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NOTES

Establishment of a Murine Cell Line Resistant to Arabinosyladenine and Devoid of Adenosine Deaminase Activity

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A population of cells stably resistant to a concentration of arabinosyladenine 100-fold greater than that tolerated by wild-type cells was selected over a period of 15 months. The cells are not cross resistant to arabinosylcytosine and may have at least one kinase with altered substrate specificity.

Vidarabine (arabinosyladenine, ara-A, VIRA-A) is a clinically useful antiviral drug effective in the therapy of several diseases caused by human herpesviruses (2, 15). Although it is known that ara-A inhibits a number of biochemical pathways, it is not clear which actions are primarily responsible for the cytotoxic and antiviral effects of the drug (J. C. Drach, *in* R. T. Walker and E. De Clerq, ed., *Targets for the Design of Antiviral Agents*, in press).

As a stratagem for unraveling which effects are critically involved in the mode of action of ara-A, we set about to establish a mammalian cell line resistant to the drug. Further, since studies in vitro on the mechanism of action of ara-A are complicated by the catabolism of ara-A to arabinosylhypoxanthine by adenosine deaminase, we used a murine cell line (B-mix K-44/6 [12]) previously shown by us to be devoid of detectable adenosine deaminase activity (10).

Wild-type B-mix K-44/6 cells will tolerate 2 μM ara-A (4). By gradually increasing the concentration of the drug in the medium over a 15-month period, it was possible to adapt cells to grow in medium containing 215 μM ara-A (Fig. 1). Twice the cells died (Fig. 1, downward arrows), apparently because the concentration of drug was increased too rapidly, and cells at a lower passage had to be retrieved and initiated from storage in liquid nitrogen (Fig. 1, upward arrows).

Cells resistant to 215 μM ara-A were cloned by sparsely seeding 60-mm tissue culture dishes and carefully removing isolated clones in a drop of warm 0.02% EDTA–0.05% trypsin in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (pH 7.2 at 37°C [9]), using a micropipette. All clones were tested for genetic stability by passaging them 10 times in the absence of drug and then verifying their resistance to 215 μM ara-A. One clone (A-1) was selected for further evaluation; this clone has a population doubling time similar to that of the wild-type cells (ca. 25 h).

To establish a dose-response relationship between the drug concentration and cell growth, 60-mm tissue culture dishes were seeded with wild-type B-mix K-44/6 and clone A-1 cells. After overnight incubation in a humidified, carbon dioxide-enriched atmosphere at 37°C, replicate cultures were trypsinized and cell numbers were determined using techniques previously reported (11). Medium from the remaining dishes was decanted and replaced with growth medium containing selected concentrations of ara-A. After 24 h of additional incubation, cultures (in duplicate) were trypsinized and viable cells were enumerated. Figure 2 represents the dose-response relationship between the drug concentration and the number of viable cells expressed as a percentage of the control value.

Similar experiments were performed using arabinosylcytosine (ara-C) (Fig. 3). In contrast to what was seen with ara-A, wild-type cells and clone A-1 cells were equally inhibited by ara-C. Both wild-type cells and clone A-1 cells were refractory to additional inhibition by concentrations of ara-C higher than 1 μM. Experiments

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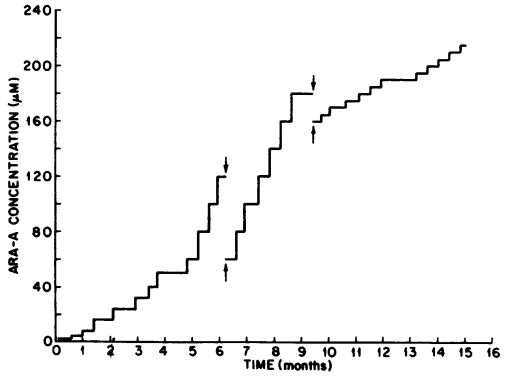

FIG. 1. Selection of B-mix K-44/6 cells increasingly resistant to ara-A. On two occasions the cells died (↓), and it was necessary to reinitiate cultures (↑) from previous passages which were frozen and stored in liquid nitrogen.

were carried out at concentrations as high as 200 μM ara-C, and both cell types were able to multiply at levels of 25% of control values (data not shown).

There are at least six possibilities which could explain the resistance of clone A-1 B-mix K-44/6 cells to ara-A. These are listed below, with an example of each mechanism taken from the literature. (i) Membrane mutants which no longer readily transported ara-A were selected. Clones of Chinese hamster ovary cells selected for resistance to tunicamycin have been shown to be membrane mutants (13). (ii) The resistant cells could have an altered ribonucleotide reductase, resulting in either expanded pools of deoxynucleotides (1, 3) or decreased sensitivity to arabinosyladenine triphosphate (3), the active fraudulent nucleotide. (iii) The substrate specificity of the replicative DNA polymerase could be altered so that arabinosyladenine triphosphate is markedly less inhibitory (6). (iv) Overproduction of the target enzyme (DNA polymerase) could result in an increased resistance of cells to ara-A. A series of 5-fluorodeoxyuridine-resistant mouse 3T6 cell lines which overproduce thymidylate synthetase by up to 50-fold compared with the parental cells have been described recently (8). (v) Adenosine deaminase activity could be reestablished with a concomitant catabolism of ara-A to arabinosylhypoxanthine. The basis for the resistance of deoxycoformycin-resistant rat hepatoma cells is a marked increase in the synthesis of adenosine deaminase (5). (vi) Levels of deoxycytidine or adenosine kinase or both could be substantially reduced with a corresponding decrease in the phosphorylation of ara-A (14).

Because clone A-1 B-mix K-44/6 cells are resistant to ara-A but not ara-C, it is unlikely that possibility i, ii, iii, or iv (above) is operative. Using methodologies developed to test the parent cell line (10), we were unable to detect adenosine deaminase activity in either clone A-1 B-mix K-44/6 cells or cytosol preparations derived from these cells (data not shown); thus, possibility v can be excluded also. Therefore, the most likely possibility is that clone A-1 cells represent a population of cells having at least one kinase with an altered substrate specificity.

Cells resistant to ara-A have been reported before. These include an ara-A-resistant protozoan (*Toxoplasma gondii*) (7) and three classes of ara-A-resistant baby hamster kidney (BHK-21/C13) cell lines (3), both of which were selected after chemical mutagenesis. In addition, a murine tumor cell line showing spontaneous resistance to the drug (6) and a human T-lymphoblastoid cell line selected by exposing cells to sublethal concentrations of ara-A plus the adenosine deaminase inhibitor deoxycoformycin (14) also have been reported. The protozoan, one class of the mutant BHK-21/C13 cell lines, and the lymphoblastoid cell line were shown to be kinase mutants. It was reported, but has not been confirmed, that the murine tumor cell line is a DNA polymerase mutant. The mechanism of resistance of the second class of BHK-21/C13 mutant cell lines was attributed to a possible ribonucleotide reductase mutation, and the third class was attributed to an unknown mechanism expressed by the cells as extreme adenosine sensitivity.

Clone A-1 B-mix K-44/6 cells are unique in two ways. First, like the parental B-mix K-44/6 line, they lack adenosine deaminase activity, a key enzyme in the degradation of ara-A. Second, the cells were selected for resistance to ara-A in the absence of an adenosine deaminase inhibitor. Cells selected in the presence of these potent inhibitors may have additional biochemi-

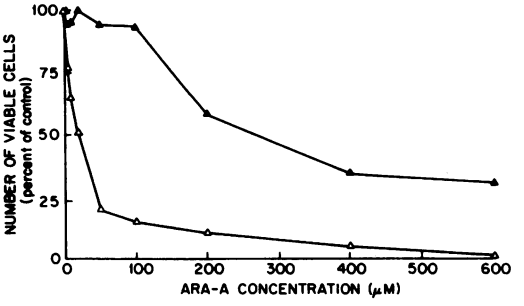

FIG. 2. Dose-response relationships between the concentration of ara-A in the culture medium and the growth of wild-type (△) or clone A-1 (▲) B-mix K-44/6 cells. Numbers of viable cells after growth for 24 h in the presence of drug are expressed as a percentage of the number of viable cells counted in the absence of drug.

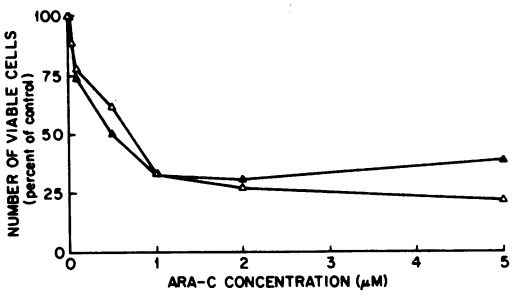

FIG. 3. Dose-response relationships between the concentration of ara-C in the culture medium and the growth of cloned wild-type (△) or clone A-1 (▲) B-mix K-44/6 cells. Numbers of viable cells after growth for 24 h in the presence of drug are expressed as a percentage of the number of viable cells counted in the absence of drug.

cal lesions (as may cells selected after chemical mutagenesis).

The metabolic pathway for the activation of ara-A in mammalian cells remains controversial (14). We anticipate that the characterization of clone A-1 B-mix K-44/6 cells not only will yield valuable information about the mechanism of action of ara-A but also will be helpful in elucidating pathways of anabolism for this interesting and clinically valuable drug.

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