Phenobarbital Modifies Antitumor Effect of Cyclophosphamide Depending on the Type of Tumor Cell Death Caused by It

V. I. Kaledin, V. P. Nikolin, T. Yu. Baimak, M. R. Galyamova*, N. A. Popova*, and E. M. Andreeva*

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Antitumor effect of cyclophosphamide on LS and P388 tumors is realized via apoptosis and on HA-1 and Krebs-2 tumors resistant to apoptosis via necrosis of tumor cells. Phenobarbital induction of cyclophosphamide-metabolizing enzymes decreases and cimetidine inhibition potentiates the effect of cyclophosphamide on LS and P388 cells and does not modulate the effect on HA-1 and Krebs-2 cells. Presumably, apoptosis and necrosis of tumor cell are induced by different cyclophosphamide metabolites.

Key Words: *transplanted tumors; cyclophosphamide; phenobarbital; apoptosis; necrosis*

Cyclophosphamide (CP) is an alkylating antitumor drug most widely used in the treatment of cancer. Its antitumor effect manifests after activation with cellular enzymes of the liver, leading to the formation of alkylating metabolite, phosphoramide derivative mustard gas (PM), which is believed to be responsible for antitumor effects of CP and acrolein [9]. Activities of enzymes metabolizing CP are many-fold increased by some cytochrome P-450 inductors (for example, barbiturates [12]). The possibility of increasing the antitumor effect of CP by induction of these enzymes, primarily by phenobarbital (PB), was investigated. Some authors observed an increase of antitumor effect of CP after phenobarbital induction [10]. However, in the majority of other studies the inductor did not modulate the antitumor effect of CP or even decreased it [4,6,8]. These contradictory results can be explained by the fact that reactions leading to the formation of activated CP metabolites (4-hydroxylation) are paralleled by reactions leading to its inactivation (dechlorethylation, conjugation with glutathione, *etc.*), and both activating and inactivating enzymes are present in tumor and can be induced by PB [14]. Hence, the result in each case is determined by enzyme activities in the tumor and whole organism.

It seems however that there is another cause of ambiguous effects of PB on antitumor activity of CP, explained by different mechanisms of its effect on different tumors. The cytostatic (and in high doses cytotoxic) effect of CP inhibits the growth of some tumors, but in doses even lower by one order of magnitude it causes complete regression of other tumors via induction of apoptosis [3]. Since apoptosis is triggered not only by DNA damage [13], it is possible that CP induces it through processes other than genotoxic. If so, stimulation of the metabolic pathway leading to the formation of genotoxic derivatives of CP can reduce, but not increase its apoptogenic effect, if it is realized by other (alternative) metabolites. This problem was not discussed previously.

We investigated the effect of PB on antitumor activity of CP realizing the antitumor activity through tumor cell apoptosis or necrosis.

MATERIALS AND METHODS

The study was carried out on CBA, A/Sn, and DBA/2 mice from Laboratory of Experimental Animals of Institute of Cytology and Genetics. Experiments were

Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk; *Novosibirsk State University

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carried out on tumor cell lines obtained by us (lymphosarcoma [3] and hepatocarcinoma HA-1 [2]) and P388 leukemia and Krebs-2 nonspecific carcinoma. The tumors were maintained in ascite form in CBA (LS and Krebs-2), DBA/2 (P388), and A/Sn mice (HA-1).

Tumor cells were transplanted to animals into the hip muscles. Visible tumors were measured and the mice were divided into experimental groups depending on tumor size.

PB (Fluka) was injected intraperitoneally in a dose of 80 mg/kg 3 times at 24-h intervals, cimetidine (Pharmachim) was administered in a single dose of 300 mg/kg through a gastric tube, and CP (OAO Biokhimik) was injected intravenously or intraperitoneally in doses of 10, 50, or 100 mg/kg 24 h after the last injection of PB or 1 h after cimetidine.

Starting from the day of CP injection, the dynamics of tumor growth was evaluated (three perpendicular diameters were measured). the antitumor effect was evaluated by tumor regression and growth inhibition.

For histological and cytological studies, 4 animals from each group were decapitated 24-72 h after CP injection, the tumors were removed; cell suspension was prepared from one part of the tumor and the other part was fixed in 10% formalin. Cell suspensions were fixed in Carnoy fluid, stained with carmine in 45% acetic acid, and the percentage of cells with fragmented nuclei (apoptotic) was counted under a microscope. Paraffin sections were stained with hematoxylin and eosin and examined under a microscope; the numerical density of necrose was evaluated by the morphometric methods [1].

RESULTS

Tumors showed different sensitivity to CP. A 75-85% suppression of tumor growth could be attained by a single CP dose of 50 mg/kg in P388 leukemia and 100 mg/kg in Krebs-2 and HA-1 tumors. LS tumor was the most sensitive to CP: it completely regressed in 10-40% mice after administration of 10 mg/kg CP. Previous histological study and electrophoresis of DNA showed a pronounced apoptogenic effect of CP on these tumor cells at a qualitative level [3]. In the present study we evaluated this effect quantitatively on cell suspensions. Two days after injection of 10 mg/kg CP more than half of cells in biopsy specimens of LS tumor were in a state of apoptosis; after CP dose of 100 mg/kg the percentage of apoptotic cells surpassed 80 (Fig. 1, *a*). By contrast, in HA-1 tumor CP in doses of 50 and 100 mg/kg inducing necrosis of an appreciable part of the cells just negligibly increased the percentage of cells in apoptosis in comparison with the control (Fig. 1, *b*). P388 leukemia was sensitive (though less than LS tumor) to apoptosis induction (up to 20% apoptotic cells at CP dose of 50 mg/kg), while Krebs-2 tumor was highly resistant to this effect: administration of 100 mg/kg CP induced apoptosis in only solitary cells (like in the control). Hence, the therapeutic effect of CP on LS and P388 tumors is realized mainly or to a great measure through apoptosis induction, while the effects on HA-1 and Krebs-2 tumors are realized through cytostatic and necrogenic activity towards tumor cells.

Treatment with PB according to a routine protocol ensuring induction of liver microsomal enzymes [12] did not modify the therapeutic effect of CP on HA-1 and Krebs-2 tumors (Fig. 2, *c*, *d*), but appreciably reduced it in LS and P388 tumors (Fig. 2, *a*, *b*). Regression of these tumors in PB-treated animals was attained later and was more transient than in mice treated with CP alone. PB injected 10 min before CP stimulated, but not suppressed its inhibitory effect on

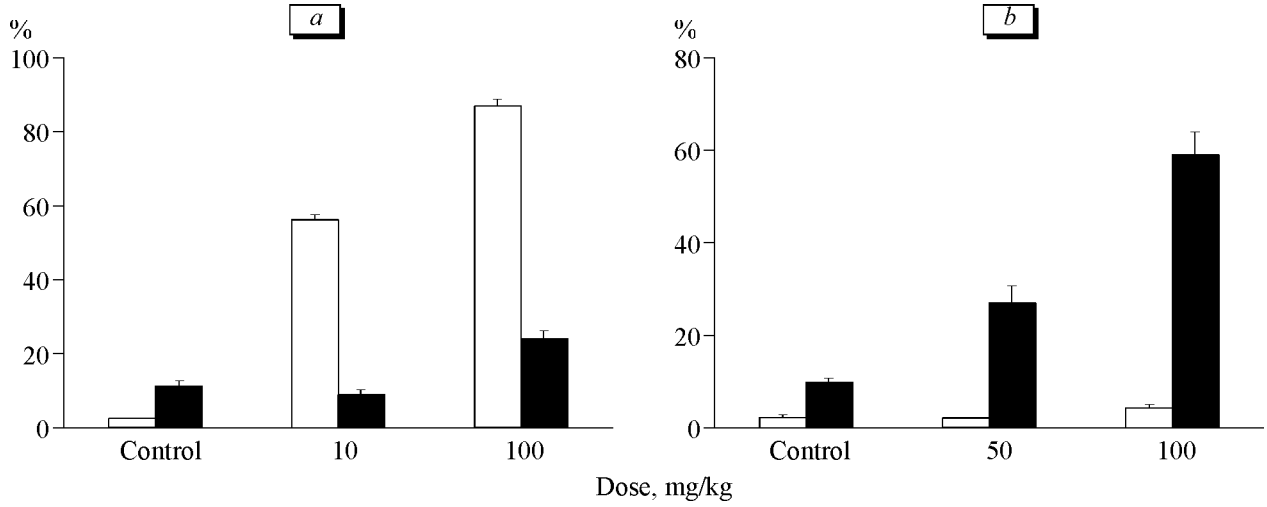

Fig. 1. Percentage of apoptotic cells (light bars) and quantitative density of necroses (dark bars) in LS (*a*) and HA-1 (*b*) tumors after cyclophosphamide treatment *in vivo*.

tumor growth. Cimetidine injected 1 h before CP produced a similar effect. It is noteworthy that in mice induced with PB cimetidine completely abolished the effect of PB on CP activity (Fig. 2, *a*).

PB induction markedly decreased apoptogenic activity of CP towards LS tumors (Fig. 3), which determined weak therapeutic effect of CP on this tumor and on P388 tumor in PB-induced animals. One more confirmation of this hypothesis is the absence of PB effect in HA-1 and Krebs-2 tumors, in which CP is unable to trigger apoptosis. Hence, opposite effects of PB [4, 6,8,10] on antitumor activity of CP on different models are largely determined by the mechanism of CP effect on cells of this or that tumor: whether it causes injuries leading to necrosis or induces apoptosis.

It is now believed that as a pro-medicine, CP inert *per se*, is activated in the organism by 4-hydroxylation realized by PB-induced forms of the liver cytochrome P-450 2B and 2C forms and subsequent nonenzymatic formation of highly reactive alkylating PM metabolite [5,9,12]. PM forms adducts with purine DNA bases, especially with those that neighbor guanine residues, and leads to the formation of cross-links in their molecules [5]. These DNA injuries are considered to be the cause of tumor cell death both by apoptosis and necrosis, depending on the intensity of DNA injuries and the state of the pro- and antiapoptotic systems in tumor cell. This explains the incapacity of cells with mutant *p53* gene to undergo apoptosis in response to DNA-damaging effects of numerous drugs and radiation [11]. However apoptosis can be induced in many tumors despite the presence of mutant *p53* variant [7].

If apoptosis and necrosis are induced by the same CP metabolite (PM), stimulation of its formation in

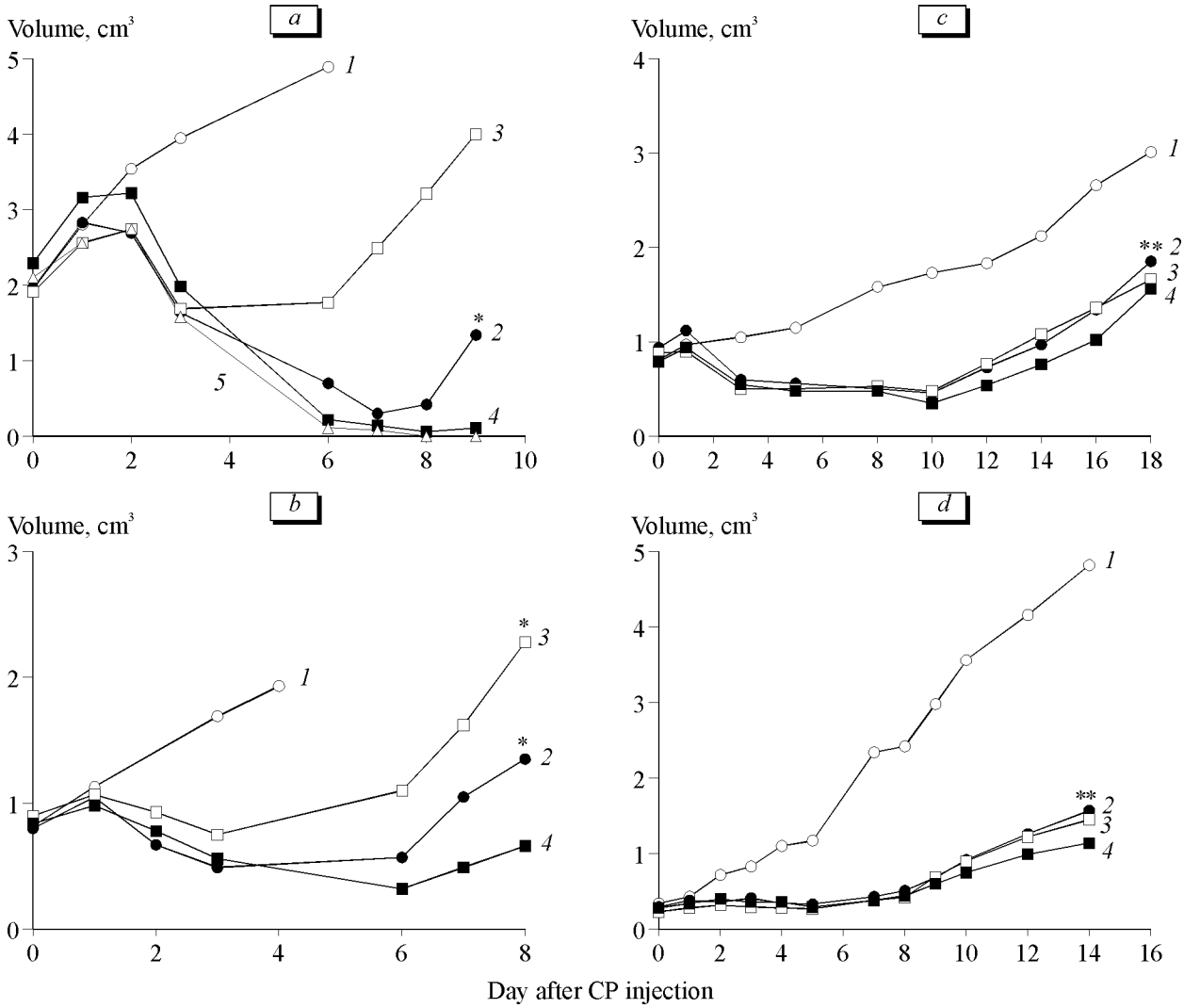

Fig. 2. Dynamics of LS (*a*), P388 (*b*), HA-1 (*c*), and Krebs-2 tumors (*d*) in control mice (*1*) and in mice treated with cyclophosphamide (CP) (*2*), CP after phenobarbital induction (*3*), cimetidine and CP (*4*), cimetidine and CP after phenobarbital induction (*5*). Means of two experiments are presented for each tumor. Number of animals: *a*) 19; *b*) 11; *c*) 16; and *d*) 20. $p<0.001$: *compared to other groups; **compared to the control.

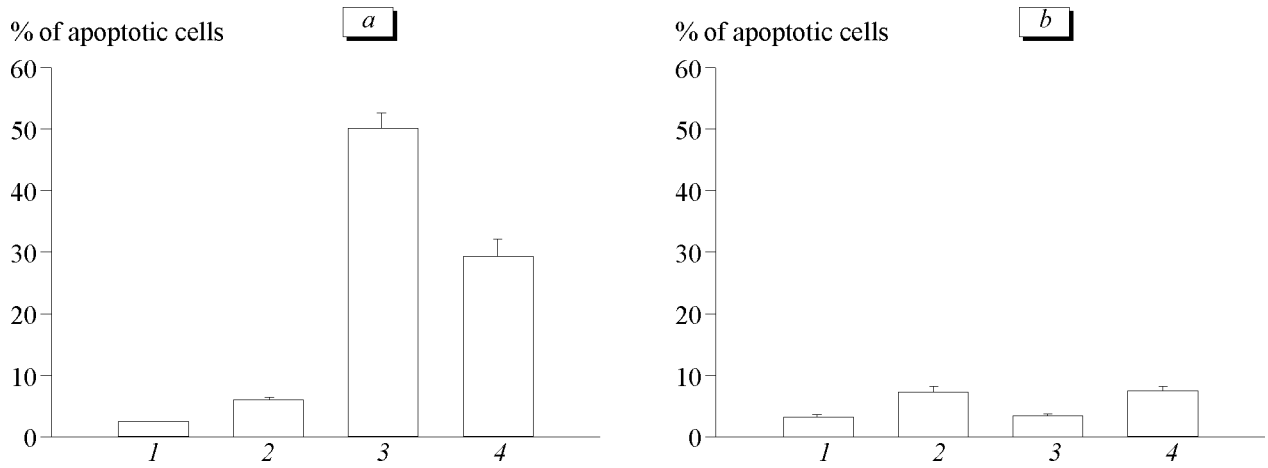

Fig. 3. Effect of P-450 induction with phenobarbital on apoptogenic effect of cyclophosphamide (CP) in LS (*a*) and HA-1 tumors (*b*). *1*) control (no induction); *2*) phenobarbital induction without CP; *3*) CP without induction; *4*) CP after phenobarbital induction.

tumor carriers, the first stage of which is 4-hydroxylation of CP, will potentiate the therapeutic effect of CP, no matter whether this effect is realized through tumor cell apoptosis or necrosis. However, after PB induction the effect of CP remained virtually unchanged in mice with HA-1 and Krebs-2 tumors and decreased significantly in mice with LS and P388 tumors. The latter phenomenon can be explained as follows: by stimulating CP 4-hydroxylation through Cyp2b induction, PB still more intensely induced activity of CP inactivating enzymes (*e. g.* glutathione-S transferase) and of Cyp3a realizing oxidative chlorodeethylation of CP and thus making it unable to activation [12]. However the fact that after PB induction Cyp2b activity in the liver of mice used in our study increased more than 17-fold while the activity of Cyp3a increased only 2.3 times rejects this hypothesis. The fact that the influence of PB induction on CP effect was abolished by cimetidine inhibiting activity of cytochrome P-450, but not glutathione-S transferase, also contradicts this hypothesis. On the other hand, without preinduction the therapeutic effect of CP towards LS and P388 tumors increased due to cimetidine blocking of CP microsomal oxidation and due to PB, if it was injected immediately before CP (Fig. 2,). Presumably, PB, as a Cyp2b substrate, inhibited CP 4-hydroxylation, competing for the enzyme.

Hence, it seems that stimulation of 4-hydroxylation, the chief reaction during PM formation, is responsible for the decrease of CP therapeutic effect on tumors, in which it induces apoptosis. Therefore, an alternative CP metabolite, but not PM, is responsible for apoptosis induction. All CP metabolites known by today are inferior to PM in alkylating activity. However, if such a metabolite possesses steric affinity for a pro- or antiapoptotic protein, it can induce apoptosis much more effectively than nonspecifically acting PM.

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