SENSITIVITY OF CYTOSTATIC-DAMAGED BONE MARROW CELLS TO THE CYTOTOXIC ACTION OF NORMAL SPLENIC EFFECTOR CELLS

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Disturbance of the genetic apparatus of cells is a most important step in the mechanism of the damping action of cytostatic preparations on cells. Investigations in this field have dealt mainly with the study of the character of cytogenetic disturbances induced by the action of cytostatics [1-3]. Meanwhile the discovery of the mechanisms of elimination of genetically damaged cells from the body is of great interest.

The aim of this investigation was to study the sensitivity of bone marrow cells with cytogenetic disturbances, induced by the action of the anthracycline antibiotic doxorubicin, the vinca-alkaloid vinblastine, and the alkylating compound cyclophosphamide, to the cytotoxic action of normal splenic effector cells.

EXPERIMENTAL METHOD

Experiments were carried out on 150 sexually mature CBA mice. Immediately before the use the preparations were dissolved in sterile isotonic NACl solution and injected intraperitoneally once, in the mean damaging dose (MDD), calculated by graphic probit analysis [7]: doxorubicin (adriablastin, Finland) in a dose of 6 mg/kg, vinblastine (Hungary) 2 mg/kg, and cyclophosphamide (USSR) 250 mg/kg. Material for investigation was taken on the 2nd, 3rd, 4th, and 10th days after injection of the cytostatics. Preparations of metaphase plates were obtained by a modified Ford's method [5], stained with azure II-eosin, and examined under the MBI-15-2 microscope. In each case at least 50 metaphase plates were analyzed: chromosomes with structural disturbances were discovered and analyzed, and cells with an altered number of chromosomes were recorded. The sensitivity of bone marrow cells, damaged by cytostatics, to the cytotoxic action of normal splenic effector cells was studied by a method based on testing the change in membrane permeability of target cells for ribonuclease [6]. Mouse bone marrow cells on the 2nd, 3rd, 4th, and 10th days after a single injection of the cytostatics were used as target cells. The target cells ($2 \cdot 10^6$/ ml) were incubated with ^{3}H-uridine (3-5 μCi/ml, specific radioactivity 24 Ci/ml) for 1 h at 37°C in medium RPMI-1640 with the addition of 10% fetal calf serum, 5 mM glutaimine, 10 mM HEPES, and 40 μg/ml of gentamicin. To prevent re-utilization of the degradation products of ^{3}H-uridine by the effector cells, the latter were pretreated with actinomycin D (1 μg/ml, 1 h, 37°C). Next, 10^4 labeled target cells in 0.1 ml of culture medium with the addition of 1 μg/ml of pancreatic RNA ("Serva") and 10^5 effector cells in 0.1 ml of culture medium were introduced into the experimental wells of a 96-well planchet. Only target cells were added to the control wells. The planchets were incubated for 14 h at 37°C in an atmosphere of 5% CO_2, after which the cells were transferred with the aid of a 12-channel fraction harvester onto filters ("Flon" No. 78-105-06). Radioactiviy was measured on a Mark III beta-counter. The sensitivity of hematopoietic cells, damaged by the cytostatics, to the cytotoxic action of the effector cells was judged by the index of membrane toxicity (IMT), calculated by the equation:

$$\text{IMT, \%} = \left(1 - \frac{\text{Number of pulses in experimental wells}}{\text{Number of pulses in control wells}}\right) \cdot 100.$$

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Table 1. IMT (in %) in Mice after a Single Injection of Doxorubicin, Vinblastine, and Cyclophosphamide

Time of investigation, days	Doxorubicin	Vinblastine	Cyclophosphamide
Control	16,50±3,51	16,50±3,51	16,50±3,51
2	18,30±8,47	(−58,00)±27,52	(−15,66)±11,47
p	>0,1	<0,05	<0,05
3	39,00±4,24	(−14,00)±2,00	13,33±18,04
p	<0,05	<0,01	>0,1
4	7,30±4,22	3,66±3,82	30,33±8,99
p	>0,1	>0,05	>0,2
10	(−56,30)±14,00	18,33±12,04	31,66±7,36
p	<0,01	>0,5	>0,1

EXPERIMENTAL RESULTS

A single injection of doxorubicin in MDD was shown to induce the formation of cells with cytogenetic disturbances in the bone marrow of the mice. The maximal "yield" of genetically damaged cells was observed on the 3rd day after injection of the antibiotic, when they numbered 21.20 ± 3.6% ($p < 0.001$) (4.16 ± 1.55% in the control). By the 10th day the number of cells with cytogenetic disturbances induced by doxorubicin had fallen to 12.00 ± 1.89%, but it remained significantly higher ($p < 0.001$) than the control level. The most characteristic type of disturbance of the chromosomal apparatus of the bone marrow cells in the early period after injection of doxorubicin were chromatid aberrations. The largest number of cells with single fragments was recorded on the 3rd day (6.80 ± 1.62%, $p < 0.01$) after injection of the cytostatic. Later during the investigation the number of cells with chromatid deletions gradually diminished, and by the 10th day it was the same as in the control (0.33 ± 0.32%). As regards cells with an altered number of chromosomes, their number differed significantly ($p < 0.01$) from the initial values (3.66 ± 0.81%) on the 2nd, 4th, and 10th days after injection of doxorubicin: 8.80 ± 1.01, 11.00 ± 1.68, and 9.66 ± 1.08% respectively.

The study of the membrane-toxic action of cells of the splenic fraction on mouse bone marrow cells after administration of doxorubicin showed that maximal sensitivity of the target cells to the splenic effector cells was observed on the 3rd day after injection of the preparation (39.00 ± 4.24%, $p < 0.01$). Bone marrow cells obtained from mice on the 4th and 10th days after injection of the cytostatic were resistant to the cytotoxic action of effectors of the natural resistance system (Table 1).

The maximal number of aberrant bone marrow cells on the 3rd day after injection of doxorubicin thus corresponds to a peak on the curve of sensitivity of hematopoietic cells, damaged by cytostatics, to the cytotoxic action of normal spleen cells. The results are evidence that splenic effector cells can distinguish cells with structural disturbances of their chromosomes, induced by the action of doxorubicin. Structural disturbances of the chromosomal apparatus probably evoke changes in the antigenic structure of the cell membrane, which are recognized by lymphocytes and induce their cytolytic activity.

The study of the cytostatic action of vinblastine in MDD showed that this agent does not induce disturbances in the structure of the chromosomes of the hematopoietic cells of the bone marrow, but stimulates the appearance of polydiploid cells (mainly with a tetraploid set of chromosomes), the number of which throughout the period of observation vary between 9.33 and 21.60%, significantly ($0.001 < p < 0.01$) above their spontaneous level. The sensitivity of mouse bone marrow cells, damaged by vinblastine, to the cytotoxic action of normal splenic effector cells fell significantly ($p < 0.05$) on the 2nd day after injection of the cytostatic, after which it gradually recovered, to reach the control values by the 10th day (Table 1).

Analysis of the results enables us to propose that the change in the number of chromosones in the bone marrow cells is not recognized by the effector cells and increases resistance of the hematopoietic cells to lysis.

The results of investigation of the cytostatic action of cyclophosphamide show that a single injection of the compound in MDD caused an increased "yield" of hematopoietic cells with damaged nuclear genetic structures in the mice. The maximal number of cells with cytogenetic disturbances in this case (61.60 ± 6.65%, $p < 0.001$) was recorded 24 h after injection of cyclophosphamide. On the 3rd day the value of this parameter fell sharply (more

than threefold) to 19.20 ± 3.26%, which was still significantly ($p < 0.01$) above the control values. By the 10th day of the investigation the difference between the control and experiment was no longer statistically significant ($p > 0.5$). Analysis of structural disturbances of chromosomes induced by the action of cyclophosphamide showed that this compound stimulates the formation of cells with single and paired fragments, and with chromatid and chromosomal exchanges, i.e., it acts on the cell in the G_1-, S-, and G_2-phases of the mitotic cycle.

As a result of this investigation of the sensitiviy of bone marrow hematopoietic cells, damaged by cyclophosphamide, to the cytotoxic action of normal splenic effector cells, a sharp decrease in IMT was discovered on the 2nd day after injection of the cytostatic (Table 1). An intact apparatus for protein synthesis in target cells is probably essential for the state of the membrane of the target cell to reflect existing disturbances in the chromosomal apparatus of the nucleus and to be recognized by splenic effector cells. The action of cyclophosphamide, however, in a dose above the therapeutic level inhibits protein synthesis by about 48%, lowers the acceptor activity of tRNA [4], and, evidently, it thus reduces the number of sites recognized by effector cells. On the 3rd day of the investigation the value of IMT came close to the control values, and later (on the 4th and 10th days), although it exceeded them in mean values (30.33 ± 8.99 and 31.66 ± 7.36% respectively), it did not differ significantly from normal ($p > 0.2 > 0.1$).

Hematopoietic cells of mouse bone marrow, with cytogenetic disturbances induced by cytostatics with different mechanisms of action, are thus characterized by unequal sensitivity to the cytotoxic action of normal splenic effector cells. For instance, injection of doxorubicin stimulates the appearance of cells with single fragments in the bone marrow, which exhibit maximal sensitivity to the cytotoxic action of normal splenic effector cells on the 3rd day of the investigation. The change in the number of chromosomes in the bone marrow cells, induced by vinblastine, is not recognized by the effector cells and increases the reistance of the hematopoietic cells to lysis. The action of cyclophosphamide, stimulating the appearance of chromosomal and chromatid aberrations in the bone marrow cells, leads to a decrease in their sensitivity to the damaging action of normal splenic effector cells on the 2nd day of the experiment. Differences in the sensitivity of cells, damaged by cytostatics, to the cytotoxic action of normal splenic effector cells must be taken into account when rational schemes of tumor chemotherapy are devised, with the inclusion of immunomodulators, namely activators of macrophages and of the T and NK systems of immunity, in the treatment program.

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