# CHANGES IN SENSITIVITY OF TUMOR CELLS GROWN IN THE PRESENCE OF FOLIC ACID TO METHOTREXATE

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UDC 615.356:[577.164.17.042.2+577.164.17].015.44:616-006

Cultivation of tumor cells (L cells) in the presence of folic acid in gradually increasing concentration led to increased resistance of the population of these cells to methotrexate. During subsequent cultivation when the folic acid concentration was not increased, the population of these cells became more sensitive to methotrexate, even than the original L cells.

KEY WORDS: L cells; folic acid; methotrexate.

Habituation of tumor cells to chemical agents is a very important problem. First, habituation to a chemotherapeutic drug during treatment deprives it of its efficacy. Second, by habituation and the creation of increased resistance to one drug, increased sensitivity to another drug can be obtained.

The writers attempted with the aid of habituation of tumor cells to a certain metabolite to modify their sensitivity to the antimetabolite: The dynamics of changes in sensitivity of tumor cells to methotrexate was studied initially during cultivation in the presence of increasing concentrations of folic acid, and later during deprivation of these cells with respect to folic acid

## EXPERIMENTAL METHOD

The L cells were cultivated in 10-ml flasks in medium No. 199 with 10% bovine serum, previously inactivated for 30 min at 56°C on a water bath. The cells were seeded in a dose of $5 \cdot 10^5$ per flask. A 0.25% solution of trypsin was used to transfer the cells. Folic acid in various concentrations (from 0.01 to 0.5 mg/ml) was added during subculture. Subculture was carried out on the third to fifth day, when a monolayer was present in the flask.

The folic acid was made up as follows: The appropriate quantity of folic acid was dissolved in 10 ml of 2.1% sodium bicarbonate and 90 ml of medium No. 199. The solution was neutralized with acetic acid and the pH adjusted to 7.2.

Methotrexate (Lederle, USA) was dissolved in 5 ml of sterile distilled water. The solution was kept at 4°C for not more than 2 weeks.

Between 24 and 18 h before the experiment the cells were seeded into test tubes at the rate of $1 \cdot 10^5$ cells in medium No. 199 with 10% inactivated bovine serum. The tubes were incubated slantwise at 37°C, and before the experiment they were examined under the microscope and tubes containing uniform growth of cells on the glass were selected. Before the experiment the culture medium was poured off and the tubes were washed with medium No. 199; medium No. 199 was then added to the control tubes and medium No. 199 and methotrexate to the experimental tubes. After incubation for 48 h in the presence of medium No. 199 and methotrexate the number of living cells was counted. Living cells were identified by Schrek's method

Laboratory of Virology, Institute of Experimental and Clinical Oncology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR, L. M. Shabad.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 81, No. 4, pp. 470-472, April, 1976. Original article submitted July 22, 1975.

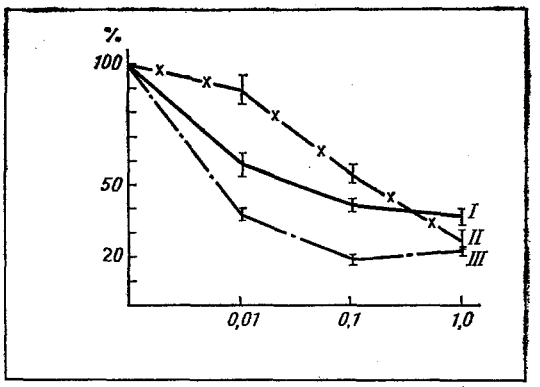


Fig. 1. Comparative sensitivity of L, L(f), and L(f)-A cells to different doses of methotrexate: I) L cells; II) L(f) cells; III) L(f)-A cells. Abscissa, logarithm of dose of methotrexate; ordinate, number of surviving cells (in %).

TABLE 1. Cultivation of Cells in Increasing Concentrations of Folic Acid

| No. of subculture | Concentration of folic acid (in mg/ml) |
|---|---|
| 1—4 | 0,01 |
| 5—7 | 0,03 |
| 8—10 | 0,04—0,06 |
| 11—12 | 0,08 |
| 13—15 | 0,1 |
| 16—26 | 0,15 |
| 27 | 0,16 |
| 28—53 | 0,2 |
| 54 and later | 0,5 |

[3] in the modification described in [1]. For this purpose 0.1 ml of the cell suspension was mixed with 1.9 ml of a mixture of 0.1% eosin and 0.1% trypan blue in equal volumes. The mixture of stains was made up immediately before use. Unstained cells were counted in a Goryaev's chamber. All tests were carried out with cultures grown in tubes. Each experiment included not less than three groups of tubes, of which one was a control and not less than two other groups contained different concentrations of methotrexate, starting from 0.001 up to 10 μg/ml. Each group consisted of at least six tubes. The L cells cultivated in the presence of increased concentrations of folic acid were conventionally designated L(f).

For each dose of methotrexate the relative number of cells surviving in the experimental tubes compared with the number in the control was determined. On the basis of these results a graph was plotted to show the relative number of surviving cells as a function of logarithm of dose for both L and L(f) cells. A typical experimental curve is shown in Fig. 1.

The inhibitory action of methotrexate is conveniently described by introducing the concept of the dose required to inhibit growth of 50% of cells ($ID_{50}$). The value of $ID_{50}$ was obtained from the graph of relative number of surviving cells versus logarithm of dose. The degree of resistance to methotrexate was expressed as the ratio of $ID_{50}$ for L(f) cells to $ID_{50}$ of the original L cells.

## EXPERIMENTAL RESULTS

The increase in concentration of folic acid in the culture medium during subculture of the L-cells ($5 \cdot 10^5$) is shown in Table 1. Starting from the first subculture, after every one of a number of subcultures of cells from the culture was grown in the presence of an increased concentration of folic acid, seedlings were made without folic acid and their sensitivity to methotrexate was determined parallel with that of the original cells. Tests showed that this sensitivity in L(f) cells with a dose of methotrexate of 0.1 μg/ml was reduced by 20-50% compared with that of L cells grown in medium No. 199. From the 1st to the 62nd subcultures this difference continued. It was less marked with other doses methotrexate. It is important to note that this increase in resistance occurred after the very first subculture, and that later cultivation of the cells in the presence of increasing concentrations of folic acid did not increase their sensitivity to methotrexate.

Changes in the sensitivity to methotrexate of L cells grown in the presence of folic acid are shown in Table 2. It will be clear from Table 2 that the degree of resistance of the L(f) cells to methotrexate (the ratio of $ID_{50}$ of the L(f) cells to $ID_{50}$ of the L cells) was on average 3.6 times higher than that of the L cells.

With sufficiently large doses of methotrexate (1 and 10 μg/ml), when the number of surviving cells was very small, the L(f) cells were more sensitive than the L cells.

TABLE 2. Sensitivity to Methotrexate of Cells Cultivated in the Presence of Folic Acid

| No. of expt. | No. of subculture | Dose of methotrexate (in μg/ml) to inhibit growth of 50% of cells ($ID_{50}$) | | |
|---|---|---|---|---|
| | | L(f) | L | $\frac{ID_{50} \text{ of L(f) cells}}{ID_{50} \text{ of L cells}}$ |
| 83 | 1 | 0,018 | 0,008 | 2,2 |
| 28 | 6 | 0,045 | 0,020 | 1,7 |
| 31 | 13 | 0,063 | 0,008 | 8 |
| 39 | 24 | 0,03 | 0,01 | 3 |
| 40 | 25 | 0,023 | 0,007 | 3,3 |
| 48 | 49 | 0,03 | 0,009 | 3,3 |
| 51 | 55 | 0,032 | 0,009 | 3,5 |
| | | | | 3,6 |

Starting from the 63rd subculture the L(f) cells were divided into two cell lines. One was grown as before in the presence of an increased concentration of folic acid the other was grown on the ordinary culture medium. This latter lines of cells preserved its increased resistance to methotrexate for nine subcultures, after which it changed sharply. The population of these cells, described as L(f) A, became appreciably more sensitive to methotrexate not only than the L(f) cells, but also than the original L cells (Fig. 1).

The increase in the resistance to methotrexate of L cells grown in medium with an excess of folic acid can be explained variously and, in particular, by selection of L(f) cells with increased folate reductase activity. The increase in the sensitivity to methotrexate of the population of L(f) cells after their deprivation of folic acid is in harmony with the original hypothesis, but its mechanism is uncertain.

It is interesting to note that the removal of folic acid from the culture medium only gradually (after nine subcultures) led to an increase in the sensitivity of the cells to methotrexate.

Further investigations with analysis of clones cultivated from individual L cells may perhaps shed further light on these processes.

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