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Scanning electron microscopy of human and murine melanoma cells exposed to medium chain-length (C_6-C_{12}) dicarboxylic acids in tissue culture

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Summary. Human and murine (Harding-Passey and Cloudman) melanoma cells were exposed to various concentrations (1×10^{-3}M–1×10^{-1}M) of adipic (C_6), azelaic (C_9), and dodecanedioic (C_{12}) acids for 1-6 hours in tissue culture, and the effects on shape and surface topography were examined by scanning electron microscopy. Effects, i.e., rounding up, concentration of microvilli, blebbing, and prominence of retraction fibrils were time and dose dependent, and for the same concentrations and exposure times, C_{12} had a greater effect than C_9, and both a significantly greater effect than C_6. These differential reactions to the three diacids parallel previously reported effects on cell kinetics and viability. The changes could be due to a prime effect on the cell membrane, or they might reflect phases of the cell cycle directed by action of the diacids on the nucleus; this latter seems unlikely. An effect on the cytoskeleton is possibly involved.

Key words: Dicarboxylic acids – Melanoma – Tissue culture – Scanning electron microscopy

Introduction

Clinically, when topically applied in the form of a 20% cream, dicarboxylic acids have been shown to have a beneficial effect upon hyperpigmentary disorders such as melasma, and lentigo maligna, and to cause regression of primary cutaneous melanoma (Nazzaro-Porro, et al., 1979;1980). In tissue culture, the C_9 (azelaic) and C_{12} (dodecanedioic) diacids affect growth and viability of murine and human melanoma cells probably by inhibiting key enzymes of the mitochondrial respiratory chain (Robins et al., 1958b; Breathnach et al., 1986; Passi et al., 1984) and DNA synthesis (Leibl et al., 1985). The C_6 diacid has minimal effects on these cellular functions, which shows that anti-proliferative activity is associated with chain-length. *In vivo*, normal human and murine melanocytes appear to be unaffected by exposure to dicarboxylic acids, and in *vitro,* they are significantly less damaged than hyperactive and malignant melanocytes (Robins et al., 1985a).

There is evidence that this selective effect of medium chain-length dicarboxylic acids on hyperactive and tumoural cells, as compared to the normal, may be due to differences in cell permeability (Picardo et al., 1985), and this directs attention to the plasma membrane. In a scanning electron microscopic (SEM) study, Persky et al. (1985), reported major morphological changes to plasma membranes of human melanoma cells exposed to the anti-cancer druges, adriamycin, and actinomycin D, and suggested these changes indicated some involvement, or susceptibility of the plasma membrane in their mechanism of toxicity. In the light of this it seemed of interest to carry out a similar SEM study of human and murine melanoma cells exposed to dicarboxylic acids in order to establish if similar changes in plasma membrane morphology would result. At concentrations and times of exposure which cause significant damage to mitochondrial membranes, no overt damage to plasma membranes of still viable cells was demonstrable by transmission electron microscopy (TEM) of thin sections (Robins et al., 1985; Hu et al., 1986).

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Materials and methods

Cell Cultures

Monolayer cultures of human melanoma (B008, Prof. Rona Mackie, Glasgow), and of Cloudman S 91, and Harding-Passey B16 murine melanomata were established from initial inocula of about 1×10^5 cells/ml giving confluent to near-confluent cultures within about two days. The basic culture media differenced as the three melanomata grow best in different media as follows:

a) For Human B0008 cells-RPM1 1640 (Flow), with 2 mM Hepes and 7.5% $NaHCO_3$ to final pH of 7.3-7.4.

b) For Cloudman cells-Williams E medium (Flow) with 2.2 g/litre of sodium bicarbonate to final pH of 7.1-7.2.

c) For Harding-Passey cells-Eagle's MEM medium with Hank's salts (Gibco), and 7.5% $NaHCO_3$ to final pH of 7.2-7.4.

All media were supplemented with 10% fetal calf serum, 2mM Glutamine, 100 i.u./ml penicillin, 100 mg/ml Streptomycin, and 0.25mg/ml Fungizone. The cells were grown in petri dishes (diameter, 3.5 cms.) on oblong Thermanox plastic cover slips (10.5 x 22mm No. 1 1/2 -Lux Scientific Co.). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

Dicarboxylic acids

Dicarboxylic acids used were dodecanedioic acid (C_{12}), azelaic acid (C_9), and Adipic acid (C_6). The sodium salts of the diacids were prepared as previously described (Robins et al., 1985), and added to cultures in medium to give final concentrations of 1 x 10^{-3}M to 1 x 10^{-1}M. Times of exposure were 1 and 6 hours. The pH of media with added diacids was 7.2-7.4.

Preparation for Scanning microscopy

Coverslips with attached cells were washed in PBSA, and fixed in half strength Karnovsky glutaraldehyde at pH 7.4 at 20°C for 15 minutes, and post-osmicated for 15 minutes. They were then washed for 0.5 minute in 0.1M cacodylate buffer at 4°C, then dehydrated for 10 minutes in graded concentrations of acetone up to absolute (four changes), followed by four further changes in absolute acetone. The cells were then critical point dried using CO_2 as the drying agent, and sputter coated with gold for 1.5 minutes at 0.1 Torr.

Results

Controls

Healthy cells in monolayer culture examined by SEM can exhibit a wide variety of shapes and surface topography depending upon individual cell line, density of distribution, mobility, stage of cell cycle, the nature of the medium, fixation and other processing steps, and contamination with cellular debris or mycoplasma. They can exhibit a heterogeneity of membranous configurations involving, leading lammellae, ruffled membranes, microspikes, microvilli, retraction fibrils anchoring filaments, and blebs. Since many of these features can result from drug perturbation, it is essential in any study, such as the present, that the range of surface topography of cells of control cultures be established at the outset. Figs. 1, 9, and 11, show cells typical of the majority-about 90% at a rough estimate without actual counts-of those of confluent or near-confluent, cultures of human, Harding-Passey and Cloudman, melanomata respectively. Taking into account appearances at different stages of the mitotic cycle (Wetzel et al., 1984), they are interpreted as interphase cells, and provide the main basis for comparison with cells exposed to dicarboxylic acids. Approximately 10% of cells exhibited varying degrees of rounding, numbers of microvilli, and blebbing, and were interpreted as cells at various stages of the mitotic cycle. This relative proportion of interphase and mitotic cells corresponds well with the distribution described for mono-layer cultures of other tumoural cell lines (Feren and Reith, 1984).

The human interphase melanoma cells (Fig. 1) were mostly flat, rather evenly covered with microvilli, and where not in direct contact, exhibited some anchoring filaments or retraction fibrils. Blebbing was not a prominent feature of these cells. Harding-Passey (Fig. 9) and Cloudman (Fig. 11) cells were flattened, spindle-, or dendritic-shaped, and likewise more or less uniformly covered with microvilli, and with very few blebs. Few anchoring fibrils were evident.

Experimental

The effects on cells of all three cultures of exposure to the three dicarboxylic acids at the various concentrations used were essentially the same, and the illustrations chosen to represent them are selectively representative of the overall results.

With azelaic acid (C_9), no effect was observed at, or below a concentration of 10^{-2}M for 1 hour (Figs. 2, human, and 10, Harding-Passey). At 10^{-2}M for 6 hours, the majority of cells were smaller in outline, in less close contact, with prominent retraction fibrils, more concentrated microvilli, and some blebs (Fig. 3). At 10^{-}M for 1 hour, the cells were more rounded, fewer in number and more widely spaced with numerous blebs, and with some cells, microvilli tended to be confined to an elevated central area, with the periphery exhibiting very few (Fig. 4). At 10^{-1}M for 6 hours, many fewer cells were present, and the great majority of those that were, were shrunken into bizarre shapes, with further development of the features seen at 10^{-1}M for 1 hour (Fig. 5). Such cells presented the appearance commonly accepted as dying or dead.

With dodecanedioic acid (C_{12}), blebbing, concentration of microvilli, and prominent retraction fibrils were already very evident at 10^{-3}M for 1 hour, (Figs. 7 and 12), and at 10^{-2}M for 1 hour the cells were shrunken, and clearly affected to a greater degree than with C_9 at the same concentration for 1 hour or for 6 hours (compare Figs. 3 and 8). At 10^{-2}M for 6 hours and higher, the cells resembled those exposed to Azelaic acid at 10^{-1}M for 6 hours.

With Adipic acid (C_6), no real difference as compared with controls was observed at 10^{-1}M for 1 hour (Fig. 6), or below, and even at 10^{-1}M for 6 hours, the effect was clearly less than with either C_9 or C_{12} at 10^{-2}M.

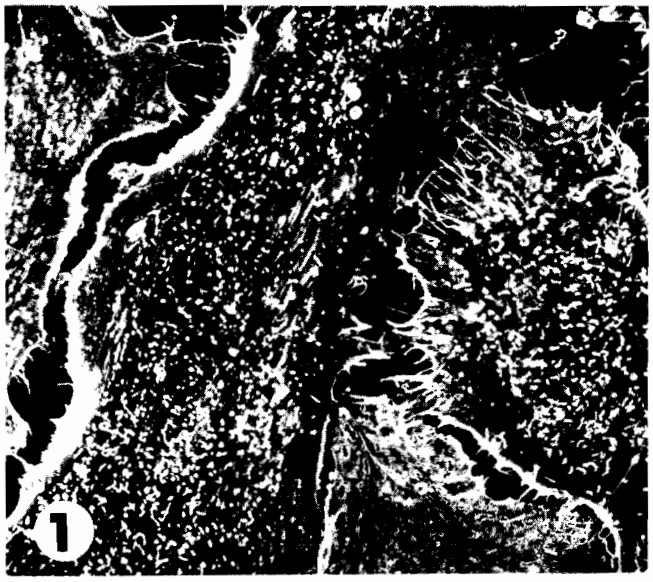

Fig. 1. Human melanoma. Control. ×3,000

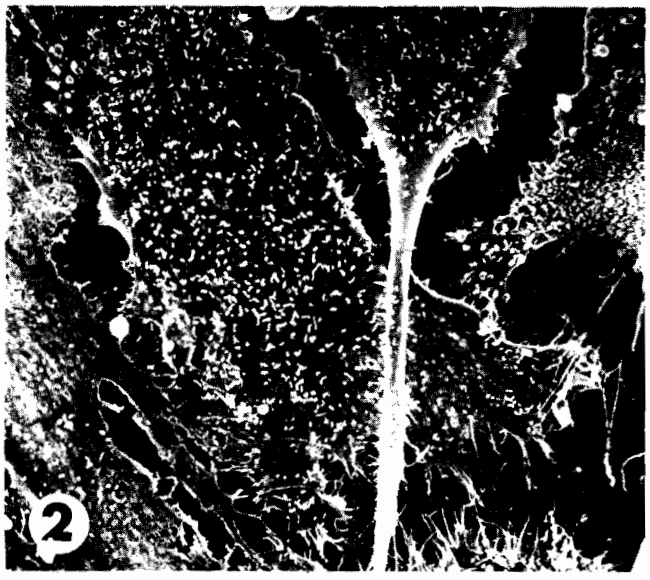

Fig. 2 Human melanoma exposed to Azelaic acid (C_9) at 10^{-2}M for 1 hour. ×3,000. Cells essentially similar to control.

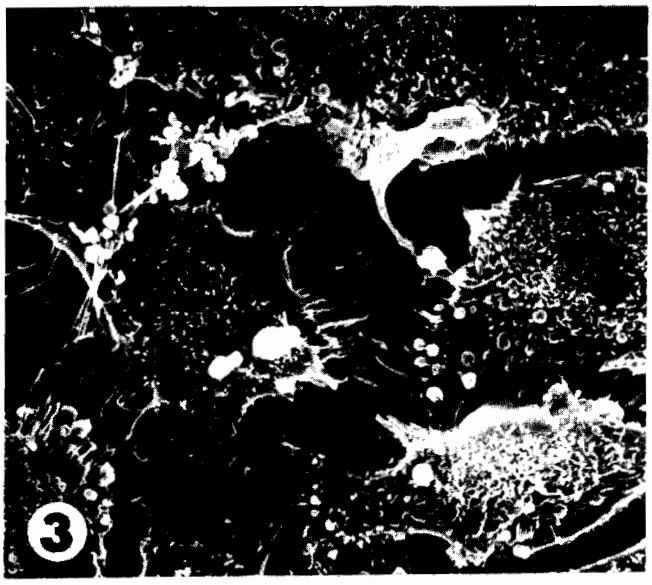

Fig. 3. Human melanoma exposed to Azelaic acid (C_9) at 1 x 10^{-2}M for 6 hours. x3,000. Cells are somewhat rounded up as compared with controls, microvilli are more concentrated, and retraction fibrils and blebs are more evident.

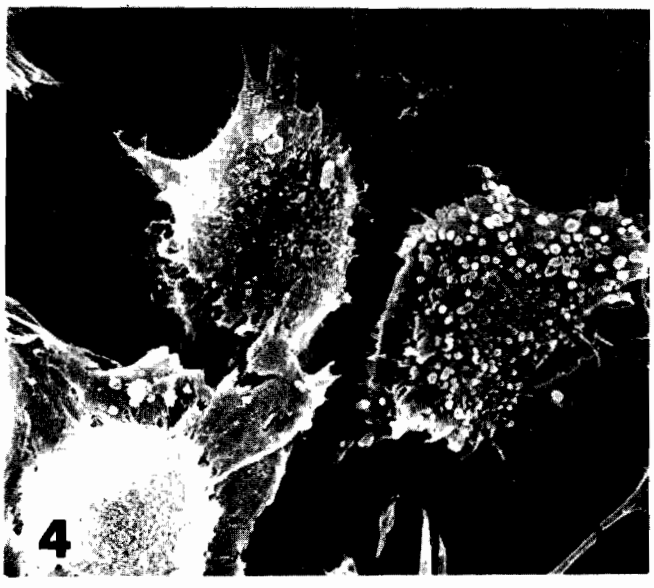

Fig. 4. Human melanoma exposed to Azelaic acid (C_9) at 1x10^{-1}M for 1 hour. x3,000. Fewer cells were present in this culture, microvilli of some cells were concentrated over a central region and blebbing was a prominent feature.

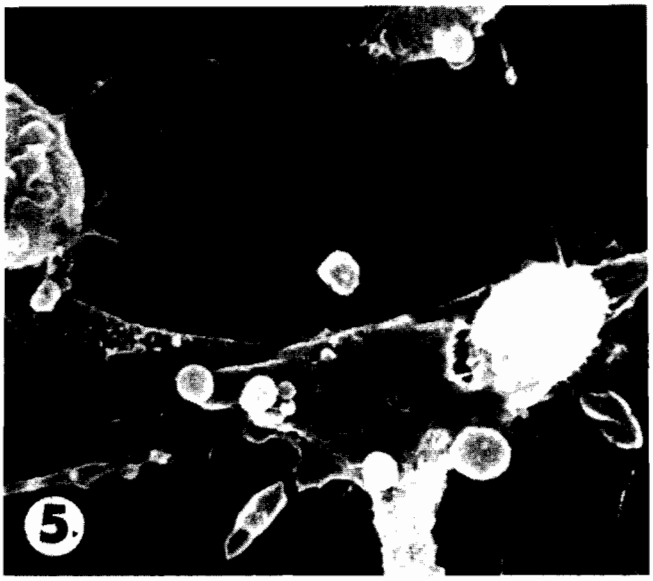

Fig. 5. Human melanoma exposed to Azelaic acid (C_9) at $1x10^{-1}M$ for 6 hours. x3,000. Many fewer cells were present in these cultures and most exhibited features of dying or dead cells as shown.

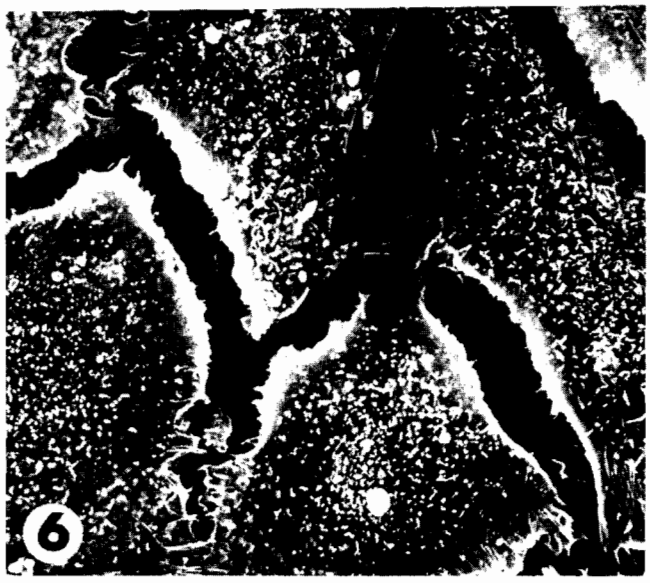

Fig. 6. Human melanoma exposed to Adipic acid (C_6) at $1x10^{-1}M$ for 1 hour. x3,000. Cells are essentially similar to controls (Fig. 1).

Fig. 7. Human melanoma exposed to Dodecanedioic acid (C_{12}) at $1x10^{-3}M$ for 1 hour. x3,000. Concentration of microvilli, blebbing, and prominent retraction fibrils are present. Compare with Figs. 1, 2, 3, and 6.

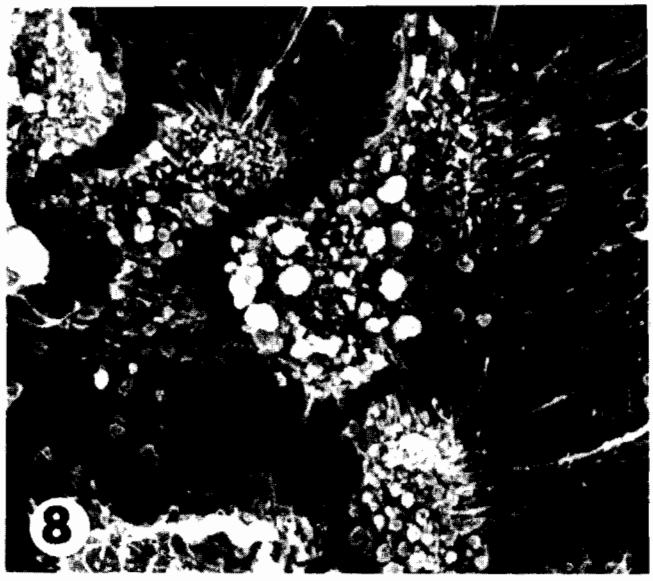

Fig. 8. Human melanoma exposed to Dodecanedioic acid (C_{12}) at $1x10^{-2}M$ for 1 hour. x3,000. Features seen in Fig. 7 are even more pronounced. Compare with Figs. 3 and 6.

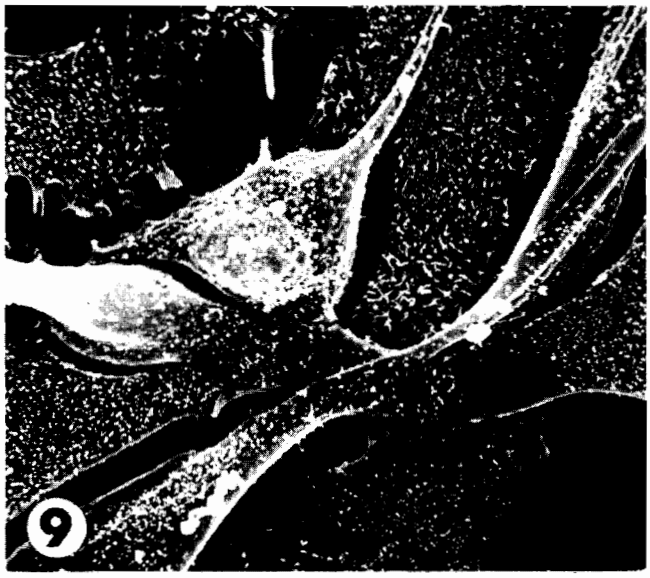

Fig. 9. Harding-Passey melanoma. Control. ×3,000

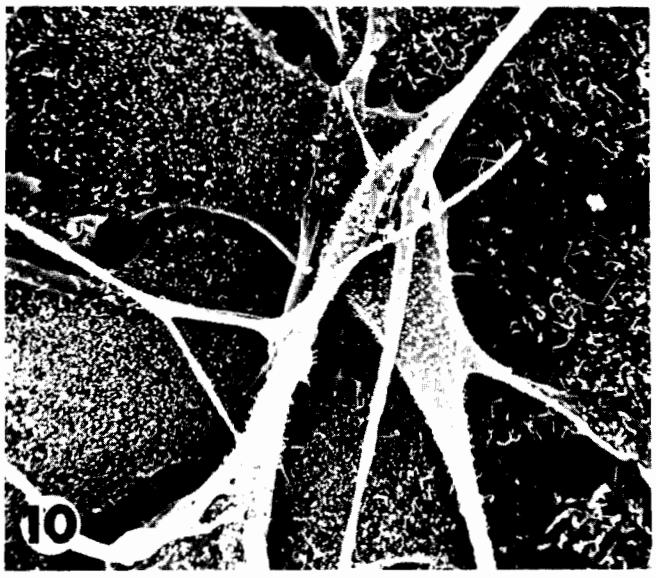

Fig. 10. Harding-Passey melanoma exposed to Azelaic acid (C_9) at 1×10^{-3}M for 1 hour. ×3,000. Cells are essentially similar to controls.

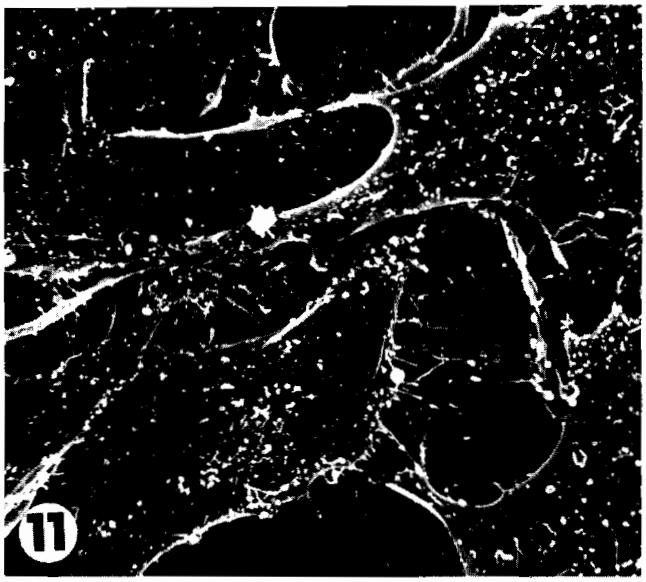

Fig. 11. Cloudman melanoma. Control. ×3,000

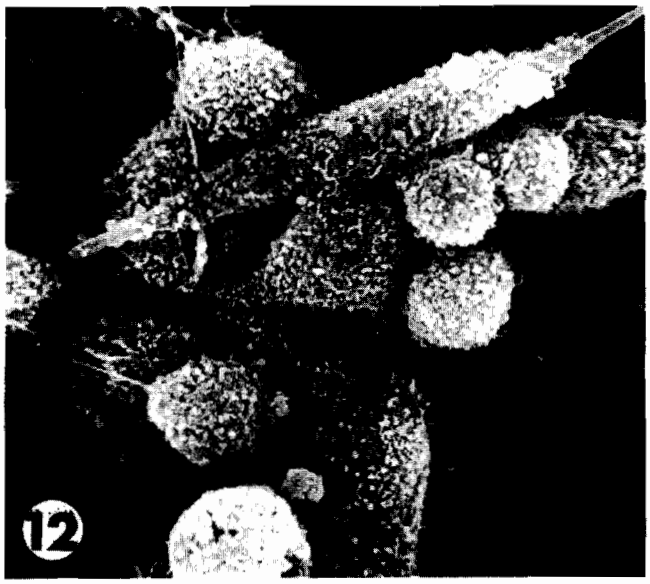

Fig. 12. Cloudman melanoma exposed to Dodecanedioic acid (C_{12}), at 1×10^{-3}M for 1 hour. ×3,000. Cells are rounded up, and blebbing and retraction fibrils are prominent features.

Discussion

This study has shown that human and murine melanoma cells exposed to medium chain-length dicarboxylic acids in culture exhibit morphological alterations of shape and surface topography which are time and dose dependent; the higher the concentration and the longer the time of exposure, the greater the effect. For the same times and concentrations, C_{12} has a greater effect than C_9, and C_6 a significantly lesser effect than the other two. These differential reactions to the three diacids parallel previously reported observations on effects on cell kinetics and viability (Robins et al., 1985b; Breathnach et al., 1986), DNA synthesis, (Leibl et al., 1985) and TEM demonstration of related selective damage to mitochondria (Hu et al., 1986). The reactions of the cells cannot be attributed to perturbations of hydrogen-ion concentration since the pH of all media with added diacids was within physiological limits. Neither can they be regarded as purely osmolar effects, since, for example, they were manifest in reaction to exposure to C_{12} at 1×10^{-3}M, but not with C_9 at the same concentration, nor even with C_6 at 1×10^{-1}M.

The nature of the surface morphological changes observed-shrinking and rounding of cells, with concentration of microvilli, blebbing, and at higher concentrations, progression of these features to those associated with cell death, closely resemble the changes reported by Persky et al., (1985) for melanoma cells exposed to Adriamycin and Actinomycin D. They speculated that with Adriamycin the changes might be due either to a direct effect on the cell membrane, or that they might reflect phases of the cell cycle direccted by Adriamycin action on the nucleus. Little is known as yet about direct effects of dicarboxylic acids on cell membrane properties, but Leibl et al. (1985) report that azelaic acid does not alter expression of Kd 200 or p 94 antigens, melanoma associated gangliosides, or fibronectin. Considering the alterations in shape, the cytoskeleton is almost certainly involved, either in itself or by virtue of its two-way associations with the cell membrane, and one outcome of the present study will be to direct further attention to these features in future, not only of tumoural melanocytes, but also of normal ones which, as established, are less affected by exposure to dicarboxylic acids, possibly due to a difference in plasma membrane permeability (Picardo et al., 1985).

It has been established that dicarboxylic acids enter melanoma cells in culture, and that they or their metabolites inhibit mitochondrial oxido-reductases and nuclear DNA synthesis (Ward et al., 1984; Passi et al., 1984). The C_6 diacid is significantly less active in these respects than either the C_9 or the C_{12} diacids. Can the present observation that C_6 has likewise a clearly lesser effect on cell surface morphology than the other two be taken as circumstantial evidence that the surface changes reflect action on the nucleus, or at any rate, the progression of the nuclear cycle?. The general effect is towards an apparent increase in cells with morphological characteristics of prophase and metaphase (Wetzel et al., 1984), as seen, for example, in Figs. 7 and 8. This is hardly what one would expect to result from inhibition of DNA synthesis per se; indeed, the reverse might be expected, i.e., a diminution in number of cells at these phases. Besides, could such numbers of cells have arrived at, and be arrested at, these stages in the time available, (1 to 6 hours), even in the absence of DNA inhibition?. It seems unlikely. Clearly, interpretation of the present morphological observations in terms of previous kinetic, biochemical, and TEM results is not easy. However, demonstration by another technique - this time SEM- of a significant difference between the effects on melanoma cells of C_6 as compared with C_9 and C_{12}, once again serves to underline the specificity of the latter two diacids as antiproliferative and cytotoxic agents.

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