATERIALS AND METHODS

Chemicals

Doxorubicin was supplied by Farmitalia Carlo Erba (Milan). Retinoic acid, MSH, L-Dopa and melanin were purchased from Sigma. Theophylline was used as a clinical preparation (Tefamin, Recordati, Milan). All drugs were dissolved immediately before use in saline, except for retinoic acid, which was dissolved in dimethylsulphoxide and then diluted in saline; the maximal final concentration of dimethylsulphoxide was 0.3%.

Cell line and experiments

The B16V melanoma cells were grown in RPMI 1640 medium (Flow) containing 10% fetal calf serum (Flow), antibiotics and 0.03 mmol/l $\text{Fe}(\text{CN})_6\text{K}_3$ [23]. For the experiments, tumour cells ($5 \times 10^4/\text{ml}$) were seeded in complete culture medium in 10 cm

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diameter petri dishes (Corning) and treated 24 h later in replicate samples. Drugs and concentrations used were: doxorubicin, 2×10^{-6} mol/l, 4×10^{-7} mol/l, 2×10^{-8} mol/l; theophylline, 2×10^{-3} mol/l, 6×10^{-4} mol/l, 2×10^{-4} mol/l; retinoic acid and MSH, 10^{-5} and 10^{-6} mol/l. Unless otherwise indicated, treatments were performed for 96 h. At the end of the experiments, light micrographs were taken on cell cultures with an inverted microscope equipped with a MPS 51S camera (Wild) on AGFA 100 ASA film, and cells were then collected in saline for all the determinations.

Evaluation of cell growth inhibition

At 96 h after treatment, cells were counted in a Coulter Counter; cell viability was determined by trypan blue dye exclusion. The drug effect was expressed as percentage of surviving cells versus control.

Evaluation of melanotic cells percentage

Aliquots of cells from each sample were smeared on albuminated slides, air dried and fixed in 4% buffered formaldehyde at room temperature for 5 min. The slides were then mounted in UV inert mounting aqueous medium (GURR, Chadwell Heath, Essex) and the percentage of the pigmented cells was estimated by microscopic observation at $1000\times$. About 700–1000 cells were counted in each sample.

Intracellular melanin evaluation

Biochemical method. Intracellular melanin content was evaluated according to Whittaker [26]. Briefly, aliquots of cells from each sample were washed twice in saline, then three times with trichloroacetic acid 5%, twice with ethanol: ethyl-ether (3:1) and once with ether. After removal of ether, the samples were air-dried and dissolved in 1 ml of hot 0.85 mol/l KOH. Samples were cooled and the optical density at 400 nm was determined. Melanin levels were evaluated by a standard curve prepared with commercial melanin.

Cytochemical method. The same smears used for cytologic evaluation of melanotic cells were used to measure the amount of melanin in single cells at 450 nm by means of a Vickers M 86 scanning and integrating microdensitometer: $100\times$ objective oil immersion, $314\ \mu\text{m}^2$ mask, and $1.25\ \mu\text{m}^2$ spot. The mean integrated optical density values, corrected for the blank (amelanotic melanoma cells), were then converted to absolute units by calibrating the instrument with neutral filters of known optical density.

Extracellular melanin evaluation

Extracellular melanin content was determined in 1 ml of tissue culture medium from treated cells to which $10\ \mu\text{l}$ of 2 N HCl had been added to bleach the phenol red. The optical density was read by means of a Beckman DU 8 spectrophotometer at 560 nm (wavelength at which the absorption of the medium was negligible). The amount of melanin, corrected for the blank, was calculated by means of a standard curve.

Cytochemical tyrosinase activity evaluation

To evaluate tyrosinase activity, slides obtained from the same cell preparation were incubated with L-Dopa at 37°C for 4 h [27], carefully rinsed with phosphate buffer and mounted. The optical density of the pigment obtained after this cytochemical reaction was measured by microdensitometry as reported above for cytochemical evaluation of melanotic cells.

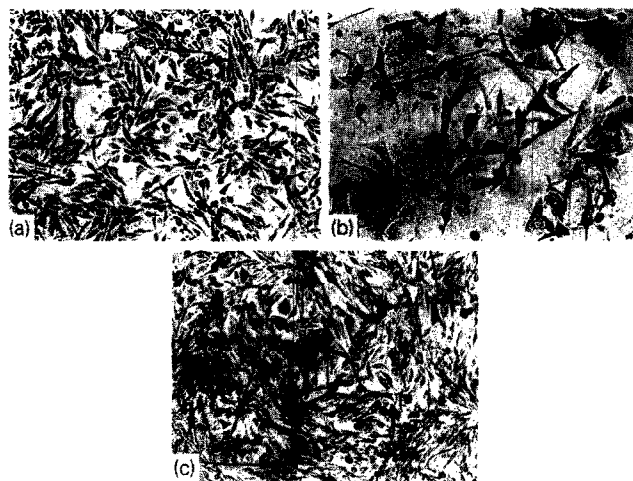


Fig. 1. Photomicrographs of cultures treated for 4 days. (a) Control; (b) doxorubicin 2×10^{-6} mol/l; (c) doxorubicin 4×10^{-7} mol/l; (d) theophylline 6.3×10^{-4} mol/l; (e) retinoic acid 10^{-5} mol/l; (f) MSH 10^{-5} mol/l. Final magnification, $250\times$ phase contrast.

Flow cytometric analysis of the cell cycle

For flow cytometric measurements of cellular DNA content, cells were fixed in 70% ethanol for 24 h at 4°C . Cells were then washed with saline solution, digested with 100 U/ml RNase type A (Sigma) for 30 min at 37°C and stained with 50 $\mu\text{g}/\text{ml}$ propidium iodide (Calbiochem) for 30 min at room temperature [28].

Fluorescence measurements were performed by means of a Leitz microscope-based flow cytometer equipped with an HBO 100 W mercury lamp [29]. The following filter combinations were used: BG 38 and 530–560 nm band pass excitation; 580 nm chromatic beam splitter; 610 nm barrier filter. The photomultiplier signals were processed and memorised by a spectroscopy modular 8000 multichannel analyser (Laben, Milan). At least 10^5 cells per sample were measured. Determination of the percentage of cells in each phase of the cycle was performed on the flow cytometric histograms by a graphic method [30].

Static cytofluorometric analysis of DNA content

DNA content on cells showing a differentiated pattern was determined on cells grown, treated and fixed with 70% ethanol on coverslips in Leighton tubes. DNA staining was performed according to the same procedure described for cell suspensions. Thereafter, coverslips were placed, with a drop of staining solution, on microscopic slides and sealed with UV-inert aqueous mounting medium.

Fluorescence measurements were performed with an MPV 2 microscope-photometer (Leitz) equipped with an HBO 100 W mercury lamp as excitation source. Excitation and emission conditions were the same used for flow cytometric measurements. At least 50 cells for each sample were measured. The photomultiplier signals were processed and memorised by a Correlatron 4096 (Laben) multichannel analyser. Fluorescence intensity histograms were obtained with a computer program.

RESULTS

Morphological examination

The morphology of B16 melanoma cells treated with different agents is shown by phase contrast in Fig. 1 for a better observation of melanin content and by light microscopy in Fig. 2. Following exposure to doxorubicin for 4 days at different

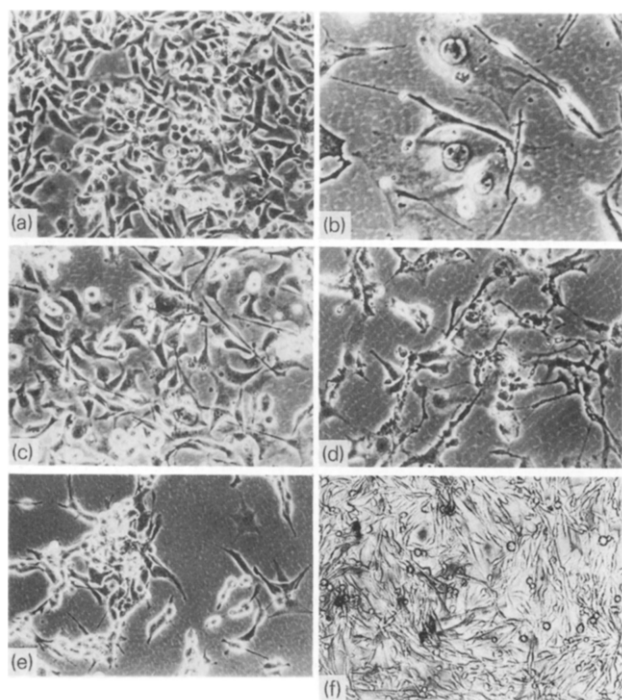


Fig. 2. Photomicrographs of cultures treated for 4 days. (a) Control; (b) doxorubicin 4×10^{-7} mol/l; (c) MSH 10^{-5} mol/l. Final magnification $250 \times$.

concentrations, cells stopped in growth appeared enlarged (an aspecific effect typical of anthracycline treatment referred to as unbalanced growth) [31] and displayed dendritic-like structures containing a terminal melanin granule. In comparison with untreated cells more, pigmented cells and a greater amount of pigment in the cytoplasm and in the dendrite-like structures were evident.

The morphology of cells treated with theophylline was quite different, since they assumed a polygonal aspect with long extensions rich in melanin granules. Very short cellular extensions could be observed after retinoic acid treatment without cell enlargement and increased melanin. MSH induced a marked increase in melanin content without cell number reduction or cell shape alterations. Many cells became a dark black, and a grey colour generally appeared throughout the cell culture.

Determination of cell viability, melanotic cells and amount of melanin

Table 1 shows the percentage of melanotic cells and the intracellular and extracellular melanin content as well as the changes in viable cell number after different drug treatments at the end of the experiment. The change of the cell number shows that doxorubicin was cytotoxic at the higher dose tested, while it was cytostatic at 4×10^{-7} mol/l, as minimal variations in cell number and no dead cells in the culture medium during the 96 h of treatment were found. At the lower dose the drug was ineffective. The number of melanotic cells in untreated samples was (mean [S.D.]) 2.2 (0.88) every 100 cells counted. Treatment with doxorubicin and theophylline induced a marked increase in the number of melanotic cells. For both drugs such an effect was paralleled by a marked reduction in cell growth, which was dose dependent and disappeared at lower doses. Retinoic acid was completely ineffective. MSH caused an even higher increase in melanotic cells which was not accompanied by a reduction in cell growth.

To verify a correlation between the number of melanotic cells and the amount of melanin synthesised, both intracellular and extracellular melanin values were determined. The amount of melanin was calculated per 10^6 cells and expressed as percentage of control. Intracellular melanin content of control cells was $0.27 (0.03) \mu\text{g}/10^6$ cells, whereas in the culture medium it was $0.80 (0.05) \mu\text{g}/10^6$ cells. Doxorubicin and theophylline at cytotoxic doses induced a marked increase in intracellular (about 940 and 830%, respectively) and extracellular (about 3110 and 1160%, respectively) melanin levels. Such an increase was dose dependent and was evident also at cytostatic concentrations: i.e. 374% and 455% for intracellular and 342% and 752% for extracellular melanin levels for doxorubicin and theophylline, respectively. In contrast, the increase in melanin induced by retinoic acid was not significant even at concentrations that reduced cell growth. MSH induced an increase in melanin content of about 500% within the cell and 200–300% in the culture medium. The extracellular level of pigment after doxorubicin and theophylline treatment was very high, whereas after MSH treatment, which induced a strong increase in melanotic cells and intracellular melanin, a minimal enhancement of extracellular melanin was observed.

Melanin content in single cells

Since the increase in total melanin (intracellular and extracellular) content in doxorubicin-treated cells was higher than the increase in the number of melanotic cells and the high heterogeneity of cell population could affect the results, melanin was measured in single cells and expressed as integrated optical density (Table 2). A variable pattern of melanin content in control and treated cells was found, since cells without melanosomes (with an optical density similar to HeLa cells), cells with only a few melanosomes present in a small region of the cytoplasm, completely black cells, and intermediate stages were

Table 1. Percentage of viable cell number, melanotic cells, intracellular and extracellular melanin content in treated cells*

Treatment (mol/l)	Change in cell number†	Melanotic cells‡	Intra-cellular melanin	Extra-cellular melanin
Untreated control	1600	100	100	100
Doxorubicin				
2×10^{-6}	40	486 (24)	943 (62)	3108 (114)
4×10^{-7}	250	336 (49)	374 (35)	342 (54)
2×10^{-8}	1300	91 (17)	145 (22)	110 (18)
Theophylline				
2×10^{-3}	100	450 (21)	829 (92)	1159 (74)
6×10^{-4}	280	404 (23)	455 (62)	752 (65)
2×10^{-4}	1400	200 (12)	180 (34)	125 (32)
Retinoic acid				
10^{-5}	460	168 (14)	195 (31)	187 (40)
10^{-6}	750	145 (7)	150 (34)	168 (2)
α -melanocyte-stimulating hormone				
10^{-5}	1300	686 (26)	496 (23)	293 (5)
10^{-6}	1600	659 (17)	537 (35)	210 (36)

* Data are expressed as percentages of controls (S.E.) Real control values are reported in the text. Each point is the mean of six observations from three independent experiments.

† % referred to no. of cells at the cell seeding.

‡ The number of melanotic cells was counted on 1000 cells for each sample as reported in Materials and Methods.

Table 2. Relative distribution of cells with different melanin content*

Treatment	(Mol/l)	IOD CLASS†		
		I	II	III
Control		50	33	17
Doxorubicin	2×10^{-6}	39	20	41
	4×10^{-7}	31	36	33
Theophylline	2×10^{-3}	42	26	32
	6×10^{-4}	11	44	45
Retinoic acid	10^{-5}	50	22	26
	10^{-6}	46	29	25
α -melanocyte-stimulating hormone	10^{-5}	10	16	74
	10^{-6}	30	9	61

* Data are expressed as percentage of cells in the different classes of integrated optical density values.

† Integrated optical density classes values: I = 0–40; II = 40–80; III = > 80.

observed. The values obtained were distributed on a wide range that was split in three classes. The frequency distribution of cells per class was therefore evaluated according to integrated optical density values.

Doxorubicin and theophylline induced a strong, only partially dose-dependent reduction in cells of classes I and II and an increase in highly melanotic cells. Retinoic acid induced only a slight shift of the percentage of cells toward classes of higher values, whereas MSH treatment resulted in a very high increase in the number of cells in class III.

Cell cycle analysis

The correlation between melanin content and cell growth was evaluated by analysing cell cycle progression. The results, expressed as percentage of cells in the different phases of the cell cycle, are presented in Table 3. For each drug (with the exception of MSH), only the effects of concentrations that caused similar effects on cell growth are reported. Comparable results were obtained after treatment with other concentrations. From the cell seeding throughout the monitored period, untreated control cells were distributed in an almost constant percentage in all the phases, with a reduction in the S compartment when cells were confluent. Doxorubicin induced an increase in the percentage of cells in the G2 + M phase already after 24 h of treatment, with a correspondent decrease in the G1 phase. The observation explains the lack of an increase in cell number. Retinoic acid and theophylline after, respectively a 72 and 96 h of treatment produced a trend of decrease in G1 and an increase in G2 + M. MSH induced a remarkable reduction of cells in the S phase at 96 h of treatment, along with an accumulation in the G1 phase that may have been due to confluency and treatment with the differentiation inducer agent.

Tyrosinase activity

Microdensitometry evaluation of the end product of the L-Dopa-reaction indicated that tyrosinase activity was present throughout the cell population and that the enzyme level increased following exposure to doxorubicin, theophylline and,

at a higher level, MSH (Fig. 3). In fact, compared with control cells, a shift of optical density toward higher values along with a decrease in the number of cells with low optical density were evident in all samples. Again, retinoic acid did not bring about significant alterations. Such data, in agreement with the evaluation of melanin in single cells, indicate that doxorubicin treatment induces an increase in tyrosinase activity in only slightly melanotic cells and an *ex-novo* melanin synthesis in amelanotic cells.

DNA content in differentiated cells

DNA content was examined after drug treatment in cells selected for a morphologically differentiated pattern (presence of dendritic-like structures and/or high intracellular melanin content). Histograms are shown in Fig. 4. Fig. 4(a) shows the DNA distribution of untreated cells randomly chosen independently of the morphology: results are in agreement with those obtained with flow cytometry. Fig. 4(b–d) report, respectively the results of treatment with doxorubicin, theophylline and retinoic acid at concentrations that acted similarly on cell growth. Compared with the results of flow cytometric analysis on the whole cell population, cells differentiated after doxorubicin or theophylline treatment showed similar but more marked alterations in cell cycle distribution, i.e. an increase of G2 + M and a decrease in G1. Cells with a tetraploid DNA content, which were absent in untreated control cultures, were always observed. Only a few DNA alterations were observed after retinoic acid treatment.

DISCUSSION

On the basis of several examined parameters, the present results strongly support the already proposed hypothesis of a

Table 3. Effect on cell cycle progression

Treatment (mol/l)	Duration of treatment (h)	Percentage distribution		
		G1	S	G2 + M
Control	0	58	22	20
	24	58	24	18
	48	59	26	15
	72	57	18	25
	96	63	12	25
Doxorubicin	4×10^{-7}	24	38	20
	48	30	23	45
	72	27	25	48
	96	26	22	52
Theophylline	6×10^{-4}	24	58	28
	48	55	22	23
	72	46	27	27
	96	46	25	29
Retinoic Acid	10^{-5}	24	65	20
	48	62	17	21
	72	53	14	33
	96	52	15	33
α -melanocyte-stimulating hormone	10^{-5}	24	46	33
	48	60	22	18
	72	63	13	24
	96	75	3	22

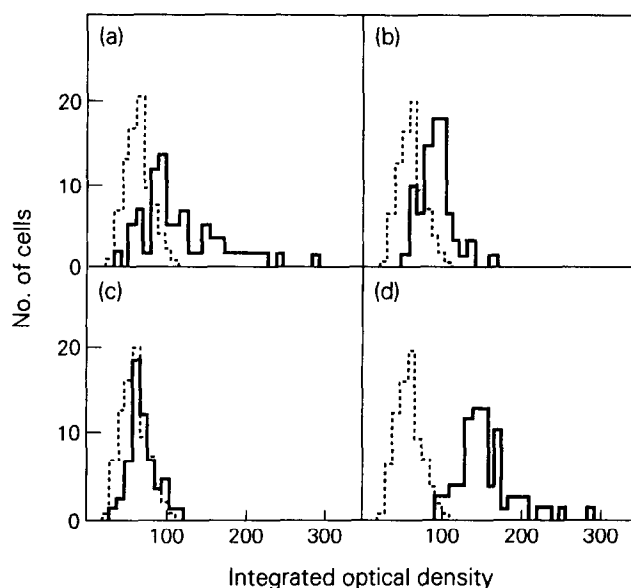


Fig. 3. Histograms of the distribution of the integrated optical density values of the L-Dopa-reaction (absolute units) (see Materials and Methods) measured on at least 50 cells in each sample. (a) Doxorubicin 4×10^{-7} mol/l; (b) theophylline 6.3×10^{-4} mol/l; (c) retinoic acid 10^{-5} mol/l; MSH 10^{-5} mol/l. Dashed area indicates the optical density value distribution of control cells.

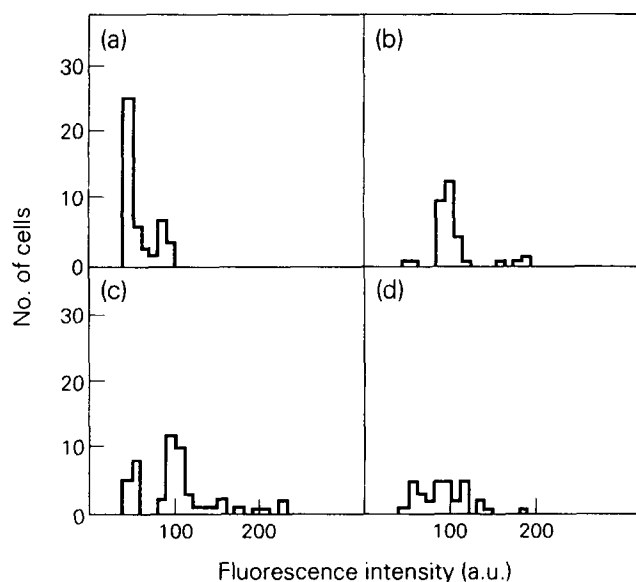


Fig. 4. Fluorescence analysis of DNA content in morphologically differentiated cells. Fluorescence arbitrary units were measured on at least 50 cells in each sample. (a) Control; (b) doxorubicin 4×10^{-7} mol/l; (c) theophylline 6.3×10^{-4} mol/l; (d) retinoic acid 10^{-5} mol/l.

differentiation inducing activity of anthracyclines on erythroleukaemia, neuroblastoma and melanoma cells and indicate that doxorubicin induces a melanoma cell maturation similarly to other differentiation inducers. Observations on the B16 melanoma cell line indicate that this population is quite homogeneous with reference to cell morphology, DNA, melanin and tyrosinase activity, and only 2% of cells appeared differentiated.

Doxorubicin treatment, besides a cytostatic effect, results

in the development of dendrite-like processes with terminal melanin granules, in an increase in the number of melanotic cells, along with an increase in melanin synthesis and tyrosinase activity (expression of melanogenetic cellular potential). Such an effect is even more pronounced than after theophylline treatment. Moreover, MSH induces an increase in melanin-synthesis without morphological differentiation and cell number reduction. Finally, retinoic acid, despite a cytotoxic effect is biochemically and morphologically ineffective in melanoma cells. Thus, morphological alterations are not correlated with the presence of more melanotic cells or to the cytostatic or cytotoxic effect of the drug.

It has been reported [32] that in melanoma cells an apparently differentiated morphology could be observed at low cell density, since the size and shape of epidermal melanocytes depend on their numerical density. At low densities, melanocytes were rich in long dendrites and were polygonal, whereas at increasing cell density they became round. However, B16 melanoma cells at low density did not appear dendritic and polygonal, thus suggesting that the differentiated morphology, that appears after doxorubicin treatment, may be an effect of the drug. Further support of a differentiation induced by doxorubicin is given by the increase in melanotic cell number, in total melanin content (intracellular and extracellular) and in melanin content in single cells where is evident that cell behaviour is heterogeneous but the majority of cells shows a similar trend. Such an effect was dose dependent, but it did not seem to be correlated with cell viability, since retinoic acid (which reduced cell viability) did not induce melanin alterations and MSH strongly increased melanin content without any reduction in cell number. A very marked increase in the amount of extracellular melanin was observed at cytotoxic concentrations of doxorubicin and theophylline, possibly due to a drug effect on the cell membrane which resulted in greater melanin-release and to the death of highly melanotic cells. At cytostatic concentrations, similar but less pronounced effects were observed. Anyway, since cell number was reduced, such a high extracellular melanin level has to be caused also by an enhancement of melanin synthesis.

Analysis of DNA content indicated a doxorubicin-induced alteration of the distribution in different phases of the cell cycle. The doxorubicin effect was cytostatic and not cytotoxic, since: (i) only a few dead cells could be found in culture medium, (ii) the number of live cells was not increased nor decreased throughout the treatment, and (iii) as previously reported [25], these highly melanotic and dendritic-like cells were alive and could be maintained in culture for a few months without cell proliferation. The result of all these effects is an accumulation in G2 + M and the appearance of polyploid cells in the differentiated population which are absent in the parental cell line. The accumulation in G2 + M does not agree with differentiation, since it is generally reported that differentiated cells accumulate in G0/G1 phase (as we observed in MSH-treated cells). However, it is possible that doxorubicin, by blocking cytokinesis, prevents accumulation in G1 and induce polyploidisation as observed with cisplatin and bleomycin [33, 34]. Moreover, Abdel-Malek *et al.* [35] recently proposed that stimulation of tyrosinase activity in melanoma cells is dependent on the cell cycle and occurs predominantly in G2 phase; a block in G2 thus seems to be essential for a differentiated function. In agreement, our data indicate a similar effect on the cell cycle after theophylline treatment, which is used as a standard differentiation inducer. However it is known that enzymatic activities (and obviously also tyrosinase) in differentiated cycling cells are generally

effective in the G1 phase (similar to the results observed in MSH-treated cells). It is therefore also possible that the very high enzyme level found after doxorubicin treatment is due to the presence of more differentiated cells with a double content of DNA in the G1 phase. Our results also indicate that tyrosinase is present throughout the cell population and that activation of the enzyme by doxorubicin, theophylline or MSH results in an increase in melanin level in already melanotic cells and in an *ex-novo* melanin synthesis in amelanotic cells [3, 8, 11].

Taken together, our results indicate that B16 melanoma cells treated with doxorubicin appear more differentiated than untreated cells. However, at the present state-of-the-art, we cannot definitively conclude whether such a phenomenon is due exclusively to an induction of differentiation or whether the presence in the parental population of few partially differentiated and less drug-sensitive cells has influenced our experiments. The differentiated pattern can probably be attributed to a multiple phenomenon where induction of cell membrane alterations, cell cycle alterations, and an attempt of differentiation involving melanin and tyrosinase activity are involved together.

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