Acta Veterinaria (Beograd), Vol. 47. No.2-3, 141–150, 1997.

UDK 619:616.155.392:636.5

THE EFFECT OF CERTAIN CYTOTOXIC AGENTS ON THE PROLIFERATION OF BONE MARROW CELLS OF CHICKENS WITH ERYTHROLEUKEMIA

GORDANA KONJEVIĆ*, Č. RUSOV** and RADMILA ŽIVKOVIĆ**

Institute of Oncology and Radiology of Serbia, ** Scientific Institute for Veterinary Medicine of Serbia, Department of Hematology ,Beograd*

(Received,11. March 1997.)

The results of an investigation of the effects of cytotoxic agents on the incorporation of ^{3}H-thymidine into DNA of bone marrow cells of normal chickens and those with acute erythroleukemia induced by C-type oncorna virus are presented. The agents included cytosine arabinoside, cyclophosphamide, mechlorethamine, doxorubicin and daunorubicin, in concentrations from 1 - 1000 mg per 2 m of bone marrow cell culture. Cytosine arabinoside, mechlorethamine, doxorubicin and daunorubicin showed a concentration dependent inhibition of DNA synthesis in normal cells, which was significantly more marked in leukemic cells. Cyclophosphamide did not induce inhibition of DNA synthesis of either normal or leukemic chicken bone marrow cells.

The two highest concentrations of cytotoxic agents, which were shown to be significantly more efficient than lower ones, did not differ in their effect on either type of bone marrow cell, although the magnitude of the effect was significantly greater on leukemic cells.

The results obtained in this in vitro study, on bone marrow cells of normal chickens and chickens with experimental acute erythroleukemia, indicate the possible usefulness of such investigations on the proliferative activity of hematopoietic cells of patients with leukemia in order to select the most suitable type and dose of cytotoxic agents.

Key words: erythroleukemia, chicken, cytotoxic agents, bone marrow, DNA, ^{3}H-thymidine

INTRODUCTION

Numerous models are employed for the study of the biological activity or mechanism of action of cytotoxic agents on leukemic cells. Most commonly used are experiments in vivo on experimental animals (Naftalovich et al., 1991) or investigations in vitro on human leukemic cells (Tazzariet al., 1994, Vogler et al., 1993, Newman et al., 1990), leukemic cell lines from experimental animals with

spontaneous or experimental leukemias (Rusov et al., 1982, Yamamoto et al., 1993, Hatta et al., 1994), lymphomas (Naftalovich et al., 1991), as well as on normal mouse and human hematopoietic cell lines and splenocytes (Naftalovich et al., 1991, Jacobsen et al., 1991). All of these models of study can give useful information for both better understandling and appropriate application of cytotoxic agents in the therapy of these types of malignancies.

Considering that erythroleukemia can be induced in a relatively simple manner in the chicken, in this study this model was used for in vitro investigation of the effect of different types of cytotoxic agents on bone marrow cell proliferation in order to estimate the dose-dependent effect on both normal and malignantly transformed bone marrow cells. Data obtained by such investigations may provide additional valuable guidelines in choosing drugs for the treatment of this type of malignancy in humans.

MATERIALS AND METHODS

Proliferation of normal and leukemic cells and their susceptibility to cytotoxic agents were determined in vitro by measuring ^{3}H-thymidine incorporation into DNA strands of bone marrow cells of normal chickens and chickens with acute erythroleukemia.

The study included an investigation of the effect of cytosine arabinoside (Mack, Germany) at concentrations of $0,3 \times 10^{-7}$ - 0.3×10^{-4} M, cyclophosphamide (ASTA, Germany) at concentrations of $1,8 \times 10^{-6}$ - 1.8×10^{-3} M, mechlorethamine (Delagrange, France) at 2.5×10^{-6} - 2.5×10^{-3} M, doxorubicin (Farmacia, Sweden) at 6.2×10^{-8} - 6.2×10^{-5} M and daunorubicin (Farmacia, Sweden) at concentrations of 0.9×10^{-8} - 0.9×10^{-5} M. The agents were dissolved in redistilled water and further diluted in isotonic NaCl or culture medium (CM) MEM (Eagle, England) supplemented with 10% chicken serum. Into flasks containing cell suspensions, 0.2 ml of dissolved agents were added, while only isotonic NaCl was added to control cultures.

Bone marrow was taken from the chicken femur and the animals were sacrificed by bleeding. Cell suspensions were prepared as previously described (Rusov et al., 1978, Volm et al., 1984). Into 8 ml glass flasks 2 ml of bone marrow cell suspensions containing 20.0×10^6 cells were added. The suspensions were kept for 60 min. in an incubator with 5% CO_2 and 95% air, after which the cytotoxic agents were added at concentrations of 1, 10, 100 i 1000 μg in 2 ml of suspension. Each concentration was examined in quintuplet.

After 2 h of incubation 37×10^3 Bq of 3H-thymidine in 0.1 ml of isotonic NaCl solution was added to each flaskh. Incubation with the radioisotope on a shaker with about 50 movements per minute lasted for 2 h. The flasks were then placed on ice, the cells were lysed in distilled water and treated with 10% tetrachloroacetic acid (TCA). The precipitate was washed out by a vaccum through a Sartorius filter with 7.5 and 5% TCA. Dried filters were then placed in vials containing 5 ml of scintillate based on toluol, and the radioactivity was

Contrary to cyclophosphamide, the other alkylating agent, mechlorethamine, gave in all studied doses a prominent inhibition ($p < 0.05 - 0.01$ for doses from 1 – 1000 μg) of ^{3}H-thymidine uptake into normal and leukemic bone marrow cells compared to controls (Figure 3). The inhibitory effect on cells

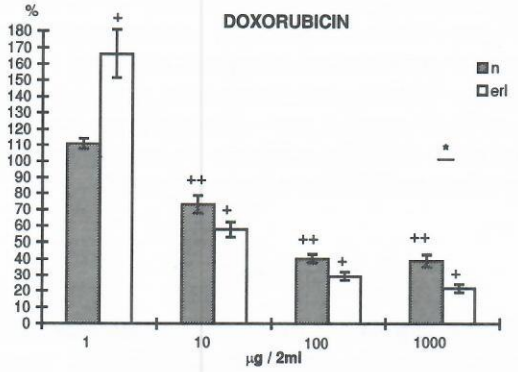

Figure 4. The inhibitory effect of different concentrations of doxorubicin on in vitro proliferation of bone marrow cells of normal chickens (n = 4) and chickens with erythroleukemia (n =4). Bars represent the average values ($\bar{x} \pm SE$) expressed as a percent of the respective control (n = 8) (+ - denotes the significance of the difference of erythroleukemic /erl/ and normal /n/ bone marrow compared to control bone marrow; * - denotes the significance of the difference between erythroleukemic and normal bone marrow, +,* - $p < 0.05$, ++,** - $p < 0.01$).

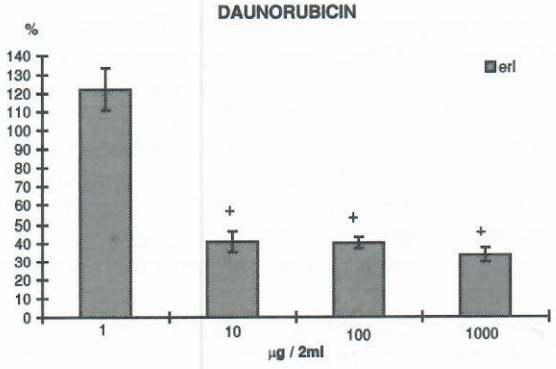

Figure 5. The inhibitory effect of different concentrations of daunorubicin on in vitro proliferation of bone marrow cells of chickens with erythroleukemia (n = 4). Bars represent the average values ($\bar{x} \pm SE$) expressed as a percent of the respective control (n = 4) (+ - $p < 0.05$ difference of erythroleukemic bone marrow compared to control bone marrow).

The studied concentrations of the alkylating agent, cyclophosphamide did not have a significant effect on 3H-thymidine incorporation into DNA in the bone marrow cell cultures of normal chickens (Figure 2). Moreover, at the lowest

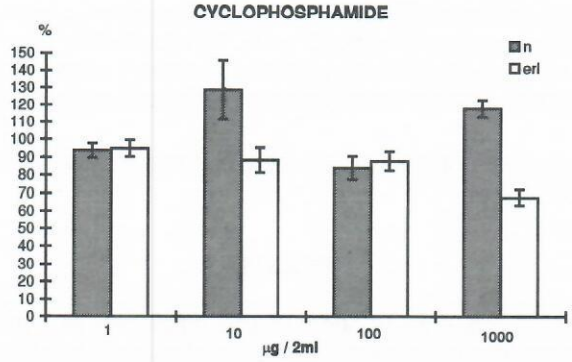

Figure 2. The inhibitory effect of different concentrations of cyclophosphamide on in vitro proliferation of bone marrow cells of normal chickens (n = 5) and chickens with erythroleukemia. Bars represent the average values ($\bar{x} \pm SE$, n = 5) expressed as a percent of the respective control (n = 10).

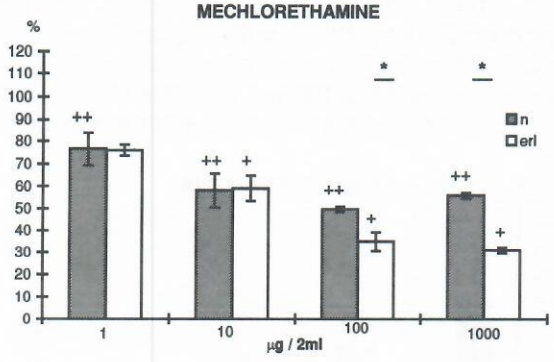

Figure 3. The inhibitory effect of different concentrations of mechlorethamine on in vitro proliferation of bone marrow cells of normal chickens (n = 4) and chickens with erythroleukemia (n = 4). Bars represent the average values ($\bar{x} \pm SE$) expressed as a percent of the respective control (n = 8) (+ - denotes the significance of the difference of erythroleukemic /erl/ and normal /n/ bone marrow compared to control bone marrow; * - denotes the significance of the difference between erythroleukemic and normal bone marrow, +,* - $p < 0.05$, ++,** - $p < 0.01$).

concentration of 1 μg it had a slight potentiating effect. Also, the effect of cyclophosphamide on DNA synthesis in bone marrow cells of chickens with acute erythroleukemia was not significant, but it was most pronounced at the concentration of 1000 μg/20 x 10^6 cells.

the cultures of normal and leukemic bone marrow cells a significant inhibition of DNA synthesis. Considering the dependence of cytosine arabinoside on the cell cycle, it is of interest that a 4 h treatment gave approximately the same effect (inhibition of proliferation of about 70%) on malignant melanoma cell cultures (Chabner et al., 1996) as it did on erythroleukemic bone marrow cultures in this study.

An alkylating agent can induce lethal lesions of DNA by producing reactive intermediates which attack nucleophilic sites. Mechlorethamine caused significant inhibition of DNA synthesis in normal and leukemic cells, while cyclophosphamide, did not influence DNA synthesis of normal cells and only in the highest concentration exhibited an inhibitory effect on 3H-thymidine uptake into leukemic cells. As it is necessary for cyclophosphamide to be activated in the liver in order to acquire cytotoxic characteristics, this agent should not exhabit any activity in in vitro conditions (Calabresi et al., 1985, Erlichman et al., 1987, DeVita et al., 1991).

Anthracycline antibiotics have a pleiotropic effect on DNA by intercalatng between DNA base pairs. In this manner they inhibit topoisomerase II, the direct consequence of which is the induction of apoptosis. These agents also activate protein kinase C and lead to the production of free radicals. In this study doxorubicin and daunorubicin showed similar inhibitory activity to antimetabolites and alkyating agents on DNA synthesis in bone marrow cells.

A common characteristic for the investigated cytotoxic agents which induced inhibition of 3H-thymidine uptake into bone marrow cells in culture, was that the concentrations of 100 and 1000 μg gave a significant further depression of DNA synthesis as compared to 10 μg/20 x 10^6 cells, in both normal and leukemic cells. However, the effect of these two highest doses did not differ significantly. At the highest concentration the cytotoxic agents were significantly better in inhibiting 3H-thymidine uptake into erythroleukemic bone marrow cells compared to normal bone marrow. This difference indicates the selectivity of action of these cytotoxic agents on erythroleukemic cells, which is of importance when dealing with drugs, especially those with a narrow therapeutic range.

These data obtained in an in vitro study on the bone marrow of chickens with erythroleukemia, should be carried on further and should be taken into consideration when analyzing the proliferative activity of hematopoietic cells of individual patients with leukemia in order to choose the most suitable types and doses of cytotoxic agents.

REFERENCES

1. *Calabresi, P., Parks, R. E.* 1985. Chemotherapy of neoplastic diseases. In: "Pharmacological basis of therapeutics" *(Ed. Goodman Gilman, A. Goodman, L. S., Rall, T. W., Murad, F.) McMillan Publ. Comp.., New York, pp.* 1240-1306.
2. *Chabner, B. A.* 1996. Cytidine analogyes, In: "*Cancer chemotherapy and biotherapy. Principles and practice*". *(Ed. B. A. Chabner, D. L. Longo)* 2nd *Edition, Lippincot-Raven Pbl., Philadelphia, New York, pp* 213-234.

of normal and pathological bone marrow was the same at the lowest doses (1 – 10 μg), while at higher doses of 100 μg it was 30%, and at the dose of 1000 μg it was 45% more pronounced ($p < 0.05$) for erythroleukemic cells.

The anthracycline antibiotic, doxorubicin, gave a significant dose dependent decrease of proliferation in the applied doses of 10 to 1000 μg for healthy and erythroleukemic bone marrow. The greatest difference in the effect was at the dose of 1000 μg, when the inhibition of erythroleukemic bone marrow prolliferation was almost 50% higher. On the other hand, the minimal dose of 1 μg gave a significant enhancement of proliferation of erythroleukemic cells (Figure 4).

Daunorubicin, also an anthracycline antibiotic, gave a significantly lower ($p < 0.05$) incorporation of ^{3}H-thymidine into leukemic bone marrow cells copared to controls at the doses of 10-1000 μg, while the lowest concentration of 1 μg did not show an inhibitory effect on DNA synthesis (Figure 5).

DISCUSSION

Chemotherapy has a significant place in the treatment of malignant diseases, although results obtained in clinical studies are not always satisfactory. Considering that hematological malignancies may be of different cellular origin the effect of many groups of cytotoxic agents are being analyzed in experimental conditions on permanent cell lines or malignantly transformed bone marrow or peripheral blood cells originating from patients in order to estimate individual sensitivity to these drugs (Dal Pozzo et al., 1989, Phillips et al., 1990). For this purpose it is possible to expose in vitro cultures of leukemic cells to cytotoxic drugs, and then by examining the inhibition of 3H-thymidine incorporation to estimate their activity. There is usually good correlation between the results obtained in in vitro studies of these drugs with their therapeutic effect (Schwarzmeir et al., 1984).

Thus different authors (Dal Pozzo et al., 1989, Phillips et al., 1990) show that for more successful treatment of leukemias it is desirable to determine chemosensitivity, i. e. individual sensitivity of patients with leukemias to cytotoxic agents by monitoring parameters of cell proliferation.

Considering the significance of altered metabolism of nucleic acids in the appearance of leukemias, one of the principal goals in the treatment is the application of drugs with an inhibitory effect on nucleic acid metabolism (Das et al., 1980). For this reason it is essential to have data concerning the proliferative activity of different types of hematopoietic cells and their sensitivity to cytotoxic agents.

In this study five cytotoxic agents were investigated, which can be divided, according to their chemical nature and mode of action, into three groups, antimetabolites (cytosine arabinoside), alkylating agents (cyclophosphamide, mechlorethamine) and anthracycline antibiotics (doxorubicin, daunorubicin).

Antimetabolites have multiple effects on DNA synthesis, the most important of which is their ability to be incorporated into this molecule. Cytosine arabinoside, an agent commonly used in the treatment of acute leukemias, exhibited both in

eritroleukozom izazvanom oncorna virusom C-tipa. Ispitivani su citozin arabinozid, ciklofosfamid, mehloretamin, doksorubicin i daunorubicin, u koncentracijama od 1 - 1000 μg na ćelije kostne srži u kulturi. Citozin arabinozid, mehloretamin, doksorubicin i daunorubicin su pokazali dozno zavisno inhibitorno dejstvo na sintezu DNK u normalnim, a značajno više u leukemijskim ćelijama. Ciklofosfamid u ispitivanim dozama nije pokazao inhibitorno dejstvo na sintezu DNK, kako na normalne tako i na leukemijske ćelije kostne srži pilića.

Dve najveće doze citotoksičnih agenasa, koje su značajno efikasnije od nižih, nisu se međusobno razlikovale po efektu na ćelije kostne srži zdravih, odnosno, bolesnih pilića, ali je efekat bio značajnije veći na leukemijske ćelije.

Dobijeni rezultati u ovoj studiji in vitro na ćelijama kostne srži normalnih i pilića sa akutnom eksperimentalnom eritroleukozom ukazuju na potrebu sličnih ispitivanja proliferativne aktivnosti hematopoeznih ćelija individualnih ljudi sa leukemijom, radi boljeg izbora vrste i doze citotoksičnih preparata.

3. *Dal Pozzo, O., Bernabel, P. A., Santini, V., Saccardi, R., Santini, S., Rossi, Ferrini, P.* 1989. A comparison of two assays for the in vitro chemosensitivity testing on human acute non-lymphoblastic leukemia cells. *J. Chemother., 1,* 318-323.
4. *Das, K. C., Garewal, G., Mohany, D.* 1980. Derangement of erythroleukemia. Normal deoxyuridine suppression and impaired thymidine incorporation in bone marrow cultures. *Acta haemat. 64,* 121-130.
5. *DeVita, V. T. Jr. Alkylating agents. In:* "Cancer, Principles and Practice of Oncology", *(Ed. DeVita, V. T. Jr., Hellman, S., Rosenberg, S. A.), J. B. Lippincot Comp., Philadelphia, 3rd Edition, 1991, pp.* 367.
6. *Erlichman, C.* 1987. The pharmacology of anticancer drugs. In: "The Basic Science of Oncology" *(Ed. Tannoch, I. F., Hill, R. P.) New York, Pergamon Press, pp.* 292-307.
7. *Hatta Y., Aizawa, S., Itoh, T., Baba, M., Horie, T.* 1994. Cytotoxic effect of extracellular ATP on L1210 leukemic cells and normal hemopoietic stem cells. *Leuk. Res. 18,* 636-641.
8. *Jacobsen, S. E., Ruscetti, F. W., Longo, D. L., Keller, J. R.* 1991. Antineoplastic Dolastatins: Potent inhibitors of hematopietic progenitor cells. *J. Natl. Cancer Inst. 83,* 1672-1677.
9. *Naftalovich, S., Yefenof, E., Eilam, Y.* 1991. Antitumor effects of ketoconazole and trifluoperazine in murine T-cell lymphomas. *Cancer Chemother. Pharmacol., 28, 384-390.*
10. *Newman, E. M., Villacorte, D. G., Testi, A. M., Krance, R. A., Harris, M. B., Ravindranath, Y., Pinkel, D.* 1990. Biochemical interactions between methotrexate l-beta-D-arabinofuranosylcytosine in hematopoietic cells of children: A Pediatric Oncology Group Study. *Cancer Chemother.Pharmacol., 2,* 60-66.
11. *Phillips, R. M., Bibbi, M. C., Double, J. A.* 1990. A critical appraisal of the predictive value of in vitro chemosensitivity assays. *J. Natl. Cancer Inst. 82,* 1457-1468.
12. *Rusov, Č.* 1978. Significance of the myelogram in the diagnosis of spontaneous leukosis in chickens. *Acta Veterinaria, 28,* 131-139.
13. *Rusov, Č. Stojimirović, E., Živković, R., Konjević, G.* 1982. Ispitivanje dejstva citostatičkih sredstava na ugradnju 3H-thymidina u ćelije kostne srži normalnih i pilića sa eritroleukozom. VII Hematološko transfuziološki dani, *Knjiga sažetaka,* 1982. pp. 36, No. 50 (Abstr.).
14. *Rusov, Č., Živković, R.* 1975. Studies of anemia in chicks with acute erythroleukosis. *Acta Veterinaria, 25,* 107-114.
15. *Schwarzmeir, J. D., Paietta, E., Mittermayer, K., Pirker, R.* 1984. Prediction of the response to chemotherapy in acute leukemia by a short term test in vitro. *Cancer, 53,* 390-395.
16. *Tazzari, P. L., Bolognesi, A., De Totero, D., Lemoli, R. M., Fortuna, A., Conte, R., Crumpton, M. I., Stripe, F.* 1994. Immunotoxins containing saporin linked to different CD2 monoclonal antibodies: In vitro evaluation. *Br. J. Hematol. 86,* 97-105.
17. *Vogler, W. R., Olson, A. C., Hajdu, J., Shoji, M., Raynor, R., Kuo, J. F.* 1993. Structure-function relationships of alkyl-l-lysophospholipid analogs in selective antitumor activity. *Lipids, 28,* 511-516.
18. *Volm, M.* 1984. Use of tritiated nucleotide incorporation for prediction of sensitivity of tumors to cytostatic agents. *Behring Inst. Mitt. 74,* 273-284.
19. *Yamamoto, J., Nagai, Y., Horie, T., Awazusx, S.* 1993. Effect of vitamin A on methotrexate cytoxicity in L 1210 murine leukemia cells in culture. *Cancer Chemother. Pharmacol., 32,* 263-267.

DEJSTVO POJEDINIH CITOTOKSIČNIH AGENASA NA PROLIFERACIJU ĆELIJA KOSTNE SRŽI PILIĆA SA ERITROLEUKEMIJOM

GORDANA KONJEVIĆ, Č. RUSOV i RADMILA ŽIVKOVIĆ

SADRŽAJ

U radu se prikazuju rezultati ispitivanja efekta citotoksičnih lekova na ugradnju ^{3}H-timidina u DNK ćelija kostne srži normalnih i pilića sa akutnom