

Formycin B-Resistant Mutants of Chinese Hamster Ovary Cells: Novel Genetic and Biochemical Phenotype Affecting Adenosine Kinase

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Stable mutants which are approximately three- and eightfold resistant to the pyrazolopyrimidine nucleosides formycin A and formycin B (Fom^R) have been selected in a single step from mutagenized Chinese hamster ovary cells. In cell extracts, the two Fom^R mutants which were examined were both found to contain no measurable activity of the enzyme adenosine kinase (AK). However, cross-resistance studies with other adenosine analogs such as toyocamycin and tubercidin show that these mutants are distinct from toyocamycin or tubercidin resistant (Toy^r) mutants which also contain no measurable AK activity in cell extracts. Studies on the uptake and incorporation of [³H]adenosine and [³H]tubercidin by various mutants and parental cell lines show that unlike the Toy^r mutants, which are severely deficient in the phosphorylation of these compounds, the Fom^R mutants possess nearly normal capacity to phosphorylate these compounds and incorporate them into cellular macromolecules. These results suggest that the Fom^R mutants contain normal levels of AK activity *in vivo*. In cell hybrids formed between Fom^R × Fom^S cells and Fom^R × Toy^r cells, the formycin-resistant phenotype of both of the Fom^R mutants behaved codominantly. However, the extracts from these hybrid cells contained either ≈50% (Fom^R × Fom^S) or no measurable (Fom^R × Toy^r) AK activity, indicating that the lesion in these mutants neither suppresses the wild-type AK activity nor complements the AK deficiency of the Toy^r mutants. The presence of AK activity in the Fom^R mutants *in vivo*, but not in their cell extracts, along with the codominant behavior of the mutants in hybrids, indicates that the lesions in the Fom^R mutant are of a novel nature. It is suggested that the genetic lesion in these mutants affects AK activity indirectly and that it may involve an essential cellular function which exists in a complex form with AK. Some implications of these results regarding the mechanism of action of formycin B are discussed.

The pyrazolopyrimidine ribosides formycin A and formycin B constitute an important class of nucleoside analogs (C-nucleosides) which, rather than having the usual C-N bond in the ribosidic linkage, are linked by a carbon-carbon bond (7, 29). As a result of this structural modification, these antibiotics are hydrolytically stable and possess very useful biochemical and medicinal properties, including antiviral and anticancer (7, 15, 25, 29). Earlier biochemical studies with these analogs have indicated that unlike formycin A and other adenosine analogs (e.g., toyocamycin and tubercidin), formycin B, which is an analog of inosine, is not phosphorylated in cells (31). However, formycin B has been reported to be a good inhibitor of human purine nucleoside phosphorylase (6, 26, 28) and polyadenosine diphosphoribose polymerase (20). On the contrary, Osborne et al. (23) have found that

inhibition of purine nucleoside phosphorylase is a secondary effect of formycin B toxicity, but the primary site of action of formycin B was not identified in these studies.

In an attempt to understand the mechanism of action of formycin A and formycin B, we have undertaken a genetic approach to study the mechanism by which resistance to formycin B may develop in mammalian cells. In this regard, stable mutants of Chinese hamster ovary (CHO) cells which show different levels of resistance to formycin A and formycin B (Fom^R mutants) have been isolated. Genetic and biochemical studies with two of the Fom^R mutants are presented in this paper and show that the extracts from both of these mutants contain no measurable activity of the enzyme adenosine kinase (AK) and that the drug-resistant phenotype of both of these mutants behaves codominantly in

cell hybrids. These Fom^R mutants are different from AK-deficient toyocamycin-resistant mutants in terms of their cross-resistance to various adenosine analogs as well as in their behavior in cell hybrids (14). These and other studies presented here show that the genetic lesion in the Fom^R mutants is of a very novel type. These results are discussed in relation to the mechanisms of action of formycin A and formycin B.

MATERIALS AND METHODS

Cell culture and cell lines. The different CHO cell lines employed in these studies are listed in Table 1. The parental CHO cell line which has been used for mutant selection is auxotrophic for proline and is referred to as wild type (WT) in our work. The cells were routinely grown in monolayer culture at 37°C in alpha minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum. However, all experiments involving various nucleoside analogs were carried out in alpha medium supplemented with 5% dialyzed fetal calf serum. In experiments in which adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) has been added (denoted by +EHNA), the final concentration of EHNA was 10 µg/ml (1). The procedure employed for cell hybridization was the same as described earlier (12, 22). The hybrids formed between two cell lines are denoted by Hyb A × B, where A and B are the two parental cell lines.

Chemicals. The sources of various drugs and chemicals were as follows. Toyocamycin (NSC 63701) was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Silver Spring, Md.; formycin A, formycin B, and tubercidin were purchased from Sigma Chemical Co., St. Louis, Mo.; EHNA was obtained from Burroughs Wellcome Co., Research Triangle Park, N.C. [³H]tubercidin was obtained by the custom labeling of tubercidin by a catalytic exchange procedure (New England Nuclear Corp., Boston, Mass.) and then was purified by paper chromatography (specific activity, 100 mCi/mmol). [³H]adenosine (specific activity, 36 Ci/mmol) was purchased from New England Nuclear.

Degree of resistance of mutant cells towards various drugs. The degree of resistance of mutants towards various drugs was determined by seeding 100 and 200 cells (in a 0.5-ml volume of growth medium) into the wells of 24-well tissue culture dishes, containing 0.5 ml of the various dilutions of the drugs at two times the final concentrations desired in the growth medium (13). The dishes were incubated for 6 to 7 days at 37°C, after which they were stained with 0.5% methylene blue in 50% methanol; then the number of colonies was counted. From the averages of the numbers of colonies observed at different concentrations of the drugs, relative plating efficiencies were determined as the ratios of the number of colonies at a given drug concentration to that obtained in the absence of the drug. The D₁₀ value of a drug towards a cell line refers to the dose of the drug which reduces relative plating efficiency of the cell line to 10%.

Selection of mutants. Selection of mutants was carried out by procedures similar to those employed earlier (10, 11). An exponentially growing culture of

TABLE 1. CHO cell lines used in the present studies

Cell line	Phenotype and origin
WT (Pro ⁻)	Proline-requiring CHO line used for various mutant selections (9, 27)
Fom ^{R2} and Fom ^{R4}	Formycin A- and B-resistant mutants selected from WT cells
Toy ^{r4}	Toyocamycin-resistant mutant selected from WT cells at 10 ng of toyocamycin per ml (14)
EOT	Multiply marked CHO line resistant to emetine, ouabain, and 6-thioguanine, selected from WT cells in earlier studies (12)
EOT-Toy ^{r2}	Toyocamycin-resistant mutant selected from EOT line in the presence of 10 ng of toyocamycin per ml (unpublished data)

WT cells was treated with 300 µg of the mutagen ethyl methanesulfonate per ml for 20 h. This treatment results in about 50% cell killing (10). The mutagen-treated cells were grown for 3 days in non-selective medium to allow time for mutation fixation. The selection of mutants was carried out by plating 10⁶ cells per 100-mm-diameter dish (P-100 dish) on several dishes in medium containing 10 µg of formycin A per ml. The plating efficiencies of the cells at the time of plating were determined by plating a known number (usually 250) of cells in nonselective medium in 60-mm-diameter dishes, and the mutation frequencies observed were corrected for this.

Cellular uptake and incorporation of [³H]tubercidin and [³H]adenosine. The cellular uptake and incorporation of [³H]adenosine and [³H]tubercidin were studied by procedures similar to those described earlier (11). In these experiments, ca. 5 × 10⁴ cells were seeded into the wells of 24-well tissue culture dishes. After approximately 2 days, when the wells were nearly confluent, the medium was carefully aspirated, and 0.25 ml of the solutions containing the desired concentrations of [³H]adenosine (10 µCi/ml) or [³H]tubercidin (0.2 µCi/ml) in growth medium (supplemented with 10 µg of adenosine deaminase inhibitor EHNA per ml to prevent deamination of adenosine and 5 × 10⁻⁵ M uridine) were added to each well. At different time intervals, the labeled medium was removed and the cells were rinsed three times with phosphate-buffered saline. The cells from each well were dissolved in 0.5 ml of a solution of 0.4% deoxycholic acid in 0.1 N NaOH. One half of the cell lysate was counted directly after the addition of aqueous counting scintillant to determine the total cellular uptake of radioactivity. The remaining cell lysate was precipitated by the addition of cold 10% trichloroacetic acid and then was filtered, washed, and counted to obtain a measure of the amount of radioactivity which had been incorpo-

rated into cellular macromolecules. At the same time, total numbers of cells in two parallel control wells of each cell line were determined by trypsinization and by counting samples in a Coulter electronic Counter. The cellular uptake and incorporation of [3 H]adenosine and [3 H]tubercidin in different cell lines were normalized for a constant number of cells (5×10^5).

AK assay. The cell extracts for measurement of AK activity were generally prepared by trypsinizing cells from three to four nearly confluent P-100 dishes and then washing them twice with phosphate-buffered saline by centrifugation and resuspension. The washed cells were suspended at a concentration of 10^7 cells per ml in 0.1 M potassium phosphate buffer (pH 7.0) and were disrupted by sonication for two 30-s-interval bursts in a Bronson sonicator. The resulting crude cell extract was centrifuged for 30 min at $30,000 \times g$ at 4°C . The supernatant from this run was dialyzed for 16 h at 4°C against 200 volumes of 0.01 M potassium phosphate buffer and then was used for measurement of AK activity.

The assay of AK activity in cell extracts was carried out as described before (4, 14). The reaction mixture (in a final volume of 250 μl) contained 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM ATP, 0.25 mM MgCl_2 , 4×10^{-5} M [3 H]adenosine (specific activity, 36 mCi/mmol) and between 25 and 50 μl of cell extracts. The reaction which was carried out at 37°C was initiated by the addition of cell extracts. At 5-min intervals, 50 μl of the reaction mixture was removed and added to 1.0 ml of an ice-cooled solution of 0.1 M lanthanum chloride, which precipitates AMP. The precipitated AMP was collected on fiber glass filters, which were washed five to six times with cold water (6 to 7 ml each time). The filter papers were then dried and counted in a toluene-based scintillation fluid. The radioactivity which was bound to the filter paper in a parallel control experiment lacking cell extract was subtracted from all experiments.

Under the conditions used, conversion of [3 H]adenosine into [3 H]AMP by WT cell extracts was linear for at least the first 15 min, and the duplicate samples generally showed less than 5% variation. The amount of [3 H]AMP produced by any cell extract was converted into nanomoles of [3 H]AMP. The specific activity of AK in various cell extracts was calculated by dividing AK activity by the concentration of proteins in the cell extracts, which was measured by the method of Lowry (17).

RESULTS

Selection of mutants resistant to formycin A/ formycin B and cross-resistance studies. While the cross-resistance of toyocamycin-resistant (Toy^r) mutants (14) of CHO cells to various adenosine analogs was being examined, it was observed that in contrast to the other adenosine analogs such as toyocamycin, tubercidin, or 6-methylmercaptopyrimidine riboside (6-MeMPR)—to which the Toy^r mutants were highly resistant (>100-fold)—these mutants exhibited only very slight (approximately twofold) cross-resistance to the two pyrazolopyrimidine analogs formycin A and formycin B. These experiments suggested

that the toxicity of formycin A and formycin B was mediated by a mechanism different from that of other adenosine analogs.

To understand the mechanism of cellular action of formycin A and formycin B, we sought to obtain cellular mutants which exhibited increased resistance to these analogs. The dose-response curve of WT CHO cells in the presence of different concentrations of formycin A showed that the plating efficiency of WT cells was reduced sharply at drug concentrations between 1 and 5 $\mu\text{g/ml}$ (Fig. 1), and at 10 $\mu\text{g/ml}$, no colonies were obtained, even when 5×10^5 cells were plated in a 100-mm-diameter tissue culture dish (data not shown). The selection of mutants resistant to formycin A was carried out in the presence of 10 μg of the drug per ml in both control and mutagen-treated cultures (see above). In the control culture, no colonies were obtained from a total of 2.5×10^6 viable cells (corrected for plating efficiency) which were plated in the presence of the drug in these experiments. However, in the culture which was pretreated with the mutagen ethyl methanesulfonate, a number of resistant colonies were observed in various dishes, and the mutant frequency was estimated to be about 1.0×10^{-5} . Some of the resistant colonies which appeared in different dishes in these experiments were picked and grown in nonselective medium; then their degree of resistance towards formycin A was determined. All six colonies which were examined proved more resistant to formycin A as compared with the parental WT cells, and the dose-response curves of two mutant clones, $\text{Fom}^{\text{R}2}$ and $\text{Fom}^{\text{R}4}$, which have been chosen for further studies, are shown in Fig. 1. Based upon

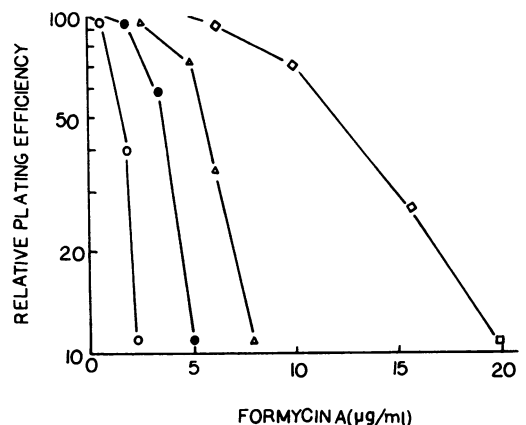


FIG. 1. Survival curves of parental and the Fom^{R} and Toy^r mutant cell lines in the presence of increasing concentrations of formycin A (in the absence of EHNA). Symbols: ○, WT; △, $\text{Fom}^{\text{R}2}$; □, $\text{Fom}^{\text{R}4}$; ●, Toy^4 .

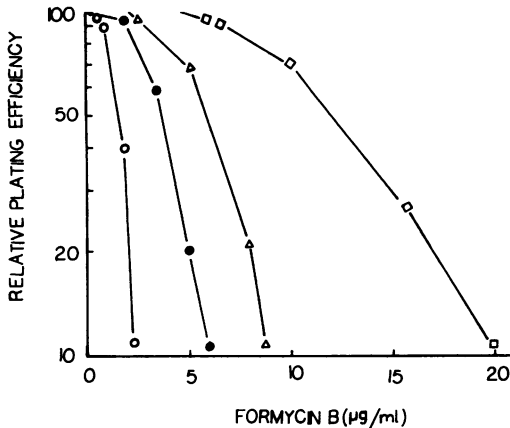


FIG. 2. Survival curves of different cell lines for formycin B. Symbols: ○, WT; △, Fom^R2; □, Fom^R4; ●, Toy⁴.

their D₁₀ values, these mutants are about three- (Fom^R2) and eight- (Fom^R4) fold more resistant to formycin A as compared with the WT cells. The different levels of resistance of these mutants to formycin A indicate that these mutants arose from independent genetic lesions. The drug-resistant phenotype of both these mutants has remained completely stable upon subcloning in the absence of formycin A and upon growth in nonselective medium for several months.

After obtaining the mutants described above, it was realized that formycin A, which is an adenosine analog, is a good substrate for the enzyme adenosine deaminase, which is present in large amounts both in cells and in fetal calf serum (16). Because of this, formycin A, under the conditions used in these experiments, was most probably rapidly converted into formycin B, which may have been the actual selective agent. This was consistent with the fact that all of the mutants selected for resistance to formycin A exhibited cross-resistance to formycin B, and the concentration responses of various cell lines towards these two analogs were virtually identical (Fig. 2). Further evidence supporting this possibility was provided by experiments in which the toxicity of formycin A was determined in the presence of EHNA, which is a very potent inhibitor of the enzyme adenosine deaminase (1). In the presence of 10 µg of EHNA per ml in the growth medium, a concentration which completely inhibits adenosine deaminase activity, the toxicity of formycin A towards WT CHO cells was greatly enhanced (Fig. 3). Interestingly, the Toy⁴ mutant which showed only slight cross-resistance to formycin A in the absence of EHNA became highly resistant to this drug when EHNA was present. This result indicated

that in the presence of EHNA, when deamination of formycin A was prevented, it behaved like other adenosine analogs such as toyocamycin or 6-MeMPR, which are phosphorylated by the enzyme AK. Furthermore, in accordance with earlier observations with toyocamycin and tubercidin (14, 24), mutants resistant to formycin A (in the presence of EHNA) can be obtained in both control and mutagen-treated CHO cell cultures at very high frequencies (10⁻³ to 10⁻⁴; data not shown). All of these observations strongly suggested that formycin A under the mutant selection conditions employed was largely converted into formycin B, and that similar mutants may also be obtained by directly selecting for formycin B.

In the presence of EHNA, although the sensitivity of WT cells towards formycin A was greatly enhanced, the degree (i.e., fold) of resistance of the two Fom^R mutants was not appreciably altered (Fig. 3). The resistance of Fom^R mutants to formycin B as well as to formycin A (+EHNA) suggested that in these mutants, a step which is common in the action of both these analogs is very likely affected. To characterize these mutants further, the cross-resistance of the Fom^R mutants towards a number of adenosine analogs was examined. The dose-response curves of the WT, the two Fom^R mutants, and a Toy^r mutant for toyocamycin and tubercidin are shown in Fig. 4 (A and B). The two Fom^R mutants showed only slight cross-resistance to toyocamycin and tubercidin (Fig. 4). In contrast, the Toy^r-4 mutant, which lacks AK, was highly resistant to these analogs. These results indicated that the lesion in Fom^R mutants was different from that seen in the Toy^r type of mutants.

Deficiency of AK in the extracts of Fom^R mutants. Earlier studies have shown that most of

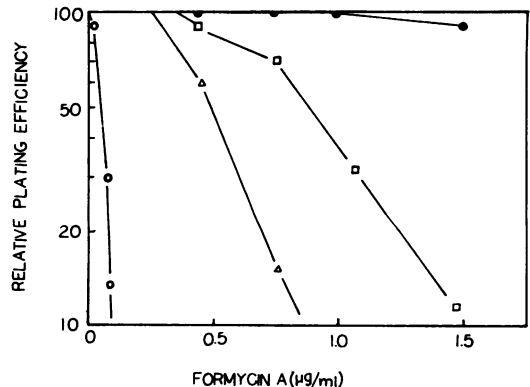


FIG. 3. Dose-response curves of different cell lines for formycin A in medium containing EHNA (10 µg/ml). Symbols: ○, WT; △, Fom^R2; □, Fom^R4; ●, Toy⁴.

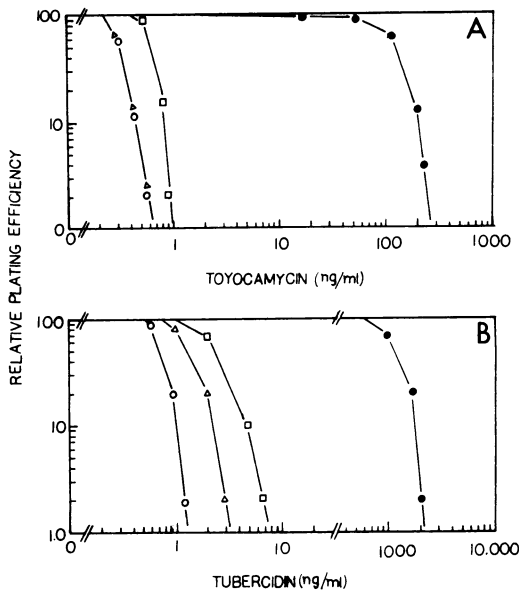


FIG. 4. Survival curves of parental and mutant cell lines for toyocamycin (A) and tubercidin (B). Symbols: O, WT; Δ, Fom^R2; □, Fom^R4; ●, Toy^r4.

the nucleoside analogs require initial phosphorylation before they become toxic to cells, and that the vast majority of mutants resistant to various nucleoside analogs are affected in the phosphorylating enzymes (2, 5, 8, 14, 18, 24). We therefore examined whether these mutants were affected in any of the purine nucleoside phosphorylating enzymes. Currently, no enzyme which specifically phosphorylates inosine or inosine analogs is known in mammalian cells. However, the major purine nucleoside phosphorylating activity that is present in CHO cells is of AK; therefore, initially the activity of AK in the parental and various mutant cell extracts was determined. The assay of AK activity in various cell extracts was carried out as described above, and the results of these studies are shown in Fig. 5. Extracts from both Fom^R mutants contained no measurable AK activity; in this regard, these mutants were very similar to the Toy^r mutants, which also lacked this enzyme (14).

In view of the sensitivity of the Fom^R mutants to toyocamycin and tubercidin, the absence of AK activity in cell extracts from such mutants was surprising. Since our routine procedure for preparation of cell extracts involved trypsinization and sonication of cells, the possibility that such treatments cause inactivation of the mutant enzyme was considered. To exclude this and related possibilities, cell extracts from various cell lines were prepared in a number of different

ways, including: (i) growth of cells in suspension culture followed by sonication, (ii) growth of cells in suspension culture and the preparation of cell extracts by swelling in hypertonic buffer followed by Dounce homogenization, and (iii) examination of AK activity in crude cell extracts before centrifugation and dialysis. Results of these studies showed that no AK activity could be detected in the extracts from Fom^R mutants under any of the conditions described above.

The possibility that the cell extracts from Fom^R mutants contain an inhibitor of AK activity was also investigated. This was done by mixing cell extracts from the WT and the mutant cells in different proportions and then measuring AK activity. Results of these studies showed that AK activity in such mixtures was exactly as much as may be expected from the relative proportion of the WT cell extract (data not shown). These studies exclude the presence of an inhibitor of AK activity in mutant cell extracts, as well as activation of the mutant cell enzyme by the WT cell extracts.

Dominant behavior of the Fom^R mutants in somatic cell hybrids. The cross-resistance pattern of the Fom^R mutants and their lack of AK activity in cell extracts indicated that these mutants were affected in AK in a manner different from the Toy^r mutants. It was, therefore, of interest to determine the behavior of Fom^R mutants in cell hybrids and to determine whether the genetic lesions in these mutants would complement the lesion in Toy^r mutants. This was carried out by constructing cell hybrids between the two Fom^R mutants and a Fom^S cell

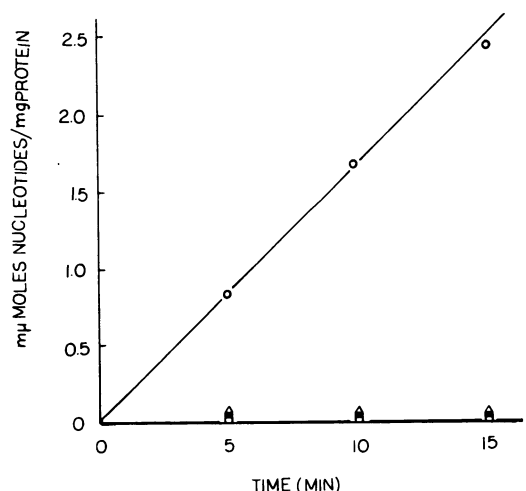


FIG. 5. AK activity in the extracts of WT and mutant cells. The assay procedure for the enzymes is described in the text. Symbols: O, WT; Δ, Fom^R2; □, Fom^R4; ●, Toy^r4.

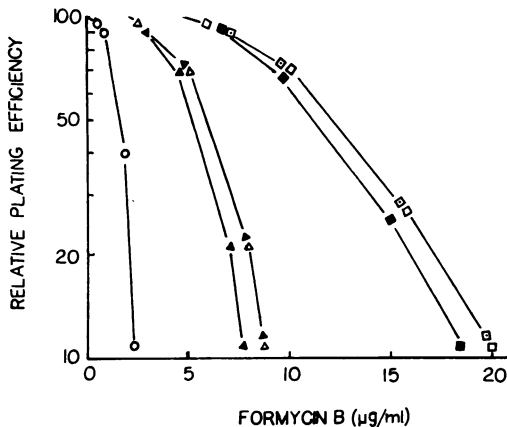


FIG. 6. Dose-response curves for formycin B for the parental and hybrid cell lines. Symbols: ○, EOT; △, Fom^{R2}; ▲ and ◻, EOT × Fom^{R2} clones; ◻, Fom^{R4}; ◻ and ■, EOT × Fom^{R4} hybrid clones.

line EOT, which contained a number of genetic markers that are very useful for the selection of hybrid clones (12). The cell lines were fused, using polyethylene glycol and dimethyl sulfoxide, by procedures described earlier (22). Subsequently, the selection for hybrid clones was carried out in HAT-plus-ouabain medium (containing 10 µg of hypoxanthine, 0.4 µg of amethopterin, and 5 µg of thymidine per ml, and 2 mM ouabain) in which none of the parental cell lines survive but the hybrids between the two cell lines are able to grow (12). As expected, the hybrid clones were obtained in these crosses at high frequency ($\approx 10^{-2}$), whereas in control crosses (e.g., EOT × EOT or Fom^{R4} × Fom^{R4}), the frequency of cells which were able to grow in the medium described above was found to be less than 1 in 10^5 .

Many of the hybrid clones from the crosses of Fom^{R2} and Fom^{R4} mutants with the EOT cell line were picked and grown in nonselective medium. The hybrid nature of these clones was ascertained by karyotype determination, and all of the clones examined were found to be pseudotetraploid, as may be expected for hybrids. Subsequently, the degree of resistance of these hybrid clones towards formycin A and formycin B was determined. The dose-response curves towards formycin B of two representative hybrid clones from these crosses are shown in Fig. 6. All of the other hybrid clones examined (at least six from each cross) showed very similar results. The hybrids formed between the two Fom^R mutants and the EOT cell line were nearly as resistant to formycin B as the resistant parent was (Fig. 6). The dose-response curves of these cell lines for formycin A (+EHNA) were also very similar (data not shown). These results

indicated that the Fom^R phenotype of these mutants expresses itself in a dominant/codominant manner.

In view of the behavior of Fom^R mutants in hybrids, it was of obvious interest to know whether the hybrids formed between the Fom^R × Fom^S cells contained any AK activity. For such purposes, cell extracts were prepared in parallel from the parental cell lines and a number of hybrid clones, and the AK activity in such extracts was measured. The results of such studies are shown in Fig. 7. The specific activity of AK in cell hybrids formed between Fom^R × Fom^S lines was about 50% of that observed for the WT cells (Fig. 7). These results indicate that the AK from the mutant cells was not being expressed in the hybrids, and they show that the AK deficiency of the Fom^R mutant was not transdominant (it is therefore referred to as codominant expression). These results also provide further evidence against the presence of an inhibitor of AK activity in the mutant cells.

We next examined whether the AK deficiency of the Fom^R mutants can be complemented upon hybridization with an AK⁻ Toy^f mutant. The cell hybrids between Fom^{R4} and a Toy^f mutant of EOT line (EOT Toy^{f2}) were constructed as before, and the AK activity in the extracts of such hybrids was measured. The results of these studies showed that none of the hybrids formed between Fom^{R4} and EOT Toy^{f2} contained any measurable AK activity (Fig. 7). The sensitivity of these hybrid cell lines towards formycin B and toyocamycin was also determined, and the representative results with two hybrid clones are shown in Fig. 8 and 9. The hybrids formed

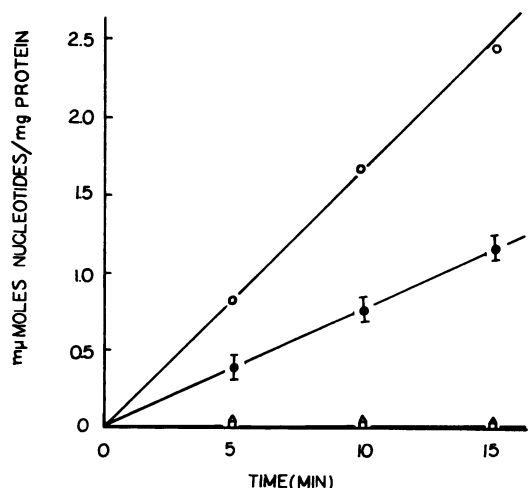


FIG. 7. AK activity in the extracts of parental and the Fom^R × Fom^S hybrid cell lines. Symbols: ○, EOT; ◻, Fom^{R4}; ●, EOT × Fom^{R4}; △, EOT Toy^{f2} × Fom^{R4}.

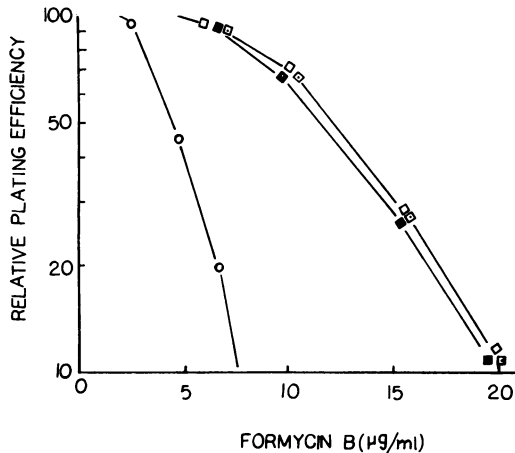


FIG. 8. Dose-response curves for formycin B for the parental Fom^R and Toy^f cell lines and for the hybrids formed between them. Symbols: \circ , EOT Toy^2 ; \square , Fom^{R4} ; \blacksquare and \square , EOT $Toy^2 \times Fom^{R4}$ hybrid clones.

between cell lines were nearly as resistant to formycin B as was the Fom^R parent, which again shows the codominant nature of the Fom^R phenotype. However, very interestingly, these hybrid cell lines were found to be as sensitive to toyocamycin as the WT cells, even though these hybrids showed no activity of AK in cell extracts.

Cellular uptake and incorporation into macromolecules of [3H]adenosine and [3H]tubercidin. Earlier studies on toyocamycin and tubercidin have shown that these analogs need to be phosphorylated by AK before they become toxic to cells (2, 14). Therefore, the sensitivity of the Fom^R mutants and the $Fom^R \times Toy^f$ hybrid cell lines to toyocamycin and tubercidin, in the absence of any measurable AK activity in the extracts, raised the question whether these cell lines contained AK activity *in vivo*, but activity which for some reason could not be measured in cell extracts. This possibility was tested by studying the cellular uptake and incorporation of [3H]adenosine and [3H]tubercidin in various cell lines. These studies were carried out as described above, and the results of these studies for [3H]adenosine are shown in Fig. 10. In the Toy^f mutant, cellular uptake and incorporation of [3H]adenosine were greatly reduced (Fig. 10). This particular mutant also showed greatly reduced uptake and incorporation of [3H]tubercidin ($\approx 5\%$) in comparison with the WT cells (data not shown). The reduced uptake of adenosine and tubercidin in the Toy^f line is consistent with the AK deficiency of this mutant and its increased resistance to various adenosine analogs. However, very interestingly, in contrast to

the Toy^f mutant, both Fom^{R2} and Fom^{R4} lines showed normal uptake and incorporation of [3H]adenosine (Fig. 10) and [3H]tubercidin (data not shown). In a separate experiment, 30 min after the addition of [3H]adenosine to various cell lines, the acid-soluble radioactivity was extracted and analyzed by paper chromatography. Results of these studies (not shown here) showed that approximately 80% of the radioactivity from both WT and the Fom^R mutant cells was present in the form of phosphorylated derivatives. These results provide evidence that both of the Fom^R mutants contain nearly normal levels of AK activity *in vivo*.

DISCUSSION

Results presented in this paper show that mutants of CHO cells selected for resistance to the nucleoside analogs formycin A ($-EHNA$) or formycin B exhibit a novel genetic and biochemical phenotype. Although the biochemical phenotype of the Fom^R mutants (i.e., lack of any measurable AK activity in cell extracts) is similar to that of the toyocamycin or tubercidin resistant (Toy^f) mutants described earlier (2, 14, 24), several different lines of evidence show that these two types of mutants involve different kinds of genetic lesions. First, in contrast to the Toy^f mutants which are highly resistant to various adenosine analogs, including toyocamycin and tubercidin, the Fom^R mutants exhibit very slight, if any, cross-resistance to such analogs. At the same time, the Fom^R mutants are much more resistant to formycin A ($-EHNA$) or formycin B in comparison with the Toy^f mutants. Second, the drug-resistant phenotype of the Fom^R mutants is expressed in a codominant manner in cell hybrids constructed between $Fom^R \times Fom^S$ or $Fom^R \times Toy^f$ cell lines. The drug-resistant phenotype of Toy^f mutants, on the other hand, behaves recessively under these

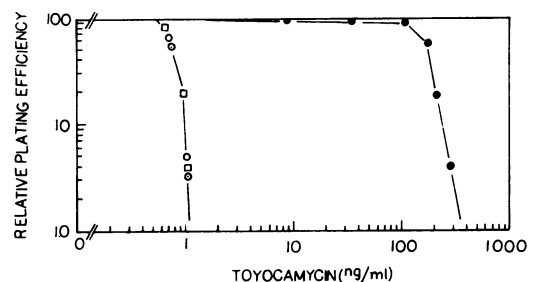


FIG. 9. Dose-response curves for toyocamycin for the parental Fom^R and Toy^f cell lines and for the hybrids formed between them. Symbols: \circ , Fom^{R4} ; \square and \odot , EOT $Toy^2 \times Fom^{R4}$ hybrid clones; \bullet , EOT Toy^2 .

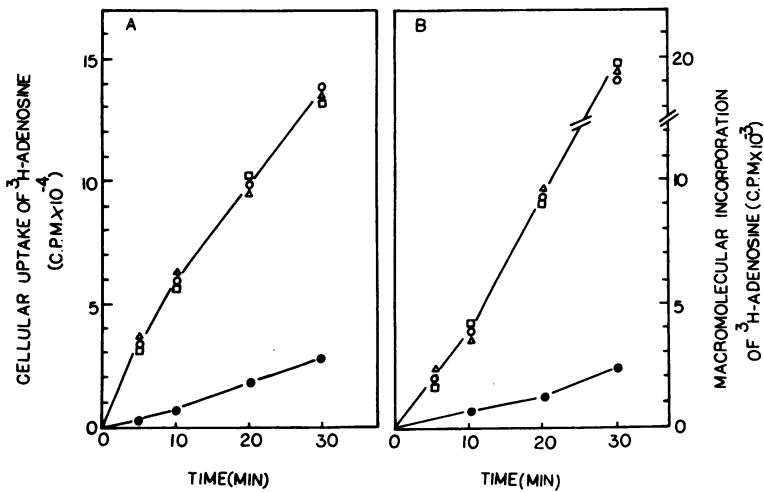


FIG. 10. Cellular uptake and incorporation into macromolecules of [³H]adenosine by various cell lines. The experiment was carried out as described in the text. (A) Total cellular uptake of [³H]adenosine. (B) Incorporation of [³H]adenosine into trichloroacetic acid-insoluble material. Symbols: ○, WT; △, Fom^R2; □, Fom^R4; ●, Toy^f4.

conditions (14, 24). Third, whereas the Toy^f mutants show a severe deficiency in the cellular uptake, phosphorylation, and incorporation of adenosine and tubercidin, the Fom^R mutants possess nearly normal capacity for the phosphorylation and incorporation of these compounds. Finally, in contrast to the Toy^f mutants which are obtained spontaneously in CHO cells at very high frequency ($\approx 10^{-3}$ [14, 24]), the spontaneous frequency of Fom^R mutants was less than 10^{-6} , and the two Fom^R mutants which have been investigated here were obtained from ethyl methanesulfonate-treated cultures at a frequency of about 10^{-5} . The various genetic and biochemical differences in the properties of Toy^f and Fom^R mutants also exclude the possibility that the Fom^R mutants arise from a second mutation in a Toy^f mutant. In fact, our observation that two independent Fom^R mutants which exhibit different degrees of resistance to formycin B behave similarly provides strong evidence that the phenotype described above results from a single genetic lesion.

The two Fom^R mutants which have been studied have both been found to contain no measurable activity of the enzyme AK in cell extracts. Since formycin B, which is an inosine analog, is not a substrate for AK either in vivo or in vitro (i.e., it has no effect on [³H]adenosine phosphorylation even at 50 μ g/ml [unpublished data]), the deficiency of AK in Fom^R mutants is quite surprising. However, results presented here indicate that the AK deficiency of these mutants is not directly responsible for their drug-resistant phenotype, and that the mutant cells may in fact contain normal amounts of AK

activity in vivo. Some of the observations which support this view are: (i) the Fom^R mutants are as proficient as WT cells in the cellular uptake, phosphorylation, and incorporation into macromolecules of [³H]adenosine and [³H]tubercidin; (ii) the Fom^R × Fom^S hybrid cells which contain about 50% AK activity are as resistant to formycin B as the parental mutant lines are; (iii) the Fom^R mutants show normal sensitivity to various toxic adenosine analogs (e.g., toyocamycin and tubercidin) to which other AK⁻ mutants reported earlier, exhibit a high degree of cross-resistance (14). These results taken together provide strong evidence that the Fom^R mutants contain AK activity in vivo but that this activity is somehow not observed in cell extracts. The possibility that cell extracts from the Fom^R mutants contain an inhibitor of AK activity has also been excluded by the experiments described here.

The enzyme AK, which is seemingly affected in Fom^R mutants, is a purine salvage pathway enzyme which is not required for cell growth under normal conditions. It is expected that cellular mutants lacking this enzyme should behave recessively in cell hybrids, as has been observed for the Toy^f mutants (14, 24). Since formycin B, even at very high concentrations (e.g., 50 μ g/ml), has no effect on the activity of AK either in whole cells or in cell extracts (unpublished data), a mutation in AK alone could not readily account for the drug-resistant phenotype of the Fom^R mutants. A mutation affecting only AK could not also explain the codominant behavior of the Fom^R mutants, especially when the AK⁻ phenotype is not trans-

dominant in hybrid cells. The codominant behavior of the lesion in the Fom^R mutants, in fact, suggests that the genetic lesions in these mutants most likely affect an essential cellular reaction which in sensitive cells is inhibited by formycin B or its derivative(s) and to which the mutant cells have become resistant. All of these observations together strongly suggest that the deficiency of AK in the mutant cell extracts is caused indirectly by the lesions.

At present, our knowledge regarding the mechanism of cellular toxicity of formycin A/formycin B is very limited, and this has presented a problem in understanding the nature of genetic lesion in the Fom^R mutants. However, cross-resistance studies with Fom^R and Toy^f have provided some insight in this regard. Our observation that Toy^f mutants are highly resistant to formycin A in the presence of the adenosine deaminase inhibitor EHNA (where it is not converted to formycin B) provide evidence that formycin A, like toyocamycin and tubercidin, is phosphorylated by AK before it becomes toxic to cells. At the same time, our observation that Toy^f mutants do not show appreciable cross-resistance to formycin B indicates that AK is most likely not carrying out the phosphorylation of formycin B. It has recently been reported that in several species of *Leishmania* (e.g., *L. donovani*, *L. tropica*, *L. mexicana*, etc.), formycin B is initially phosphorylated to its ribonucleoside-5'-monophosphate, which is subsequently converted into other metabolites (3, 21, 25). However, unlike other nucleosides and analogs, this phosphorylation reaction in *Leishmania* is reportedly carried out by a nucleoside phosphotransferase activity (3, 25). Although phosphorylation of formycin B in mammalian cells has not yet been reported, in view of the above-mentioned observation it is very likely that formycin B is initially phosphorylated before it becomes toxic to the cells.

The observation that we think is most important in understanding the nature of lesion in these mutants is that the Fom^R mutants show resistance to both formycin A (in the presence of EHNA where it is not deaminated to formycin B) and formycin B, even though these may be phosphorylated by different enzymes. This result suggests that both of these analogs, after initial phosphorylation, inhibit a common cellular reaction which in the mutant cells becomes resistant to the action of these drugs. At present, the cellular function other than AK which is affected in the Fom^R mutants remains to be identified. Since formycin B is known to be an inhibitor of the enzyme purine nucleoside phosphorylase (26, 28), the activity of this enzyme in the mutant cells has also been examined. Results of our studies indicate that both of the Fom^R

mutants contain normal levels of this enzyme, which shows sensitivity to formycin B similar to that of the enzyme from WT cells (unpublished data). More recently, studies in *Leishmania* have shown that formycin B-5'-monophosphate is an inhibitor of the enzyme adenylosuccinate synthetase (ASS [3]). Since ASS is a key enzyme of purine nucleotide biosynthesis, which should carry out the next reaction after presumed phosphorylation of formycin B, this is a potential target for mutational alteration in Fom^R mutants. Furthermore, since many of the enzymes of purine nucleotide biosynthesis exist as some sort of complex, as indicated by their copurification (19), a mutation in ASS could influence the activity of other enzymes in the pathway. It is of interest in this regard that some of the mutants of mouse lymphoma cells which are affected in ASS show increased resistance to adenosine, 6-thioguanine, and 6-mercaptopurine and contain approximately 50 to 60% higher levels of another purine salvage pathway enzyme, hypoxanthine guanine phosphoribosyl transferase (30). Although at present it is not known whether AK exists or functions in cells in association with ASS (or other purine nucleotide biosynthetic enzymes), in view of the above-mentioned observations and the properties of the Fom^R mutants, such a possibility is quite likely. Further studies on the identification of other cellular component(s) which are affected in the Fom^R mutants and how they interact with AK should provide valuable insight into the mechanism of action of formycin B and regarding interaction among various purine nucleotide biosynthetic enzymes.

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