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ACTIVITY OF AZELAIC ACID ON CULTURES OF LYMPHOMA- AND LEUKEMIA-DERIVED CELL LINES, NORMAL RESTING AND STIMULATED LYMPHOCYTES AND 3T3 FIBROBLASTS

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Abstract—Azelaic acid (C9- -dicarboxylic acid) is a competitive inhibitor of tyrosinase and some oxido-reductase *in vitro*, and *in vivo* has a beneficial effect on lentigo maligna and malignant melanoma. A definite cytotoxic effect in cultures of malignant melanocytes was also reported. In order to establish if the cytotoxic effect of the diacid is exerted equally in the absence of tyrosinase, lymphoma- and leukemia-derived cell lines were cultured for 72 hr with 10^{-3} M, 10^{-2} M and 5×10^{-2} M C9 disodium salt.

Normal resting lymphocytes, lymphocytes activated by phytohemoagglutinin, and mouse Balb/c 3T3 fibroblasts were also tested to study a possible effect of azelaic acid on DNA synthesis and cell duplication. At 10^{-3} M C9 had no effect on the viability of all the cells tested; at 10^{-2} M and 5×10^{-2} M, C9 2Na had a 50–80% cytotoxic effect on lymphoma- and leukemia-derived cell lines, while at the same concentrations it was not toxic to normal lymphocytes, either resting or stimulated, or to 3T3 fibroblasts. The experiments on cellular incorporation of (1–9 ^{14}C) azelaic acid showed that the radiocarbon uptake was two to three times higher for lymphoma- and leukemia-derived cell lines than for lymphocytes, either resting or stimulated, or 3T3 fibroblasts.

Biochemical analysis revealed that the diacid underwent beta-oxidation in all the cell cultures. Fractionated centrifugations of 3T3 fibroblasts cultured in the presence of radiolabelled azelaic acid (2×10^{-4} M) plus cold C9 2Na (10^{-2} M), showed that the radioactivity was mainly concentrated in the cytoplasm. The results, being similar to those obtained by adding azelaic acid to cultures of melanoma cells, suggest that the cytotoxic effect of azelaic acid may be due to interference with mitochondrial oxido-reductase enzymes, rather than with tyrosinase.

The difference in reaction between lymphoma- and leukemia-derived cell lines and normal or stimulated lymphocytes, and 3T3 fibroblasts, could be explained on the basis of a different degree of permeability of the cell membrane, and/or to a possible different sensitivity of reaction of mitochondrial functions. A similar argument could be used to explain the absence of an effect of dicarboxylic acids upon normal as compared with hyperactive or malignant melanocytes *in vivo*.

Dicarboxylic acids from C8 to C13 are a group of non-toxic non-teratogenic substances [1] whose biological activity has been discovered only recently. They are competitive inhibitors of tyrosinase [2] and of some mitochondrial respiratory enzymes [3] *in vitro*; the C9 and C12 dicarboxylic acids applied *in vivo*, topically in a cream, have proved effective in the treatment of melasma, post-inflammatory melanosis [4] and lentigo maligna [5, 6]. Clinically and histologically it has been demonstrated that azelaic acid (C9 dicarboxylic acid) has a definite cytotoxic effect on malignant melanocytes of primary cutaneous melanoma [7, 8], though normal melanocytes appeared not to be affected [5, 9].

As suggested for some melanocytotoxic phenolic compounds which are substrates for tyrosinase [10, 11], the melanocytotoxicity of azelaic acid might be due to its capability of inhibiting tyrosinase, the key enzyme for the formation of melanin. However, evidence has recently accumulated suggesting that the action of dicarboxylic acids is not strictly correlated with anti-tyrosinase activity. Ultrastructural autoradiography of melanoma cells exposed to labelled C12 showed no radioactivity at sites of location of tyrosinase within the cells, but rather in mitochondria and nucleus [12]. Several authors [13, 14] are at present emphasizing that an important feature of the cytotoxicity of anti-cancer drugs is their impairment of mitochondrial function, and there is significant evidence that dicarboxylic acids may exert an anti-mitochondrial effect. Their involvement with mitochondrial function was initially demonstrated by studies on the metabolism of C9–C12 dicarboxylic acids, showing that, when orally administered to rat and man, they are partially metabolized by the mitochondrial beta oxidative enzymes [15]. Beta oxidation of the diacids was also shown to occur in cultures of melanoma cells [12]. In addition, pharmacological investigations on isolated rat liver mitochondria revealed that dicarboxylic acids from C8

to C13 are capable of inhibiting cell respiration, and that they are competitive inhibitors of at least three mitochondrial oxido-reductases, i.e. NADH dehydrogenase, succinic dehydrogenase, and reduced ubiquinone cytochrome *c* oxido-reductase [3].

The present study was instituted in order to establish if the toxic activity of azelaic acid on cells is exerted equally in the absence of tyrosinase. For this purpose, the effect of the diacid has been tested on cultures of cells other than malignant melanocytes, viz. on leukemia- and lymphoma-derived cell lines. In order to study a possible further effect of azelaic acid on DNA synthesis and cell duplication, as has been suggested [12, 16, 17], normal resting lymphocytes (PBL), PBL induced to synthesize DNA *de novo*, and mouse fibroblasts 3T3 cell line were also studied. It seemed to be of interest to establish if there was a different effect of azelaic acid on leukemia- and lymphoma-derived cell lines compared with the normal cells used in the experiments.

MATERIALS AND METHODS

Cell cultures. The following cell lines were used in the present study: the Burkitt lymphoma, Epstein–Barr virus-transformed cell lines EB3, J. Joye and Raji, and the T lymphoma-derived cell line Molt 4 (kind gift of Dr. G. Aragona, University of Rome); the erytroleukemia cell line K562 (R. Herberman, NCI, Bethesda, U.S.A.); the mouse fibroblast cell line Balb/c 3T3 clone A31-1-1 (T. Kakunaga, NCI, Bethesda, U.S.A.).

The suspended cell lines were cultured in RPMI 1640 (Gibco) medium with 10% fetal calf serum (Gibco) and penicillin (100 U/ml) and streptomycin (100 μg/ml) (complete medium). For the experiments the cell lines were seeded at 2–3 × 10^4 cells/well in 96 wells plates.

Balb/c 3T3 fibroblasts were cultured in complete MEM (Gibco) with 10% fetal calf serum (Gibco) and antibiotics as above [18]. For experimental purposes, cells at passages 8 to 12 were used. Cells were seeded at concentration of 10^6 cells/ml in 60 ml Petri dishes.

Peripheral blood lymphocytes (PBL) were isolated on Ficoll hyparque gradient from normal human volunteers. They were cultured in complete RPMI 1640 at 2 × 10^5 cells/well in 96 wells plates (Falcon), in the presence or absence of Phytohemoagglutinin (PHA-P Wellcome) at 1 μg/ml.

In all the experiments, cells were cultured for 72 hr in the absence or presence of dicarboxylic acids, and pulsed for the last 4 hr of cultures with 3H-thymidine (3HT) (Amersham; spec. act. 2Ci/mmole) at 5 μCi/ml. Unpulsed cultures were used for the evaluation of cell viability by the trypan blue dye exclusion test.

Dicarboxylic acids. Azelaic acid (C9) (Fluka, 99% pure) as its disodium salt at pH 7.2 was used at final concentrations of 10^{-3} M, 10^{-2} M and 5 × 10^{-2} M. Some cultures were exposed to the same concentrations of the disodium salt of adipic acid (C6 dicarboxylic acid). This diacid does not inhibit the mitochondrial oxido-reductases [3] and its use, therefore, could serve as a control not only for any specific effect of azelaic acid, but also for any non-specific osmolar effect, especially at the higher concentrations. The osmolarity of C9 and C6 disodium salts in the cultures medium was tested by cryoscopic method, and found to be similar.

Cell uptake and metabolism of radiolabelled azelaic acid. K562 and Molt 4 tumor-derived cell lines, PBL and PHA-PBL at 10^6 cells/ml, and cells of 3T3 fibroblast line were cultured for 5, 15, 30, 60 and 90 min and 24 hours at 37°C in 5% CO_2 in the presence of 2 × 10^{-4} of (1–9 ^{14}C) azelaic acid (20 μCi; spec. act. 13.3 mCi/mmole, Radiochemical Center, Amersham, U.K.) plus 10^{-2} M cold C9 2Na. The cold diacid was added in order to obtain a high concentration of azelaic acid. The cells were washed three times with phosphate buffer pH 7.4, centrifuged at 1000 rpm for 15 min, mixed with 5 ml scintillation fluid (Unisolve), and counted in a beta counter (Beckman). Blanks were represented by the same cells to which (1–9 ^{14}C) C9 was added immediately before the harvesting.

Analysis of lipid extracts from cell cultures. In order to establish the fate of azelaic acid within the cells, the radioactive cells were suspended in 0.2 ml of 10^{-3} M HCl and extracted three times with chloroform : methanol 2 : 1. Standard lipid fractions, lecithin, triolein, oleic acid, cholesterol and cholesterol oleate (50y each) and dicarboxylic acids from C5 to C9 (10y each) were added to the extracts. The extracts were dried over anhydrous Na_2SO_4 and the solvent was evaporated under nitrogen. Each residual matter (proteins, carbohydrates, etc.) and a measured aliquot of the respective extract were mixed with scintillation fluid and counted directly by a beta counter. Another measured aliquot of each extract was fractionated by thin layer chromatography (TLC) (Stratochrom SI AP Carlo Erba) according to the method of Passi *et al.* [19]. Lipid fractions were visualized by iodine vapour; dicarboxylic acids were visualized by bromocresol green [17]. To estimate the relative amount of radioactivity present in the different lipid classes, the areas of silica gel containing the individual spots were scraped off the plates and placed in counting vials with 0.2 ml of water and 0.2 ml of 30% H_2O_2. Standard scintillation fluid was added and the vials counted.

Analysis of dicarboxylic acids. The remaining aliquot of each extract was utilized for the study of dicarboxylic acids by high pressure liquid chromatography (HPLC) as reported [15]. Briefly the extracts were dissolved in 1 ml of CH_3CN : MeOH 4 : 1 (v/v) and 1 mg of para-bromophenacylbromide and 4 μl of the catalyst *N,N*-diisopropylethylamine were added. The mixtures were heated at 50–60°C for 15 min. After evaporation of some solvent up to 0.4 ml, each sample was step-by-step completely injected into the liquid chromatograph (1084 B, Hewlett-Packard) with detector operating at 255 nm. The p.bromophenacyl diesters were separated on a reversed phase column (25 cm × 4 mm i.d.) RP18.5 μm (Browlee Labs, St. Clara, CA). An initial isocratic elution (60% CH_3CN in water adjusted at pH 3.1 with H_3PO_4) for 5 min was followed by a gradient to 100% CH_3CN in 60 min. The peaks corresponding to the dicarboxylic acids (from C5 to C9) were individually collected and counted in a beta counter. The recovery efficiencies were performed by adding from 0.01 to

0.1 μCi ^{14}C C9 plus 10^{-2} M cold C9 to untreated cell cultures immediately before the extraction procedures.

Intracellular localization of radiolabelled azelaic acid. For this study only 3T3 fibroblasts were used. 25×10^6 cells in 40 ml medium were incubated with 2×10^{-4} M (1–9 ^{14}C) C9 (500 μCi) plus cold 10^{-2} M C9 2Na at 37° in 5% CO_2. After 60 min the cells were washed three times and homogenized in hyposmotic medium (10 mM Tris–EDTA). The cellular fractions were separated by several centrifugations: the 150 *g* for 15 min pellet—"heavy fraction"—containing nuclei and plasma membrane, the 6000 *g* for 20 min pellet—"mitochondrial fraction"—containing mitochondria, and the "microsomal fraction" and "cytosol" respectively pellet and supernatant at 100 000 *g* for 60 min [20]. All the fractions were washed three times in phosphate buffer pH 7.4. The pellets and supernatants, separately collected, were separated in measured aliquots. One was directly counted by a beta counter, and the remainder was analysed by the combined HPLC-beta counter method mentioned above.

RESULTS

Effects of dicarboxylic acids on cell cultures

The results of the studies on the rate of 3HT uptake and trypan blue exclusion tests showed that C9 2Na, when added at concentration up to 10^{-3} M had no effect on the proliferative activity and viability of any of the cells tested. At concentrations 10^{-2} M and 5×10^{-2} M the C9 2Na had a markedly adverse effect on the proliferative rate and survival of lymphoma- and leukemia-derived cell lines (Figs. 1 and 2). At the same concentrations of C9 the proliferative rate and survival of resting or PHA-stimulated lymphocytes and 3T3 fibroblasts were unaffected (Figs. 1 and 2).

With adipic disodium salt, up to 5×10^{-2} M, there was no effect on the 3HT uptake and viability of any of the cells tested. At similar concentrations the osmolarity of C6 2Na was approximately equal to that of C9 2Na.

Cell uptake and metabolism of radiolabelled azelaic acid

The experiments on cellular incorporation of (1–9 ^{14}C)azelaic acid showed that the radiocarbon uptake varied with the cell type, and was proportional to the external concentration of the diacid up to 10^{-1} M and to the incubation time, peaking at 60 min. The recovery of added labelled C9 was 88 ± 5%.

In Table 1 are represented the results of the experiments on the different cell cultures (10^6 cells/ml) incubated for 60 min with 20 μCi/ml of (1–9 ^{14}C)C9 to which 10^{-2} M cold azelaic acid was added in order to obtain the concentration that proved to be toxic for the tumor derived cell lines. Table 1 shows that the radiocarbon incorporation was two to three times higher with leukemia- and lymphoma-derived cell lines than with lymphocytes ($P < 0.005$), either resting or stimulated, and with 3T3 fibroblasts.

The analysis of the cell extracts showed that about 70% of the total radioactivity was present in the lipid extracts and 30% in the residual matter. The TLC–HPLC–beta counter analysis of the chloroform methanol extracts showed that, after 60 min incubation, nearly 30% of the lipid radioactivity was detectable in the dicarboxylic acids fraction on the thin layer plate. The remaining lipid radioactivity was mainly concentrated in the phospholipid, cholesterol and triacylglycerol fractions, separated by TLC. As shown by HPLC analysis, in addition to labelled C9, radioactive dicarboxylic acids catabolites with chain lengths 2 or 4 carbon atoms shorter than C9 were detected, thus indicating that a beta-oxidation of the diacid partly took place (Table 2).

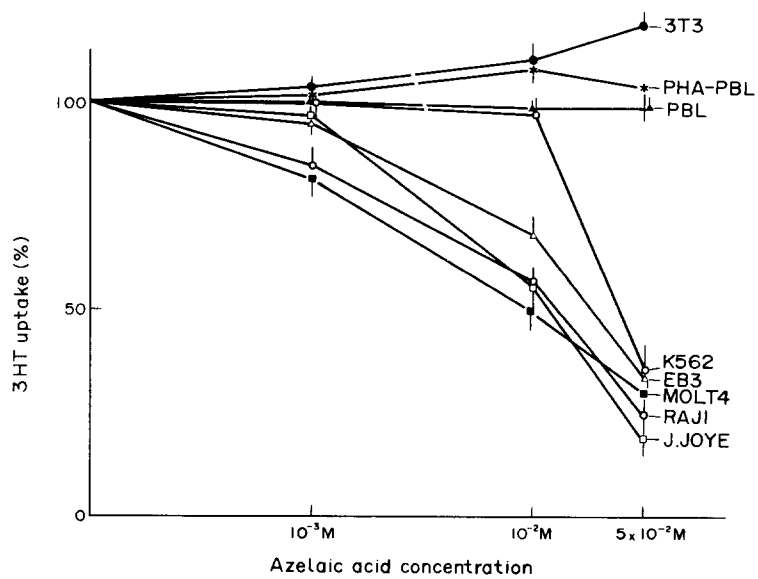

Fig. 1. Effect of increasing concentration of C9 2Na on the proliferative activity of lymphoma- and leukemia-derived cell lines. PBL, PHA-PBL, and 3T3 fibroblasts as measured by the 3HT incorporation. Each value represents the mean ± S.D. of 3 experiments.

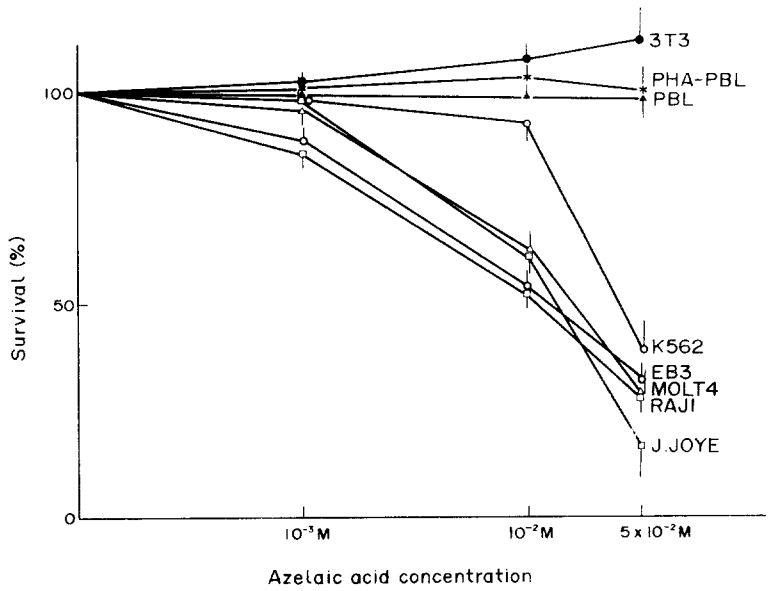

Fig. 2. Effect of increasing concentration of C9 2Na on the survival of lymphoma and leukemia cell lines, PBL, PHA-PBL and 3T3 fibroblasts, as tested by trypan blue exclusion test. Each result represents the mean ± S.D. of 3 experiments.

Table 1. Uptake (cpm) of ^{14}C azelaic acid (10^{-4} M) plus cold C9 2Na (10^{-2} M) after 60 min of incubation

Molt 4	K562	PBL	PHA-PBL	3T3
2310 ± 150	1850 ± 100	670 ± 70	810 ± 60	580 ± 80

10^6 cells/ml were incubated at 37° in 5% CO_2.
Each value represents the mean ± S.D. of 3 experiments.

Table 2. Percentage of radioactive dicarboxylic acids in the lipid extracts of cells cultured with 2×10^{-4} M 1–9 ^{14}C C9 plus 10^{-2} M cold C9 2Na

	Molt 4	K562	PBL	PHA-PBL	3T3
C9	28 ± 6	25 ± 7	20 ± 5	23 ± 5	22 ± 4
C7	0.5 ± 1	3 ± 1	4 ± 2	1 ± 2	4 ± 2
C5	3 ± 3	2 ± 2	6 ± 3	4 ± 2	5 ± 3

10^6 cells/ml were cultured for 60 min. at 37° in 5% CO_2.
Each value represents the mean ± S.D. of 3 experiments.

Table 3. Intracellular localization of radiocarbon in 3T3 fibroblast

	% of total radioactivity	% of (1–9 ^{14}C) C9 in the radioactivity of each fraction*
Heavy fraction	10 ± 3.0	5 ± 0.5
Mitochondrial	5 ± 2.0	3 ± 0.5
Microsomal	3 ± 2.0	3 ± 0.2
Cytosol	60 ± 10.0	20 ± 10.0
Washing	10 ± 5.0	10 ± 5.0

25×10^6 cells were incubated in 40 ml medium for 60 min with 500 μCi (1–9 ^{14}C) C9 plus 10^{-2} M C9.
Each value represents the mean ± S.D. of 3 experiments.
* Traces of labelled C7 and C5 dicarboxylic acids were also found.

Intracellular localization of radiolabelled C9

Table 3 shows the results obtained by fractionated centrifugations of 3T3 fibroblasts cultured in the presence of radiolabelled C9. It is evident that it was mainly concentrated in the cytoplasm. The HPLC-beta counter analysis of the lipid extracts of the different cell fractions showed that 20% of the radiocarbon detected in the cytoplasm was constituted by azelaic acid *per se*. A small amount of labelled C9 was also present in the other cell fractions.

DISCUSSION

This study has shown that there was a 50–80% mortality of lymphoma- and leukemia-derived cell lines exposed to concentrations of azelaic disodium salt (10^{-2} M, 5×10^{-2} M) close to those producing similar effect on melanoma cells in culture [6, 17, 18]. The diacid, therefore, is not specifically anti-melanocytic, but can affect also lymphoma- and leukemia-derived cell lines, and quite probably it may be shown to be toxic to other tumor cell lines in the future. The fact that C6 2Na at the same concentrations had no toxic effect proves that C9 effect is a real specific one, not due to high osmolarity *per se*.

The mitochondrial involvement in all the cell types studied was demonstrated by the investigation on the metabolism and intracellular localization of labelled C9. The TLC–HPLC-beta counter analysis of lipid extracts of the radioactive cells, was in agreement with previous results from studies on the metabolism of C9 and C12 dicarboxylic acids in rat and man [15], and of metabolism of labelled C12 in melanoma cell cultures [21]. Radioactivity was present in the dicarboxylic acid fraction which was constituted not only by C9, but also by labelled dicarboxylic acid catabolites with chain lengths 2 or 4 carbon atoms shorter than C9. This indicates that a beta-oxidation of the diacid was involved to some extent. The remaining radioactivity was essentially localized in the triacylglycerol and phospholipid fractions, an observation which further confirms that beta-oxidation of the diacid only occurs in mitochondria. In fact the complete oxidation of one molecule of azelaic acid should produce one molecule of ^{14}C malonyl CoA, which is the immediate precursor of the two carbon atoms unit in fatty acid biosynthesis, and three molecule of acetyl CoA, one of which is labelled, which enter the general metabolism.

The separate centrifugations performed on 3T3 fibroblasts showed that radioactivity was mainly concentrated in the cytosol. The presence in the cytosol of a relatively high amount of extractable ^{14}C C9, together with its metabolic products, proves that azelaic acid crosses both the cell and mitochondrial membranes. In fact, ultrastructural autoradiography has demonstrated the presence of radioactivity within mitochondria after exposure of normal [16] and malignant melanocytes [12] in culture to labelled C12.

The decrease of 3HT uptake (Fig. 1) caused by azelaic acid on lymphoma- and leukemia-derived cell lines is not likely to be attributable to the inhibition of cell proliferation but rather to a direct effect on cell viability as shown by the trypan blue exclusion tests (Figs. 1 and 2).

How might the difference in reaction between the tumoral-derived cell lines and the normal cells used?

It could be that, as suggested for other tumoral cells [13], in leukemia- and lymphoma-derived cell lines the mitochondria are deficient in some way that renders them more susceptible to the damaging effect of azelaic acid. However, we have no information on this point. What we wish to emphasize is that, according to our data, a correlation exists between the ^{14}C C9 uptake and the toxic effect of azelaic acid. In fact, we have shown that the amount of radiocarbon uptake was 2 or 3 times higher in the lymphoma- and leukemia-derived cell lines than in resting or stimulated lymphocytes and 3T3 fibroblasts (Table 1). This certainly indicates a higher degree of permeability of the cell membranes of the tumor-derived cell lines to the diacid. In this case it could be suggested that the cells are faced with an amount of azelaic acid exceeding the beta-oxidation capacity of the mitochondria, an excess therefore being available to inhibit the oxido-reductases of the respiratory chain. A similar argument could be used to explain the absence of an effect of dicarboxylic acids upon normal as compared with hyperactive or malignant melanocytes *in vivo*.

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