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HUMAN MYELOMA CELLS ACQUIRE RESISTANCE TO DIFLUOROMETHYLORNITHINE WITHOUT OVERPRODUCING ORNITHINE DECARBOXYLASE

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SUMMARY: An exposure of a human myeloma cell line to 2-difluoromethylornithine, the mechanism-based inhibitor of ornithine decarboxylase (EC 4.1.1.17), resulted in a selection of tumor cells readily growing in the presence of 4 mM difluoromethylornithine, a concentration that swiftly halted the growth of the parental cells. Determination of the intracellular polyamines revealed that there were measurable amounts of putrescine and spermidine in the resistant cells. Restriction enzyme analyses of genomic DNA isolated from the resistant cells indicated that the gene dosage for ornithine decarboxylase was not increased to any appreciable extent. Similarly, the accumulation of mRNA was unaltered. The resistant myeloma cells, however, displayed arginase (EC 3.5.3.1) activity that was roughly ten times higher than that in the parental cells.

An inhibition of ornithine decarboxylase, the rate-controlling enzyme of the biosynthesis of polyamines, leading to a depletion of putrescine and spermidine, almost invariably results in growth inhibition of animal cells (for ref. see 1). With the availability of specific and potent inhibitors of the enzyme, such as 2-difluoromethylornithine (DFMO) (2), it has, however, become increasingly evident that tumor cells can easily acquire resistance to inhibitors of ornithine decarboxylase. In almost every instance the resistance developed upon exposure to DFMO or compounds alike has been based on an overproduction of ornithine decarboxylase through an amplification of actively expressed genes in mouse (3,4,5,) and in human (6) tumor cells. In the absence of an apparent gene amplification the overproduction can also be attributable to a strikingly increased accumulation of ornithine decarboxylase mRNA (7,8) or even to an enhanced translability of normal amounts of the message (8). In contrast to these studies, Medrano et al. (9) recently reported that a short exposure of various mouse or human cells to cycloheximide resulted in a generation of cell populations resistant to DFMO without any overproduction of ornithine decarboxylase. Based on the profound polyamine depletion found in the resistant cells, the latter authors (9) suggested that the treatment may have influenced the expression of some other genes possibly fulfilling the requirements for the polyamines.

The abbreviation used is: DFMO, 2-difluoromethylornithine.

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We here report a selection (under the pressure of DFMO) of a variant human myeloma cell line resistant to the antiproliferative effects of DFMO although not overproducing ornithine decarboxylase. The resistance was in all likelihood attributable to a significant polyamine production from ornithine as resulted from a strikingly enhanced arginase activity. The resistant cells likewise showed moderately impaired accumulation of DFMO.

MATERIALS AND METHODS

Cell cultures and the selection of DFMO-resistant cells

A human myeloma cell line (Fr) originally established from the myelomatous bone marrow of a multiple myeloma patient (10) was used. The cells were grown in RPMI 1640 (Gibco Ltd., Paisley, Scotland) medium supplemented with 5% pooled human serum (Finnish Red Cross Transfusion Service, Helsinki, Finland) and with antibiotics (penicillin and streptomycin). The myeloma cells were exposed to gradually increasing concentrations of DFMO over a period of several months resulting in a generation of variant cell population able to grow in the presence of 4 mM DFMO.

Materials

DL-2-Difluoromethylornithine was obtained from Centre de Recherche Merrell International (Strasbourg, France) and DL-α-[3,4-^{3}H]difluoromethylornithine (specific radioactivity 33.3 Ci/mmol) was purchased from New England Nuclear Corporation (Dreieich, West-Germany). DL-[1-^{14}C]Ornithine (spec.act. 61 Ci/mol), [^{32}P]dCTP (spec.act. 410 Ci/mmol) and L-[U-^{14}C] arginine (spec.act. 345 Ci/mol) were purchased from Amersham International (Amersham, U.K.). The restriction enzymes Eco RI, Hpa II were obtained from Amersham International and Msp I from Boehringer Mannheim GmbH Biochemica (Mannheim, West-Germany). For hybridization studies, a human ornithine decarboxylase cDNA clone, pODC10/2H (11) (kindly provided by Dr. O.A. Jänne) was used.

Preparative and analytical methods

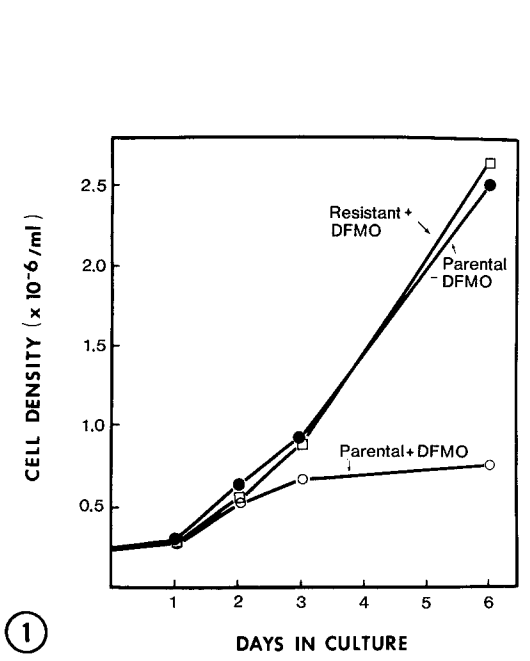
Total cellular RNA was isolated and extracted by the method of Auffray and Rougeon (12) and DNA as described by Blin and Stafford (13). RNA was fractionated by electrophoresis in 1.4% agarose gels in the presence of 1 M glyoxal, transferred onto GeneScreen filters (New England Nuclear Corporation) (14) and hybridized to nick-translated (15) pODC10/2H.

Isolated genomic DNA was digested with Eco RI, Hpa II or Msp I according to the instruction of the suppliers. The restriction fragments were fractionated by electrophoresis in 0.9% agarose gels, transferred onto nitrocellulose filters (16) and hybridized to nick-translated pODC10/2H.

Polyamines were determined by the method of Seiler (17). The activity of ornithine decarboxylase was assayed as described in (18) and that of arginase by the method of Alhonen-Hongisto et al. (15).

RESULTS

A several months' exposure of the human myeloma cells to increasing concentration of DFMO resulted in a selection of cells readily growing in the presence of 4 to 5 mM DFMO. As indicated in Fig. 1, the parental Fr cells exposed



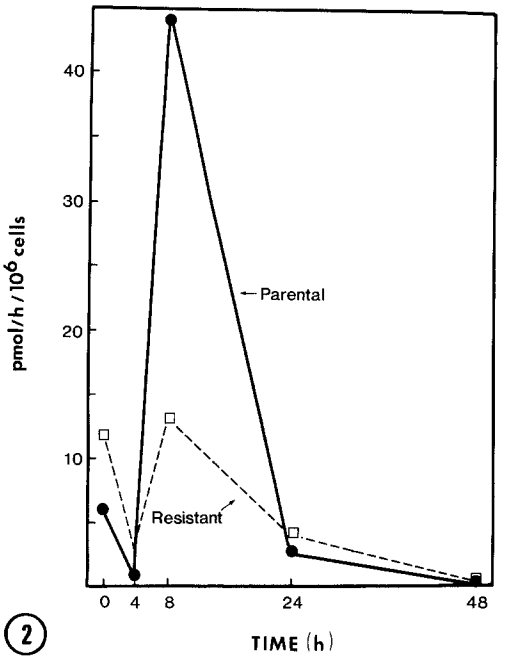


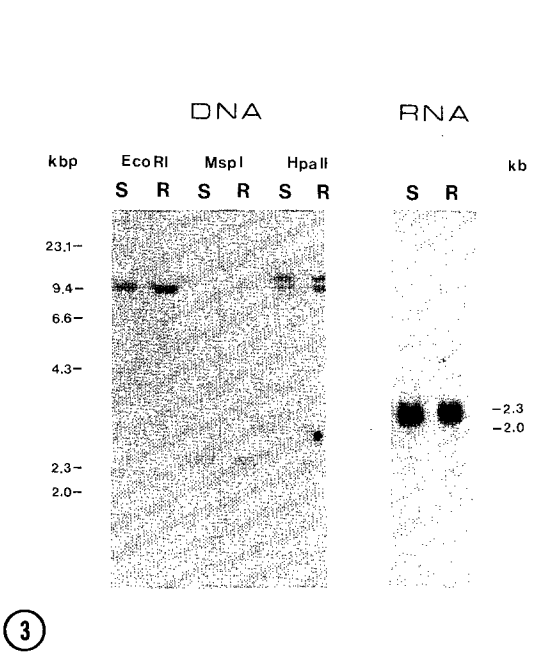
Fig. 1. Effect of DFMO on the growth of human Fr myeloma cells. Resistant cells were grown in the presence of 4 mM DFMO for several months prior to the experiment whereas 4 mM DFMO was added into the parental cell cultures (parental + DFMO) at the beginning of the experiment. Duplicate cultures.

Fig. 2. Ornithine decarboxylase activity during the growth of parental and DFMO-resistant Fr cells. The time-point 0 refers to the dilution of the cultures with fresh medium.

to 4 mM DFMO halted their growth in a few days, yet the variant cells grew in the presence of similar concentrations of the drug as fast as did the parental cells in the absence of the drug (Fig. 1).

Assay of ornithine decarboxylase activity (Fig. 2) revealed no signs of a possible overproduction of the enzyme in the resistant cells. In fact, the maximum enzyme activity was lower in the resistant cells than in the parental cells (Fig. 2). Determination of cellular polyamines indicated that, in spite of the continuous presence of rather high concentrations of DFMO, the resistant cells still contained measureable amounts of putrescine and spermidine (20% and 15% of that found in the parental cells, respectively). Spermine content was virtually unchanged (results not shown).

Restriction endonuclease analyses indicated that ornithine decarboxylase gene dosage was virtually unaltered in the resistant cells (Fig. 3). The use of the CCGG-cleaving isoschizomeric restriction enzymes, methylation-sensitive Hpa II and methylation-insensitive Msp I (20), for the digestion of genomic DNA revealed no apparent differences in the methylation status of ornithine decarboxylase sequences (Fig. 3). The slightly more intensive signals shown in the DNA isolated from the resistant cells (Fig. 3) were not reproducible in repeated experi-



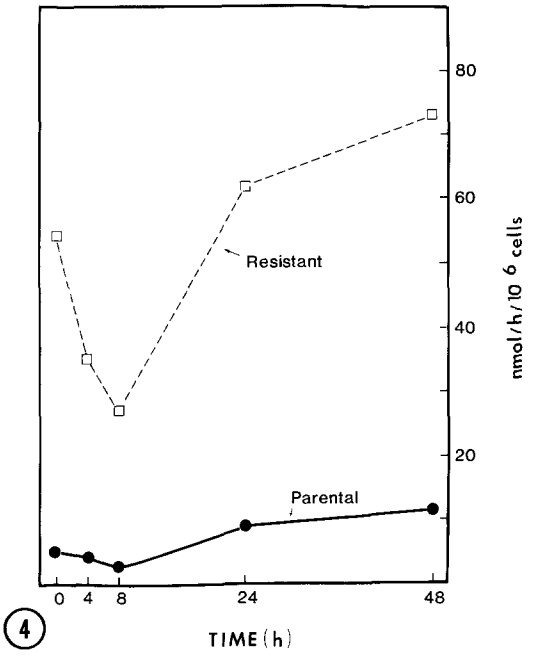


Fig. 3. Restriction enzyme analyses of genomic DNA isolated from the parental (S) and resistant (R) and the levels of ornithine decarboxylase mRNA. DNA (10 µg) was digested with the indicated restriction endonucleases, electrophoresed, blotted and hybridized with a human ornithine decarboxylase cDNA (pODC10/2H) as described under Materials and Methods. Similarly, 10 µg of total RNA was subjected to electrophoresis, blotted and hybridized with pODC10/2H. kbp, kilobasepairs; kb, kilobases.

Fig. 4. Arginase activity during the growth of parental and DFMO-resistant Fr cells. The time-point 0 refers to the dilution of the cultures with fresh medium.

ments. In any event, there was no increase in the accumulation of ornithine decarboxylase-specific mRNA, as also illustrated in Fig. 3.

In order to elucidate whether the resistance to DFMO of the variant cells was based on an impaired accumulation of the drug, we measured the uptake of DFMO by the parental and variant cells. The uptake of the drug was indeed slower in the resistant cells, however, the decrease was only 35 to 50% (results not shown) which probably is not enough to explain the resistance to 4 to 5 mM DFMO of the variant cells.

A determination of the sensitivity of ornithine decarboxylase extracted from the sensitive and resistant Fr cells to DFMO in vitro revealed no differences between the two cell lines (results not shown).

We finally addressed the question whether the resistance and the continuous production of putrescine and spermidine could be attributable to an enhanced production of ornithine, which could compete with DFMO (2). As shown in Fig. 4, the resistant cells exhibited arginase activity (catalyzing the conversion of arginine to ornithine) which was roughly 10 times higher than that in the paren-

tal cells at most of the time points tested. By comparing Fig. 2 and 4, one may also notice that the arginase activity (per a given number of cells) was more than 1000 times higher than the activity of ornithine decarboxylase.

DISCUSSION

The present findings add a further mechanism to the bewilderingly long list of the compensatory reactions triggered by a blockage of putrescine and spermidine formation. We recently (6) selected a DFMO-resistant variant derived from an other human myeloma (Sultan) cell line. In these cells the resistance was exclusively based on a striking overproduction of ornithine decarboxylase through an amplification of apparently transcriptionally active genes (6). In the case of the present variant Fr cells, the drug-resistance seemingly resulted from an overproduction of ornithine, which probably competes with DFMO and weakens its inhibitory action towards ornithine decarboxylase. In fact, the moderately decreased uptake of DFMO by the resistant cells may likewise be a reflection of increased intracellular (and extracellular?) ornithine pools.

The total polyamine content in the resistant cells was 48% of that found in the parental cells. In comparison with the parental cells, the resistant cells contained 20% of putrescine, 15% of spermidine and 90% of spermine. Is this enough for normal cell proliferation to occur? Probably yes, as Porter and Bergeron (21) recently showed that minimum requirement for cell proliferation is 30 % of normal spermidine and 60% of normal spermine. Although only containing 15% of spermidine, our resistant Fr cells contained still 20% of putrescine and near normal levels of spermine. However, if this is not the case, i.e. these levels of the polyamines are not sufficient for cell proliferation to occur, one has to go for further thinking along the lines proposed by Algranati and his coworkers (9); the requirement for the polyamines can be fulfilled by an expression of other growth-related genes.

There are several pieces of experimental evidence indicating that arginase in fact functions as a biosynthetic enzyme of the polyamines in non-ureotelic cells and could be co-ordinately induced with ornithine and adenosylmethionine decarboxylases (22, 23). We likewise showed that a replacement of the natural polyamines (putrescine, spermidine and spermine) by cadaverine-based polyamines (cadaverine and aminopropylcadaverine) resulted in a profound reduction of arginase activity in a mouse tumor cell line (19). Hölttä and Pohjanpelto (24) described a arginase-deficient cell line the growth of which was dependent on polyamine supplementation in serum-free medium.

The present finding revealing that arginase activity is enhanced under the pressure of DFMO links the enzyme even tighter to the biosynthetic pathway of the polyamines.

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