Chromosomal Constitution and Amethopterin Resistance in Cultured Mouse Cells

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SUMMARY

The chromosomal constitutions of four amethopterin-resistant sublines of cultured Sarcoma 180 cells were compared with those of two sensitive sublines. No correlation between amethopterin resistance and chromosomes was observed. The chromosomal character of a resistant subline was completely retained in the sensitive subline derived from it after 9 months of continuous cultivation in the absence of the drug. The folic acid reductase content increased with increasing resistance and was 300–400 times higher in the 3000-fold resistant cell line than in the sensitive parent cells. Extensive selection is shown to be involved in the development of the amethopterin-resistant cell lines. This selection is most probably based on the capacity of a cell to rapidly increase the content of folic acid reductase.

Previous investigations on the correlation between amethopterin resistance and possible changes in chromosomal constitution in mammalian cells have yielded varied results. Hauschka (12) studied two cases of amethopterin resistance—namely, mouse ascites plasma cell tumor 70429 and lymphatic leukemia P-288. In the latter case, a decrease in the number of minute chromosomes was observed in the resistant cells. Cailleau (2) did not detect any chromosomal changes in amethopterin-resistant L-4946 cells of mice. Biesele et al. (1), on the other hand, found a clear correlation in L1210 mouse leukemias. In this case resistance was associated with the absence of one large submetacentric chromosome. Harris and Ruddle (11) developed an amethopterin-resistant line of pig kidney cells in culture and observed a decrease in the number of chromosomes. Similarly, a change in the karyotype was observed in three lines of aminopterin-resistant HeLa cells (23). In none of these studies were any biochemical changes considered, and the degree of resistance was known only for pig kidney (fivefold) and HeLa cells (up to sevenfold).

The present study concerns several lines of cultured mouse Sarcoma 180 cells which have been made from three- to 3000-fold resistant to amethopterin. The chromosomal constitution of the resistant lines is compared with that of two sensitive lines. One of these is the original parent S-180, and the other one was derived from a resistant subline by growing it in the absence of the drug until it became sensitive again. This required a period of 9 months. One biochemical change which has been shown by Hakala et al. (10) to accompany amethopterin resistance consists of an increase in the folic acid reductase in the cell. Thus, the present study elucidates not only the relationship of chromosomes and amethopterin resistance but also the relationship of chromosomes and the amount of folic acid reductase in mammalian cells.

Although the development of amethopterin-resistant cell lines was accompanied by chromosomal changes, these changes persisted even when the resistance was lost. Thus, these chromosomal changes proved not to be associated with amethopterin resistance or with the ability of the cells to synthesize excessive amounts of folic acid reductase.

EXPERIMENTAL PROCEDURE

Origin of cells.—Sarcoma 180 cells, isolated by Foley and Drolet (8), were purchased from Microbiological Associates, Inc., Bethesda, Maryland. Development of amethopterin-resistant cell lines.

* This investigation was supported in part by research grants (CY-4175 and CS-9749) from the National Cancer Institute of the United States Public Health Service.

Received for publication April 9, 1962.
—The 67- and 174-fold resistant lines of S-180 cells were developed in T-60 culture flasks over a long period of time (several months) by gradually increasing the concentration of amethopterin from 0.02 to 0.5 μM (10). The media used were modifications of Eagle’s medium as described in Table 1. The 3000-fold resistant cell line was similarly developed from the one which was 174-fold resistant by gradually increasing the concentration of amethopterin from 0.5 to 50 μM. The threefold resistant cell line resulted when the 67-fold resistant one was grown in the absence of the drug for 6 months.

Maintenance of cultures.—The various cell lines were maintained in modified Eagle’s media (Table 1) supplemented with 10 per cent of horse serum. Each cell line was also preserved in a frozen cell bank (13).

Preparation of cell extract.—To determine the free and amethopterin-bound folic acid reductase, cell extracts were prepared as follows. Cells were grown in Roux bottles in their respective maintenance medium (Table 1). They were removed from the glass with a rubber scraper and were suspended into amethopterin-free maintenance media in homogenizer tubes. The suspension was centrifuged for 5 min. at 500 r.p.m. The supernatant medium was discarded, and the cells were washed once with a twofold volume of cold solution of inorganic salts (6) and centrifuged for 10 min. at 2000 r.p.m. The weight of these “packed cells” was recorded, and cold saline was added for homogenization. The homogenate was rinsed quantitatively with saline into a centrifuge tube, and the cell debris was removed by centrifugation for 20 min. at 18,000 r.p.m. in an International refrigerated centrifuge. One ml. of the cell extract corresponded to 100–500 mg. of cells.

Determination of free folic acid reductase.—Free folic acid reductase was determined by titrating the enzyme in the cell extract with amethopterin, as described previously (10). Time of incubation was 15 min. in studies of the resistant cell lines and 30 min. in studies of the sensitive lines.

Determination of amethopterin-bound folic acid reductase.†—The estimation of the amethopterin-bound folic acid reductase was performed by determining the amount of the heat-liberated amethopterin in the cell extract. To release the bound amethopterin, the cell extract described above was placed in a boiling water bath for 10 min. This treatment denatures all of the protein, and the drug is released. To determine the content of amethopterin in the extract, folic acid reductase prepared from the 3000-fold resistant cell line was titrated with this heated extract. Simultaneously, a titration was also performed with a solution of amethopterin of known concentration (standard). The time of incubation was 15 min.

Study of chromosomes.—The cells from one T-60 flask (10–20 million cells) were transferred into a test tube and suspended into 10 cc. of the corresponding maintenance medium. One ml. of 0.05 per cent colchicine in isotonic saline was added to the suspension, which then was incubated for 18 hr. at 37°C. After scraping the attached cells from the walls of the tube with a rubber scraper the suspension was transferred into a centrifuge tube and centrifuged for 5 min. at 700 r.p.m. The supernatant was carefully decanted, and the cells were suspended in 5 ml. of 1.12 per cent sodium citrate solution. After this suspension had stood, with occasional stirring, for 30 min. at room temperature, the centrifugation was repeated and the supernatant was carefully decanted. To this sediment freshly prepared acetic acid-alcohol (glacial acetic acid: absolute ethyl alcohol = 1:3) was added as a fixative. After this mixture had stood for 30 min. the fixative was decanted, and 45 per cent acetic orcein stain as described by Sandberg et al. (21). Only intact metaphases were examined, and exact chromosome counts were made on all cells in which

1) The method for the determination of free and bound amethopterin in cell extracts was developed by Dr. Sigmund F. Zakrzewski. All the amethopterin which is found in bound folic acid reductase (M. T. Hakala and S. F. Zakrzewski, unpublished data.)
the chromosomes were well spread without much overlap. A detailed analysis was made placing emphasis on marker chromosomes (metacentric, submetacentric, and minute chromosomes) which usually are not found in the normal karyotype of the mouse (12).

RESULTS

Genetic aspects of the amethopterin-resistant lines.

—Earlier studies left no doubt that a selection was involved in the development of the amethopterin-resistant cell lines (10). Evidence for a gradual process was found in the fact that the resistance disappeared gradually in the absence of the drug, and no definite “steps” could be distinguished. In about 1 month (twelve to eighteen cell generations), the degree of resistance and also the folinic acid reducítase content decreased to one-half of the original.

An attempt was made, therefore, to determine the degree of selection involved in the development of these highly resistant S-180 cell lines. This was done by estimating through trials the minimum number of parent cells which was necessary to permit the eventual outgrowth of resistant cells in each final maintenance medium. Depending on the size of the starting population, either T-30 or T-60 culture flasks were used, and the medium was changed 3 times weekly. The maximum number of cells that could be used was 20 million in a T-60 flask. Even though this study does not allow an exact estimation of “mutation” frequency, it indicates that the amethopterin-resistant cells which are able to grow at these high concentrations of amethopterin are so rare that conventional methods (3, 16, 22) could not be used. Even in a population of 20 million S-180 cells, there did not exist enough cells to initiate growth in AH/67 medium (Table 2). All these cells were dead in 10–11 days in several experiments consisting of two T-60 cultures each. In AT/174 medium, on the other hand, from an inoculum of 20 million S-180 cells, a few clones were growing after a lag period of about 6 weeks. Further increase in resistance from AT/174 to AT/3000 proved to be relatively easy. Only 10⁶ cells and a rather short lag period of 3½ weeks were necessary.

Biochemical characteristics of the cell lines.—Previous work showed that the increase in amethopterin resistance in sublines AH/67 and AT/174 was accompanied by an increase in folinic acid reductase (10). This enzyme serves as a specific physiological inactivator of the drug by binding it very tightly (24). Enough of the enzyme remains free, however, to carry on its normal function—i.e., as a catalyst for the formation of tetrahydrofolinic acid. The characteristics of the different resistant cell lines are listed in Table 3. As can be seen, our present selection of resistant cell lines covers a wide range, from threefold resistant AH/3 up to 3000-fold resistant AT/3000. Even though the content of folinic acid reductase rises with increasing resistance, these two parameters become less proportional when the level of resistance increases.

The rate of influx of amethopterin into these cells was found to be directly proportional to the concentration of amethopterin in the medium (9). The rate constant of influx for AH/67, AT/174, and AT/3000 is the same, 0.46/hour/kg of packed cells. An increase in folinic acid reductase above the initial level occurs both in sensitive as well as in resistant cell lines exposed to amethopterin (9).

### Table 2

**One-Step Selection of Amethopterin-Resistant Cell Lines**

<table>
<thead>
<tr>
<th>Starting Cell Line</th>
<th>Selective Medium</th>
<th>Culture Conditions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type†</td>
<td>Amethopterin (μM)</td>
<td>Inoculum</td>
</tr>
<tr>
<td>S-180</td>
<td>AH</td>
<td>0.5</td>
<td>2×10⁷</td>
</tr>
<tr>
<td>S-180</td>
<td>AT</td>
<td>0.5</td>
<td>2×10⁷</td>
</tr>
<tr>
<td>AT/174</td>
<td>AT</td>
<td>50</td>
<td>10⁸</td>
</tr>
</tbody>
</table>

* See Table 1 for details.

### Table 3

**Characteristics of the Various Sarcoma 180 Cell Lines**

<table>
<thead>
<tr>
<th>Sarcoma 180 Cell Line</th>
<th>50% Inhibitory Amethopterin (μM)</th>
<th>Folic Acid Reductase† (μmoles/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td>0.054 ± 0.0016</td>
<td>0.38 ± 0.065</td>
</tr>
<tr>
<td>AH/3</td>
<td>0.076 ± 0.0122</td>
<td>0.6</td>
</tr>
<tr>
<td>AH/67</td>
<td>3.6 ± 0.41</td>
<td>17.3 ± 0.85</td>
</tr>
<tr>
<td>AT/174</td>
<td>9.4 ± 1.1</td>
<td>25.8 ± 0.90</td>
</tr>
<tr>
<td>AT/3000</td>
<td>160</td>
<td>121 ± 11.5</td>
</tr>
</tbody>
</table>

† Concentration of amethopterin required for 50 percent inhibition of growth in 7 days in folinic acid medium.

* Expressed as molar equivalents of amethopterin/kg of packed cells. This was determined as the sum of free and amethopterin-bound enzyme which were found in the cells grown in maintenance medium.
This increase is rapid in the first few hours and then levels off. The rate of this increase is dependent on the initial enzyme content, and, therefore, the actual amount of the induced enzyme is small in the sensitive cells. It is to be noted that the 50 per cent inhibitory concentration of amethopterin listed in Table 3 for each of the cell lines reflects the increases in folate acid reductase which might have occurred in the test condition used.

Marker chromosomes.—The chromosomes of the normal mouse cell are telocentric (12). Thus, all the chromosomes which are not of this type are considered markers. These include metacentric, submetacentric, and minute chromosomes.

Five types of marker chromosomes were found in the sensitive parent S-180 cells, as well as in the resistant AT cell lines (Table 4 and Figs. 1–7). These are designated as A, B, C, D, and m (for minute). In all AH lines, whether resistant or sensitive, only three types are found—namely, B, C, and m. All the other chromosomes in these cells were telocentric and could not be differentiated from those found in a normal mouse cell.

The marker chromosomes A and D were similar in sensitive S-180 and in both resistant AT lines but were missing in all the AH lines (sensitive and resistant). In all the three main lines of cells (S-180, AH, and AT) the marker chromosome B seemed to be different (Table 4). Whether the differences are significant cannot be stated with certainty, since standard errors were not determined. Marker chromosomes C were similar in AH and AT lines whether sensitive or resistant but seemed to differ from C present in the sensitive S-180 cells (Table 4). The minute chromosomes were similar in all cell lines. The only difference was that AH hypotetraploid mode of 75 chromosomes per cell, and part of the distribution curves overlaps that of the sensitive parent S-180.

All the AH cell lines (sensitive and resistant)
had a hypertriploid mode of 66 chromosomes (Chart 1). Table 5 shows that the homogeneity of AH/67 cell line has been perfectly retained in AH/s during 9 months of growth in the absence of amethopterin.

**DISCUSSION**

Mammalian cells resistant to purine and pyrimidine analogs contain lowered amounts of the enzymes necessary for the formation of the corresponding nucleotides (17, 20). Amethopterin resistance, on the other hand, is associated with an increase in an enzyme, folic acid reductase (7, 10). The inconsistent relationship between chromosomal constitution and resistance to drugs in general was clearly demonstrated by Hauschka (12).

In several instances (15) the enzyme content per cell has been shown to be proportional to chromosomal ploidy. Usually in these cases the content of the enzyme in the cell protein is unchanged, because the individual cells have increased correspondingly in size (12). However, such correlation does not always apply. In a tetraploid lymphoma cell, the content of glucose-6-phosphate dehydrogenase was 5–10 times higher than in a diploid cell (15). In our amethopterin-resistant S-180 cells, the increase in folic acid reductase has been measured per total cell mass and increased 300–400-fold over that found in the parent S-180 cells. However, the number of chromosomes is independent of these changes.

From clinical experience, it is well known that leukemias in some individuals are inherently resistant to treatment with amethopterin (14). If such resistance were specifically associated with a somatic "mutation" of the leukemic cells, recognizable on the chromosomal level, resistance could be predicted before any treatment is started. The inconsistency of correlation between amethopterin resistance and chromosomal constitution has puzzled several investigators (1, 2, 11, 12). Their studies were made mostly on in vivo material from mice and on pig kidney cells grown in vitro. Either no grossly recognizable change or a decrease in the number of chromosomes, including markers, has been associated with resistance.

In our hands the initial development of the amethopterin-resistant S-180 cell lines was accompanied by radical changes in chromosome number as well as in marker chromosomes. However, the sensitive line AH/s, which originated from the resistant AH/67, retained the chromosomal constitution of its resistant parent, in every respect, instead of reverting to the constitution of the original sensitive S-180. In this case, therefore, no correlation exists between chromosomal constitution and amethopterin resistance.

Although the chromosomal constitution of AH cell line stays unchanged during continuous cultivation in the absence of the drug, the level of resistance gradually decreases and is completely lost in 9 months. Thus, if the change in sensitivity to amethopterin can be considered a "mutation," it is not recognizable on the chromosomal level but could possibly involve genes. Induction of folic acid reductase by amethopterin in these cells has also been observed (9). The other induced enzymes which have been demonstrated in cell culture disappear rapidly in the absence of the inducer (4, 5). The slow disappearance of folic acid reductase (and resistance) can be explained by the retention of amethopterin in the cells, since this drug is firmly bound by the enzyme (24). The only case of enzyme induction which resembles the present one is that of penicillinase in bacteria (18, 19). In that...
case, the slow disappearance of the induced enzyme in drug-free medium was also attributed to tight binding of the drug by the cells. The growth studies and the chromosomal data support the fact that extensive selection has been involved in the development of the 67-, 174-, and 5000-fold resistant cell lines from the parent S-180 cells. These findings and the inducibility of folic acid reductase suggest that the selection is most probably based on the capacity of a cell to rapidly induce this enzyme. Such capacity for folic acid reductase induction, however, is not specifically reflected in the chromosomal constitution of the cells.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Mrs. Lois Crosswhite, Miss Luella Puccetti, and Miss Edythe Taylor.

REFERENCES

Figs. 2-7.—Metaphases of the sensitive and resistant sublines of cultured Sarcoma 180 cells. Figs. 2 and 5, sensitive parent and AH/s; Figs. 3 and 4, resistant AH/67 and AH/3; Figs. 6 and 7, resistant AT/174 and AT/8000.
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