THE USE OF AUTORADIOGRAPHY TO STUDY THE ACTION OF THIO-TEPA ON CULTURES OF HUMAN TUMOR CELLS

E. A. Timofeevskaya and E. N. Tolkacheva

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The sensitivity of cultures of human tumor cells (carcinoma of the ovary CaOv, carcinoma of the stomach CaVe, mesenchymoma Sa19) to the action of thio-TEPA was studied with the use of various criteria to assess the action of the compound. Autoradiography proved to be more sensitive than other methods (counting the number of surviving cells and the mitotic index). Determination of the intensity of DNA synthesis (with the use of thymidine-H^3) demonstrated differences in the sensitivity of the cell lines to the action of the compound in small doses. Calculation of the index of labeled cells showed a significant difference between the responses of sensitive (CaOv) and resistant (Sa19) lines.

Tumors of the same histological structure and in the same localization may differ in their sensitivity to the action of the same compound. Existing morphological criteria for assessing the action of compounds in tissue culture are not sufficiently sensitive: agreement between positive results and a clinical effect does not exceed 50% [5]. Wright and co-workers [6] showed that positive correlation between results in vitro and the results of clinical treatment occurred in 35.6% of the cases when the compounds were used in high doses causing death of all the cells in the culture, in 48% of cases when the compound had a cytotoxic effect, and in 64.2% of cases if low doses were used, causing only slight changes in the cells.

Agreement between the experimental data and the results of clinical treatment is thus increased if the doses of the compounds used in vitro are reduced. It is therefore important to be able to determine the minimal dose for cultures which would allow differences in the sensitivity of cells of different tumors to the action of the same compound to be determined in agreement with their sensitivity in vivo.

The object of the present investigation was to determine the sensitivity of cells of different tumors to thio-TEPA using different criteria to assess the action of the compound.

EXPERIMENTAL METHOD

Cell lines obtained from human tumors were used: carcinoma of the ovary (CaOv), carcinoma of the stomach (CaVe), and mesenchymoma (Sa19). The cells were grown under standard conditions. After 48-72 h (logarithmic phase of growth) the medium was changed and thio-TEPA was added in different concentrations (from 3200 to 6.25 μg/ml) each one twice the next. Contact with the compound lasted 24 h. The cells were counted in a Goryaev's chamber after the addition of 1:1000 erythrosin solution to the cell suspension.

In the autoradiographic experiments, after removal of the compound the cells were washed twice with Hanks' solution and medium with thymidine-H^3 (0.05 μCi/ml, specific activity 0.478 Ci/mmole) was added. After incubation with thymidine-H^3 (45 min, 37°C) the medium was poured off and the coverslips were washed twice or three times with Hanks's solution and once with medium No. 199. The cells were then

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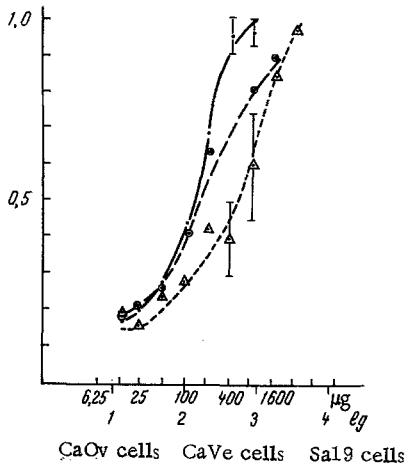

Fig. 1. Curves of number of cells of different lines versus dose of thio-TEPA. Abscissa, dose of thio-TEPA in μg/ml and log of dose; ordinate, coefficient of inhibition.

fixed with acetic alcohol. Autographs were prepared with type M (NIIKhim) fine-grain liquid emulsion. The specimens were exposed at 4°C for 3 days, then developed and fixed in the usual way and stained with aceto-orcein. The intensity of DNA synthesis and the index of labeling were determined in the experimental and control series. The intensity of DNA synthesis for each test dose of thio-TEPA was determined by counting the number of silver grains in the nuclei of 300-450 labeled cells. The labeling index and mitotic index were determined among 5000 cells for each dose of the compound. Statistical analysis of the results was carried out by the Fisher – Student method.

The action of thio-TEPA on the CaOV, CaVe, and Sal9 cultures was assessed by the use of the following criteria: counting the number of surviving (unstained) cells and calculation of the mitotic index, and determination of the intensity of DNA synthesis and the index of labeling.

For convenience when comparing the different criteria in each case, the relative index of the effect (the coefficient of inhibition) was calculated in each case.*

EXPERIMENTAL RESULTS

In the experiments of series I the relationship between the number of cells and dose of thio-TEPA was studied. Dose curves for all cell lines tested are shown in Fig. 1. Comparison of the curves revealed differences in the sensitivity of the CaOv, CaVe, and Sal9 cells. CaOv was most sensitive, Sal9 was resistance, and CaVe occupied an intermediate position. The values of LD_{50}, calculated by the method of weighing coefficients [4], also confirmed this difference. For the CaOv cells, for instance, LD_{50} was 104.2 μg/ml (5.5×10^{-4} M), for CaVe it was 129.4 μg/ml (6.9×10^{-4} M), and for the mesenchymoma 402.5 μg/ml (2.12×10^{-3} M). This difference in sensitivity was not detected if the compound was given in small doses.

The doses of the thio-TEPA used for studying its effect upon mitotic activity and DNA synthesis were selected on the basis of the first series of compound experiments. The compound was used in LD_{50}, and also in doses of 12.5 and 6.25 μg/ml. From Table 1 it can be seen that thio-TEPA greatly suppresses the mitotic activity of the cell lines studied. Thus, the compound in a minimal dose, i.e., 6.25 μg/ml (3.31×10^{-5} M) greatly decreases the mitotic index (coefficient of inhibition 0.635-0.750). Despite the high sensitivity of the test, differences in thio-TEPA action on the cell lines could not be detected.

In the next series of experiments the effect of thio-TEPA on DNA synthesis was studied. As Table 1 shows, when the compound was used in LD_{50} the intensity of DNA synthesis in the experimental series was significantly lower than in the control, but at the same time it was roughly equal for all lines (the coefficient of inhibition varied from 0.830 to 0.856). These results are in agreement with those of the cell counting. If the compound was used in a dose of 12.5 μg/ml a small but significant difference was found in the sensitivity of the cells of the lines studied ($P < 0.001$), while if a dose of 6.25 μg/ml was used the sensitivity of the CaOv and CaVe cells differed from that of the Sal9 cells ($P < 0.001$).

* Coefficient of inhibition = $\frac{\text{Absolute value in control} - \text{Absolute value in experiment}}{\text{Absolute value in control}}$

TABLE 1. Action of Thio-TEPA on Stable Cultures of Human Tumor Cells

Dose of thio-TEPA (in μg/ml)	Test	Cell line		
		CaOv	CaVe	Sa19
0 (control)	Mitotic index:			
	absolute value	5.19 ± 0.32	5.64 ± 0.32	3.20 ± 0.25
	coefficient of inhibition	0	0	0
	Mean number of grains per nucleus:			
	absolute value	56.90 ± 0.12	38.03 ± 0.14	30.2 ± 0.07
	coefficient of inhibition	0	0	0
	Number of labeled cells:			
	absolute value	38.1 ± 0.69	59.9 ± 0.70	45.4 ± 0.70
	percent of control	100	100	100
6.25	Mitotic index:			
	absolute value	1.38 ± 0.17	2.06 ± 0.23	0.80 ± 0.13
	coefficient of inhibition	0.735 ± 0.036	0.635 ± 0.061	0.750 ± 0.023
	Mean number of grains per nucleus:			
	absolute value	15.95 ± 0.10	10.65 ± 0.09	14.1 ± 0.05
	coefficient of inhibition	0.720 ± 0.002	0.721 ± 0.003	0.533 ± 0.002
12.5	Number of labeled cells:			
	absolute value	56.2 ± 0.70	7.01 ± 0.65	72.6 ± 0.63
	percent of control	147.5	117.2	160.0
	Mitotic index:			
	absolute value	1.04 ± 0.14	1.12 ± 0.16	0.60 ± 0.11
	coefficient of inhibition	0.801 ± 0.033	0.802 ± 0.040	0.812 ± 0.044
	Mean number of grains per nucleus:			
	absolute value	15.00 ± 0.11	11.15 ± 0.17	9.70 ± 0.07
	coefficient of inhibition	0.734 ± 0.002	0.707 ± 0.005	0.666 ± 0.003
	Number of labeled cells:			
	absolute value	68.5 ± 0.64	72.7 ± 0.62	67.9 ± 0.66
	percent of control	179.9	121.3	149.2
LD_{50}	Mitotic index:			
	absolute value	0.14 ± 0.04	0.16 ± 0.02	0.0
	coefficient of inhibition	0.975 ± 0.009	0.972 ± 0.005	1.0
	Mean number of grains per nucleus:			
	absolute value	9.70 ± 0.15	6.30 ± 0.13	430 ± 0.10
	coefficient of inhibition	0.830 ± 0.002	0.832 ± 0.004	0.856 ± 0.002
	Number of labeled cells:			
	absolute value	69.1 ± 0.65	32.1 ± 0.66	19.2 ± 0.56
	percent of control	181.6	53.7	42.3

The coefficient of inhibition of DNA synthesis for the CaOv and CaVe cells was 0.720 and 0.721, respectively, while for the Sa19 cells it was 0.533. The Sa19 cells were thus more resistant to the action of small doses of thio-TEPA than the CaOv and CaVe cells.

To determine the sensitivity of the tumor cells to the action of the compound it is important not only to know the changes in the intensity of DNA synthesis, but also the fraction of the cells in the population in which this synthesis takes place. In this way the reaction of the whole cell population can be determined. As Table 1 shows, thio-TEPA in doses of 6.25 and 12.5 μg/ml led to an increase in the number of labeled cells in all the lines. This effect is evidently due to a certain synchronizing action of the small doses of thio-TEPA and not to stimulation of DNA synthesis, as might be supposed if there were no evidence to show

a decrease in the intensity of incorporation of thymidine-H^3 with these doses. This increase in the index of labeling is perhaps due to the accumulation of cells in the S phase on account of an $S-g_2$ block.

During the action of thio-TEPA in LD_{50} a significant difference was observed in the reaction of the tested lines: in the cells of the sensitive line CaOv the index of labeling was increased by comparison with the control (181.6%), whereas for the more resistant CaVe and Sa19 cells the index of labeling was reduced below the control level (53.7-42.3%).

To confirm the difference between the reactions of the CaOv and Sa19 cells to thio-TEPA in the interval between LD_{50} and the small doses, the action of the compound was further investigated in doses of 50 and 200 μg/ml. For the CaOv cells the index of labeling was 130.8 and 213.7%, respectively, and for the mesenchymoma cells 93.1 and 69.7% of the control.

The results indicate a regular difference in the response of cells of the sensitive and resistant lines. The sensitive line was characterized by an increase in the index of labeled cells accompanied by a decrease in the intensity of DNA synthesis. For the resistant line the initial increase in the index of labeling accompanying an increase in the dose was replaced by a decrease in the index, and this also was accompanied by a decrease in the intensity of DNA synthesis.

Presumably the cause of the decrease in the percentage of labeled cells in the resistant line during the action of thio-TEPA in increasing doses was not only $S-g_2$ block, but also the additional appearance of a g_1-S block. As a result of this the passage of the cells into the stage of DNA synthesis was inhibited, i.e., the proportion of cells most sensitive to the action of the alkylating agent was reduced [1].

These results reflecting the sensitivity of the CaOv line are in agreement with those obtained by Kas'yanenko [2], who used the method of autoradiography with thymidine-H^3 to assess the sensitivity of animal tumor cells to sarcolysin. Later, however, Merkulov and co-workers [3] did not confirm Kas'yanenko's findings. However, these workers themselves mention certain serious difficulties with which they had to contend when using the method of autoradiography on surviving tissue fragments and which could have caused the variation in the results. By carrying out the investigations on cell cultures it was possible to avoid most of the technical difficulties associated with the uneven incorporation of thymidine-H^3 into the different layers of cells of the tissue fragment. By the use of autoradiography it was thus possible to reveal differences in the sensitivity of tumor cells to the action of compounds in small doses, which is impossible by the usual morphological method (counting the number of surviving cells, determination of the mitotic index). This is a matter of great importance to clinical medicine, for agreement between the results obtained by the use of tissue cultures and the results of treatment of patients is increased when smaller doses of the compounds are used experimentally.

LITERATURE CITED

1. N. P. Dubinin, Yu. A. Mitrofanov, and E. S. Manuilova, Izvest. Akad. Nauk SSSR, Ser. Biol., No. 4, 477 (1967).
2. V. K. Kas'yanenko, Vopr. Onkol., No. 1, 51 (1968).
3. M. F. Merkulov, Z. F. Shelaputina, N. P. Yavorskaya, et al., Farmakol. i Toksikol., No. 6, 715 (1970).
4. D. Sepetliev, Statistical Methods in Scientific Medical Research [in Russian], Moscow (1968), p. 119.
5. J. C. Wright, J. P. Cobb, S. L. Gumport, et al., Cancer, 15, 284 (1962).
6. J. C. Wright, D. G. Walker, M. M. Lyons, et al., Abstracts of Papers of the Ninth International Cancer Congress, Tokyo (1966), p. 451.