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APHIDICOLIN SENSITIVITY OF VARIANT 3T6 CELLS SELECTED FOR CHANGES IN RIBONUCLEOTIDE REDUCTASE

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SUMMARY: Two mouse fibroblast lines (3T6-HU-11 and 3T6-CA/dA) selected earlier for changes in either of the two subunits (M1 or M2) of ribonucleotide reductase showed an increased resistance to aphidicolin, a known inhibitor of DNA polymerase α. In both lines resistance is in all probability caused by overproduction of dNTPs. HU-11 overproduces an active M2 subunit, has an expanded dATP pool and shows a 2-fold increase in resistance to aphidicolin. CA/dA is mutated in the regulatory M1 subunit, overproduces both dATP and dCTP and shows a sevenfold increased resistance. Addition of aphidicolin resulted in a rapid contraction of the dCTP pool, but not of other dNTP pools. We speculate that these effects might be linked to a regulatory function of dCTP, or a compound derived from it.

INTRODUCTION

The dipterpene aphidicolin (1) interferes with eucaryotic DNA replication by specifically inhibiting DNA polymerase α (2). Inhibition of the purified polymerase is competitive with dCTP (3) and possibly dTTP (4). In contrast, inhibition of the enzyme in isolated nuclei was reported to be noncompetitive with respect to all four dNTPs (5).

Aphidicolin-resistant cell lines of Drosophila melanogaster overproduced α-polymerase or contained a resistant form of the enzyme (6). On the other hand, the development of resistance in FM3A mouse cells was not linked to changes in the α-polymerase. Such cells contained expanded dATP and other dNTP pools (7,8), probably as a result of mutations affecting the

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regulatory subunit of ribonucleotide reductase. The activity of the reductase was not inhibited by aphidicolin.

We are interested in regulatory aspects of DNA synthesis linked to the function of ribonucleotide reductase and have suggested earlier that the reductase may not only supply building blocks for DNA replication but also serve a second, regulatory role linked to dCTP or a compound derived from dCTP (9,10). It appeared possible that the inhibition of DNA replication by aphidicolin might be related to such an effect. Here we report experiments concerning the effects of aphidicolin on two mutant cell lines, selected for changes in either of the two subunits of ribonucleotide reductase.

MATERIALS AND METHODS

Aphidicolin was obtained from Dr. B. Hesp., ICI Pharm. Div., Macclesfield, England. The drug was dissolved in dimethyl sulfoxide. Before addition to medium it was diluted in water and sterilized by filtration. Poly d(A-T) and poly d(I-C) used for the determinations of dNTP pools were synthesized as described (11,12). E. coli DNA polymerase I was obtained from Boehringer, Mannheim, labeled dNTPs from The Radiochemical Centre, Amersham and non-labeled dNTPs from Sigma, St. Louis. 3T6, HU-11 (13) and CA/dA (14) cells were grown at 37^{o} in Dulbecco's modified Eagle's medium, containing 10% heat inactivated horse serum.

dNTP pools were measured in extracts of cells prepared by treatment of cell monolayers with 60% methanol as described earlier (11,12). DNA synthesis was measured by the incorporation of 1 μM [^{3}H]thymidine during a 30 min. pulse.

RESULTS

Mammalian ribonucleotide reductase consists of two non-identical subunits, named M1 and M2 (15). The two cell lines used in this work contained changes in either of the two subunits. HU-11 overproduces M2 activity 30-fold, has a 5-10-fold increased capacity to reduce ribonucleotides and a 4-5-fold increased dATP pool (13). This line was selected by us

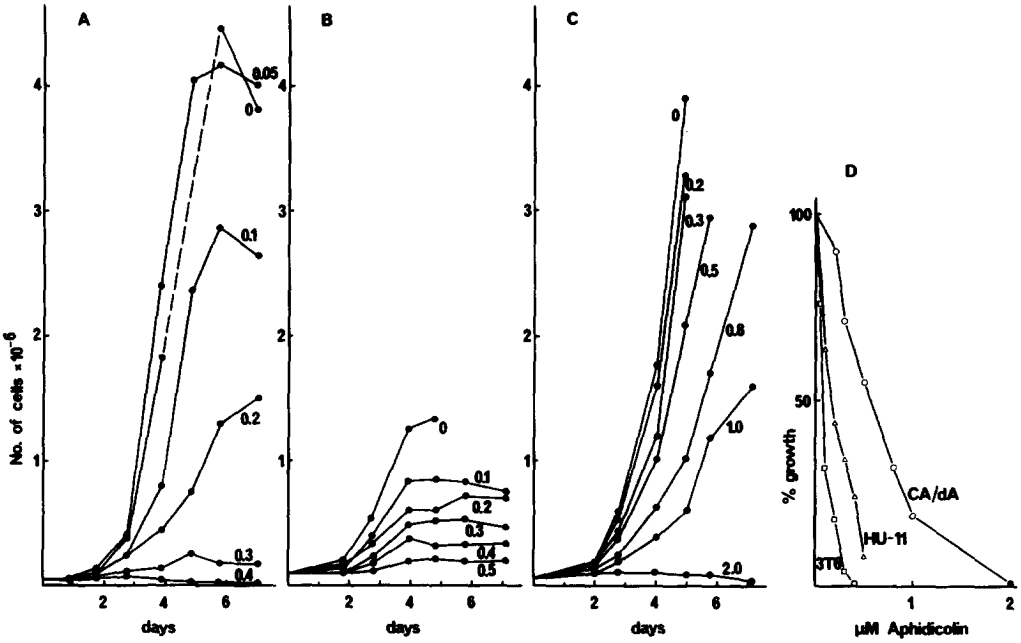

Fig. 1. Effect of aphidicolin on cell growth. 3T6 (panel A), HU-11 (panel B) or CA/dA cells (panel C) were seeded on day 0 on 5 cm Petri dishes and grown for the indicated times at 37° at the concentrations (μM) of aphidicolin shown with each curve. The cell monolayers were trypsinized on consecutive days and the cells were counted. Panel D gives the effect of aphidicolin on the relative growth of the three cell lines on the fourth day.

for resistance against hydroxyurea, a specific inhibitor of the reductase. The second line, CA/dA, is desensitized to negative allosteric control by dATP (14), in all probability because of a mutation in the M1 subunit, and contains increased pools of dATP, dCTP and possibly dGTP (16). This line was selected by Meuth and Green (14) for resistance against arabinosyl cytosine and deoxyadenosine.

Inhibition of growth of the two cell lines and the parent 3T6 line by aphidicolin is shown in Fig. 1. In the absence of the drug, HU-11 cells grow at a slower rate and reach a lower final density than 3T6 cells, while CA/dA is similar to the parent line in both respects. With all three cell lines aphi-

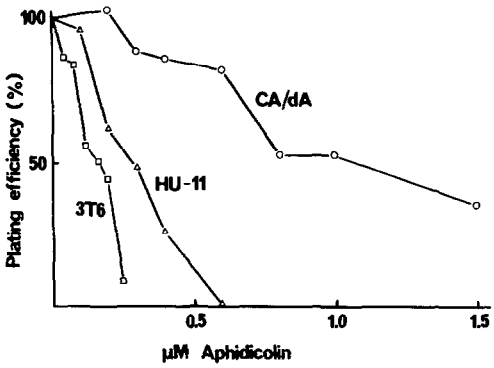

Fig. 2. Effect of aphidicolin on relative plating efficiency. Two hundred cells were plated on 5 cm dishes at various concentrations of aphidicolin and allowed to grow either for 8 days (3T6 = □—□), 11 days (CA/dA = o—o) or 13 days (HU-11 = Δ—Δ). Colonies were counted after staining with orcein.

dicolin inhibited both the rate of cell growth and the final cell density. HU-11 and CA/dA cells had attained a varying degree of resistance in both respects. When inhibition was calculated from the cell number on the fourth day of growth in the presence of the drug as percentage of that in the absence of the drug (Fig. 1D), 50% inhibition occurred at 0.08 μM and 0.2 μM of aphidicolin for 3T6 and HU-11 cells, respectively. The same inhibition with CA/dA cells was found at 0.6 μM aphidicolin. When, instead, the plating efficiency of the three cell lines was compared at different drug concentrations (Fig. 2), 50% inhibition of CA/dA cells occurred at a 6-fold higher concentration of aphidicolin compared to 3T6 cells while HU-11 cells showed a 1.7-fold increase in resistance. Taken together, our results demonstrate an about two-fold increase in resistance for HU-11 cells and a 6-8-fold increased resistance for CA/dA cells.

We next determined the effect of inhibiting concentrations of aphidicolin on dNTP pools in the 3 cell lines (Fig. 3). The

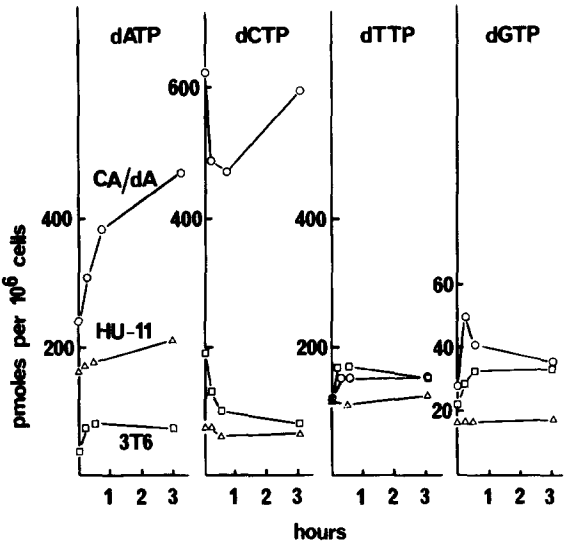

Fig. 3. Effect of aphidicolin on size of dNTP pools. Cultures were grown for 3 days as described in Fig. 1. Aphidicolin (0.5 µM for 3T6 and HU-11, 10 µM for CA/dA) was added and incubation was continued for an additional 3 hours. At different time points the cultures were extracted with 60% methanol and dNTP pools were determined. 3T6 = □—□ ; CA/dA = Δ—Δ ; HU-11 = o—o .

outcome of such determinations depends greatly on the state of growth of the culture (13). In particular the size of the dCTP pool is related to DNA synthesis. In synchronized cell cultures this pool may increase more than 5- to 10-fold when the majority of cells enter S-phase and will drop again when DNA synthesis decreases (17). The comparison of the effect of aphidicolin on pool sizes depicted in Fig. 3 was made 2 days after explantation of cells, when the cultures were maximally engaged in DNA synthesis. Pool sizes for all 4 dNTPs were determined before and up to 3 hours after addition of aphidicolin. The aphidicolin concentrations chosen for the experiments shown in Fig. 3 depressed DNA synthesis to 20% or less.

A comparison of the pool sizes before addition of the drug shows that the dATP pools of HU-11 and CA/dA cells were expand-

ed 4.5- and 7-fold, respectively. The dCTP pool was expanded 3-fold in CA/dA cells but was decreased to 40% in HU-11 cells. The dTTP and dGTP pools showed only small differences between the three lines. Addition of aphidicolin expanded the dATP, dTTP and dGTP pools, more pronounced in 3T6 and CA/dA cells than in HU-11 cells (Fig. 3). In contrast, the dCTP pool showed an immediate drop.

DISCUSSION

Our results establish that changes in either of the two subunits of the reductase may lead to an increased resistance against aphidicolin. Pool measurements demonstrate that the *in vivo* activity of the reductase is not affected confirming earlier conclusions about the polymerase being the primary target for the drug. We visualize two major alternatives for how changes in the reductase may indirectly lead to a decreased sensitivity of the polymerase: protein-protein interaction in a multi-enzyme complex (18) or increased dNTP pools. The *in vitro* experiments (3-5) favour the second alternative while *in vivo* work with previously described mutant cell lines does not distinguish between the two alternatives. These lines were selected for resistance to aphidicolin and contained mutations in the regulatory M1 subunit (7,8). In our experiments the selection procedure was not based on resistance to aphidicolin. Changes in either subunit resulted in an increased resistance to the drug making protein-protein interaction unlikely and supporting the concept that the resistant phenotype of reductase mutant cell lines depends on effects of increased dNTP pools.

It then follows that the limited resistance of the HU-11 line is caused by the expansion of a single dNTP pool, namely

a 4.5-fold increase in dATP. In the CA/dA line we find, in addition, a 3-fold expansion of the dCTP-pool and this then confers a greatly increased degree of resistance to the cells. As a possible explanation for these and earlier (9,10) results we would like to advance the hypothesis that aphidicolin might interfere with the binding of dCTP to an allosteric site of the replicase, regulating the affinity of the system for dNTP substrates. The inhibitory effect of aphidicolin could then be counteracted both by competition with dCTP at the allosteric site or by increased concentrations of one or several dNTPs, minimizing the requirement for allosteric activation.

Finally, we wish to call attention to the changes in dNTP pools occurring immediately after addition of aphidicolin (Fig. 3). With all 3 lines we observed a dissociation between the behaviour of the dCTP pool and that of the other 3 dNTPs. Similar results were found with other inhibitors of DNA replication (unpublished), demonstrating a rapid drop of the size of the dCTP pool as a consequence of blocked DNA synthesis. In our opinion these results further stress the close relationship between DNA synthesis and dCTP.

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