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EFFECTS OF CYTARABINE AND CYCLOCYTIDINE ON ACTIVATION OF HUMAN T CELLS

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ABSTRACT

Both cytarabine and cyclocytidine are used in the treatment of acute myeloid leukemia. Well known that cytarabine and other related cytosine-based nucleoside analogues are being toxic to tumour cells by increasing levels of cellular oxidative stress as it could be abrogated by antioxidants. However, very little is known both about both the effects of combinations of antimetabolites with anioxidants on the cytotoxic innate and adaptive immune cells and whether lymphocytes toxicity affects its anticancer efficiency.

Materials and methods. T cells derived from blood donors were activated in vitro in cell culture medium alone or supplemented with cytarabine 0.1-10.0 μM or cyclocytidine 0.1-10.0 μM. Cell characteristics were assessed by flow cytometry.

Results. Only cytarabine 1.0-10.0 μM had both antiproliferative and proapoptotic effects. Additionally, these cytarabine concentrations increased the γIFN-producing by CD3+CD4+ T cells and did not affect the release of this cytokine by CD3+CD8+ T cells. In contrast, the lowest concentration (0.1 μM) did not have or showed minor antiproliferative or cytotoxic effects, did not alter the release of γIFN. Cyclocytidine did not affect viability of normal peripheral blood mononuclear cells but decreased the proliferative capacity of activated normal T cells in dose-dependent manner. Additionally, cyclocytidine altered the percentage of γIFN-producing proliferative CD3+CD8+ cytotoxic T cells for any concentration tested (0.1, 1.0, 1 and 10.0 μM) meanwhile highly suppressed the number of the whole amount of CD3+CD8+ cells and did not affect the release of cytokines by CD3+CD4+ T cells.

The study of the expression of the CD107a marker showed a significant stimulating effect of 10 μm of citarabine on the activation of subpopulations of T-lymphocytes (CD3+) and cytotoxic T-lymphocytes (CD3+CD8+).

Keywords: cytarabine; cyclocytidine; oxidative stress; T cells.

1. INTRODUCTION

One of the most important groups of drugs in oncology is cytosine-based nucleoside analogs. Cytarabine (cytosine arabinoside, Ara-C) is extensively used since the late 1960s to treat a variety of oncohematological diseases, in particular acute myeloid leukemia (AML), acute lymphocytic leukemia and different types of lymphomas. High-dose Ara-C is a classical induction regimen aimed at eradicating residual leukemic cells, used to treat AML patients. Cyclocytidine is an anhydro form of ara-C which resists enzymatic deamination due to undergoing slow hydrolysis to ara-C what allows effective plasma levels of ara-C to be maintained for 6 hours following cyclocytidine administration [1].

Although in recent years progress has been made in improving cancer therapy, the treatment for each patient remains a complex problem, in this case for selecting optimal personalized treatment approach needs to be taken into account numerous parameters of the patient's condition and course of malignant process [2-7]. Last years the phenomena of involvement of the immune system in the implementation of the antitumor effect of cytostatic therapy have been experimentally identified, theoretically justified and proved on clinical material [8-10]. Chemotherapeutic drugs can enhance an immune response of the host against the tumor in addition to killing cancer cells by direct cytotoxicity. The immune system makes a crucial contribution to the effectiveness of treatment, involving mechanisms of innate and adaptive immunity and a wide range of cytokines that provide cytotoxic effects on tumor cells. Numerous preclinical and clinical studies have shown that the effectiveness of many chemotherapy drugs depends on the preservation of the functional well-being of the immune system [11-13].

An important aspect limiting the use of cytostatic drugs is that these drugs have undesirable side effects due to possible impact on the host cell's genetic apparatus. In this regard, it is attractive to search for substances or their combinations (with antioxidants, in particular), the use of which will lead to a decrease in intoxication [14]. Numerous original research articles have focused on the topic of whether supplemental antioxidants administered during chemotherapy can protect normal tissue without adversely influencing tumor damage. Due to variation in study design, intervention protocol, type of cancer, timing of observation, inclusive criteria, statistical analysis, and chemotherapy scheme develops uncertainty to make definitive conclusion regarding the risk of decreased tumor control because of administering supplemental antioxidant during chemotherapy. Previous *in vitro* studies have shown that cytarabine and other related cytosine-based nucleoside analogues are being toxic to tumour cells by increasing levels of cellular oxidative stress as it could be abrogated by antioxidants [15]. On the contrary recent review definitely concludes that that antioxidant when given concurrently (a) do not interfere with chemotherapy, (b) enhance the cytotoxic effect of chemotherapy, (c) protects normal tissue and (d) increases patient survival and therapeutic response [16-18].

However, very little is known both about both the effects of combinations of antimetabolites with anioxidants on the cytotoxic innate and adaptive immune cells and whether lymphocytes toxicity affects its anticancer efficiency.

In the present study we therefore investigated the *in vitro* effects of various cytarabine and cyclocytidine concentrations on activated T cells.

2. MATERIAL AND METHODS

2.1 Cell donors and preparation of peripheral blood mononuclear cells

The studies were approved by the local Ethics Committee (Committee on Bioethics of the Republic of Belarus) and buffy coats were derived from healthy blood donors after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Histopaque-1077; «Sigma», Germany; specific density 1.077) from buffy coats from 15 healthy blood donors (median age 39 years; 7 male and 8 female).

2.2 Drugs

Cytarabine (cytosine β-D-arabinofuranoside) and cyclocytidine (cytosine 2,2′-anhydro-β-D-arabinofuranoside) were synthetized [19].
Cytarabine, cyclocytidine, were dissolved in ddH2O to obtain a concentration of 10 mM before aliquoted. All drugs were stored at -80 °C. Drugs were thawed on the same day they were used in experiments and based on studies of *in vivo* levels the drugs were tested at the following concentrations that are relevant to low-toxicity treatment: cytarabine and cyclocytidine 0.1 μM [20-22] and at 10 μM and 1 μM corresponding to high-dose therapy [23,24].

2.3 Cell culture

PBMC were suspended in pre-warmed medium RPMI-1640 (Bio-Whittaker, USA), with 10% FBS (Gibco, Germany), 2 mM L-glutamine (Bio-Whittaker, USA), 1% antibiotic-antimycotic solution (Gibco, Germany) alone or supplemented with cytarabine 0.1-10.0 μM or cyclocytidine 0.1-10.0 μM and cultured in 96-well culture plates at a final concentration of 2.0×10^5 cells/well during 48 hours (viability and proliferation analyses), or during 72 hours (intracellular γIFN production analysis) or at a final concentration of 1.0×10^7 cells/mL during six days (mitogen-induced proliferation analysis).
T lymphocytes were activated with 2.5 μg/mL of phytohemagglutinin (PHA, Sigma, Germany). Drugs were prepared from frozen stock solutions the same day as the experiments. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 before cells/supernatants were harvested.

2.4 Flow cytometric analysis of viability, proliferation and γIFN production

Flow cytometry was performed by Cytoflex (Beckman Coulter, USA). For each sample at least 10 000 cells were counted. All results were analyzed by CytExpert Software (VWR International, LLC, USA).

2.5 Viability and proliferation assay

PBMC dissolved in PBS were stained strictly according to the manufacturer's instructions in the Annexin A5 FITC/7-AAD Kit (BeckmanCoulter, CШA); thereafter cells were washed and cultures prepared as described above. The cells were harvested after 48 hours and stained for 15 minutes with 10 μL Annexin A5-FITC and 20 μL 7-AAD. Cells were further incubated for 15 minutes in dark and thereafter washed in ice-cold 1% BSA/PBS before two-color flow cytometric analysis.
To assess the proliferative capacity after stimulation with PHA cells were stained with 7 μM carboxyfluorescein succinimidyl ester (CFSE, Fluka, Germany) for 5 minutes in dark at room temperature. The staining reaction was stopped by 2-fold centrifugation in a cold medium RPMI-1640 with 25 mM HEPES, 2 mM L-glutamine, 1% streptomycin-penicillin-neomycin and 10% inactivated FBS.
The number of proliferating and non-dividing T-cell subpopulations was registered on the 6th day of culture by flow cytometry using PC7-conjugated anti-CD3, PC5-conjugated anti-CD8 (BeckmanCoulter, USA). The proliferation of T-lymphocytes and their subpopulations was estimated as the percentage of non-dividing (CFSEhigh) and proliferating (CFSElow) T-cells.

2.6 Intracellular γIFN production assay

Spontaneous and PHA-induced intracellular production of γ-interferon (γIFN) was evaluated after 72 hours of cells culture. To quantify the level of intracellular production of γIFN, 10 ng/mL of phorbol 12-myristate 13-acetate (Sigma, Germany), 1 μg/mL of calcium salt of ionomycin (Cayman Chemicals, USA), and 10 μg/mL of brefeldin A (Cayman Chemicals, USA) were added 4 hours before the end of cultivation. Thereafter the following anti-human antibodies were added: PC7-conjugated anti-CD3, PC5-conjugated anti-CD8 (BeckmanCoulter, USA). Cells were fixed for 10 minutes with a 4% solution of *p*-formaldehyde in saline.

Cells were further centrifuged for 5 minutes at 1500 rpm and thereafter PE-conjugated anti-γIFN (Beckman Coulter, USA) was added.

2.7 Cytotoxicity assay

Cytotoxic capacity of PBMC was evaluated with target human tumor cell line K562. K562 target cells were labeled with CFSE at 7 μM concentration to discriminate target cells from effector cells. Then, effector cells (PBMC) were incubated with CFSE labeled K562 target cells at effector-to-target (E : T) ratio 5 : 1 in 96-well plates. The cells were cultured in 150 μL culture media with interleukin-2 (IL-2, Fluka, Germany) as cytotoxity stimulator alone or supplemented with cytarabine 1.0 mM or cyclocytidine 1.0 mM. After coculture for 4 hours at 37 °C, 5% CO_2, the cell mixture was stained with 5 μL of propidium iodide (PI, Invitrogen, Germany) for 15 min in the dark. The non-viable tumor cells were identified as CFSE+PI+K562 cells. PBMC cytotoxicity was calculated as as the ratio of the percentage of cell death K562 in co-culture with IL-2-stimulated PBMC to non-stimulated cells.

2.8 CD107a Degranulation Assay

CD107a expression on effector cells (PBMC or NK cells) was measured to analyze lymphocytes degranulation. Lymphocytes were incubated with or without K562 cells as described above. Following a 4-hour culture, cell mixture was stained with monoclonal antibodies against CD8-FITC, CD107a-PE, CD3-APC, and CD56-PC7 (R&DSystems, Beckman Coulter, USA). The NK and T cells were gated as $CD56^+$ and $CD3^+$ cells respectively, and cytotoxic T lymphocytes were further enumerated as $CD3^+CD8^+$ cells. To determine the CD107a expression of cells, CD107a positive rate of effector cells was analyzed.

2.9 Statistics

Statistical analysis performed using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria). The median, lower and upper percentiles (25th and 75th percentiles) were used for descriptive statistics of the study groups. The statistically significant differences between the compared groups were determined using nonparametric U-Mann-Whitney criterion and Wilcoxon criterion. The differences were considered statistically significant at $P<0.05$.

3. RESULTS AND DISCUSSION

Cyclocytidine do not affect viability of normal peripheral blood mononuclear cells, whereas cytarabine has a small, but statistically significant, antiproliferative and proapoptotic effect.

PBMC derived from healthy blood donors ($n = 15$) were cultivated *in vitro* during 48 hours in medium alone or medium supplemented with cytarabine 0.1-10.0 μM or cyclocytidine 0.1-10.0 μM. The viability (Figure 1; Annexin-AAD assay) of PBMC were then analyzed by flow cytometry. Cyclocytidine did not cause any statistically significant alteration of peripheral blood mononuclear cells viability.

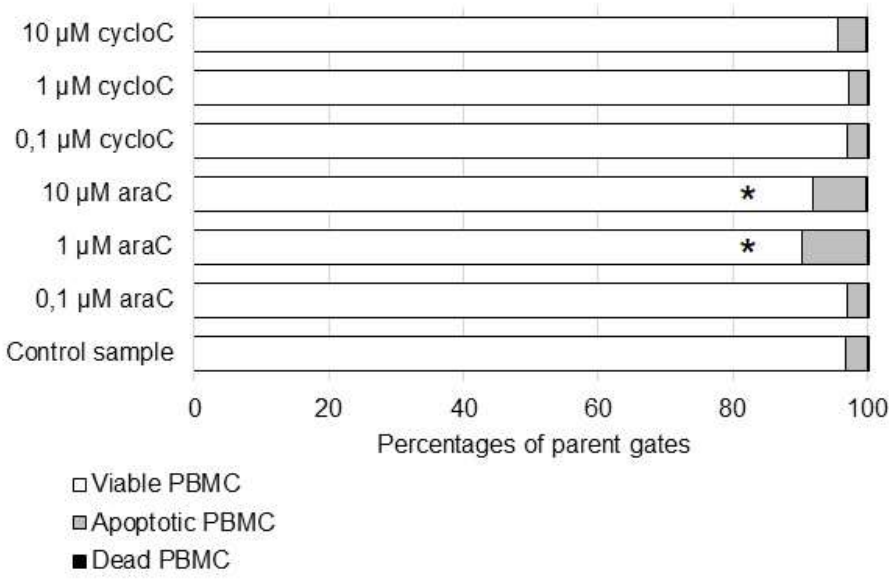

Fig. 1. Viability of peripheral blood mononuclear cells after exposure to cytarabine or cyclocytidine
*Significant from normal control, * P < 0.05*

A small, but statistically significant, decrease in viability was detected after exposure to cytarabine (1.0-10.0 µM).

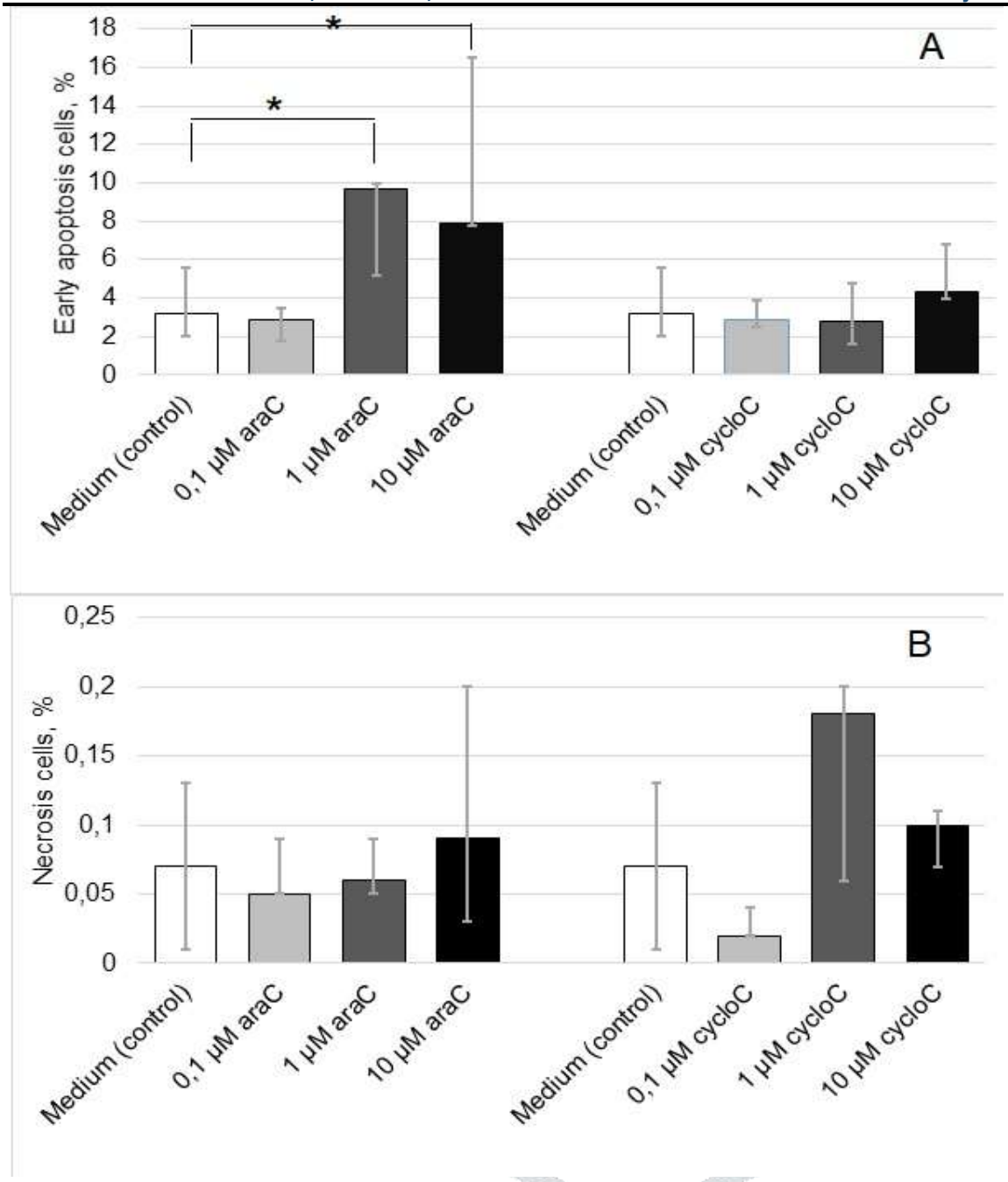

Fig. 2. Number of apoptotic (A) and necrotic (B) cells (%) after exposure to cytarabine or cyclocytidine
*Significant from normal control, * $P < 0.05$*

An increased fraction of early apoptotic cells was then detected together with the decreased viability in the cytarabine-containing cultures (Figure 2A); an observation suggesting that the decreased viability is caused by drug-induced apoptosis. Cyclocytidine at 0.1-10.0 μM concentrations did not cause any statistically significant alteration on PBMC viability.

Both cytarabine and cyclocytidine affect proliferative capacity of activated normal T cells in dose-dependent manner

PBMC derived from healthy blood donors ($n = 15$) were activated *in vitro* culture with anti-CD3 plus anti-CD8 during six days of culture in medium alone or medium supplemented with cytarabine 0.1-10.0 μM or cyclocytidine 0.1-10.0 μM.

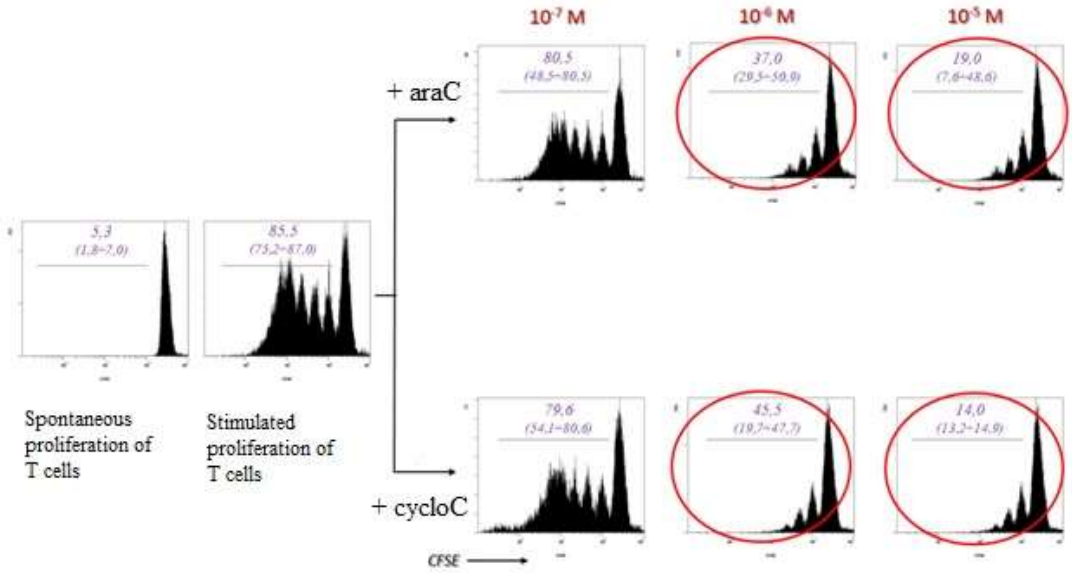

Fig. 3. Proliferation of activated T lymphocytes after exposure to cytarabine or cyclocytidine

The proliferation (Figure 3; the CFSE proliferation assay) of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were then analyzed by flow cytometry. Both cytarabine and cyclocytidine at 1.0-10.0 μM concentrations showed a dose-dependent suppressive effect on stimulated T cell proliferation. A strong and statistically significant decrease in proliferation from 85.5 (75.2-87.0)% to 19.0 (7.6-48.6)% was detected after exposure to 10.0 μM cytarabine. Similarly, when cyclocytidine at a higher concentration of 10.0 μM was present in medium the decrease in proliferation from 85.5 (75.2-87.0)% to 14.0 (13.2-14.9)% was detected.

The suppressive effect on stimulated T cell proliferation was shown both for $CD3^+CD4^+$ T helper lymphocytes as well as $CD3^+CD8^+$ cytotoxic T cells (Figure 4).

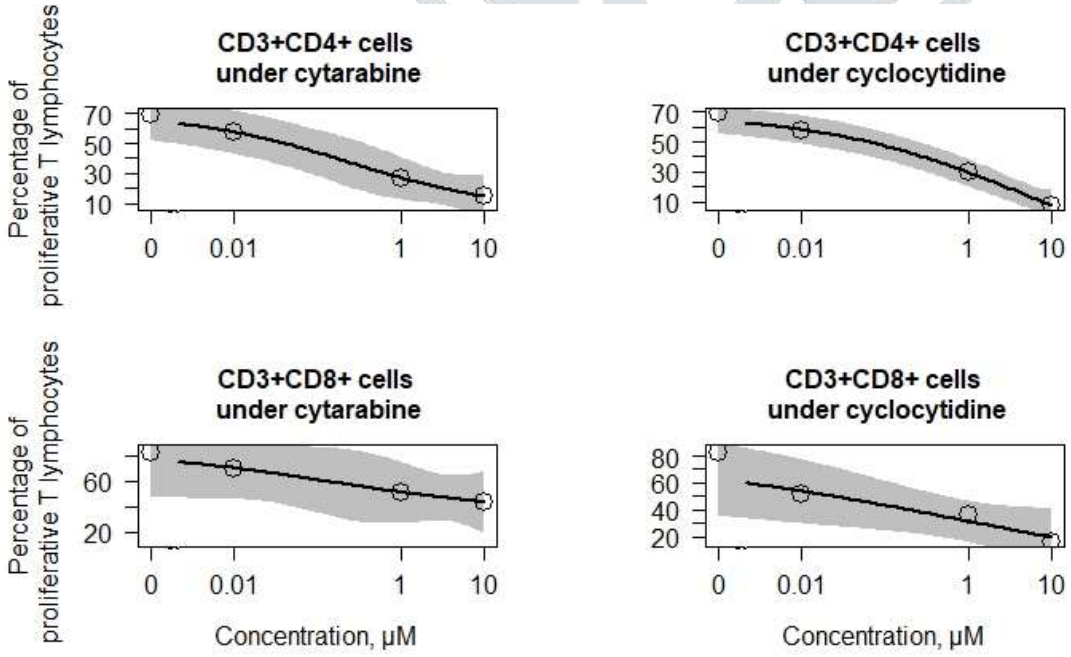

Fig. 4. Proliferation of activated subpopulations of T lymphocytes after exposure to cytarabine or cyclocytidine

The suppression of stimulated proliferation of peripheral blood lymphocytes, be it observed, is developed against the background of a pronounced cytopathic effect of cells, manifested in 97.4-98.8% of cell death; an observation suggesting that the decreased viability is caused by activation-induced apoptosis.

The intracellular γIFN production by stimulated T cells is altered by both cytarabine and cyclocytidine

Normal PBMCs derived from 15 healthy individuals were cultivated *in vitro* during 72 hours in medium alone or medium supplemented with drugs. Production of γIFN was not altered for non-stimulated cultures containing various concentrations of cytarabine and cyclocytidine. Only after PHA-stimulated activation the number of γIFN-producing $CD3^+$ T cells showed an expected dose-dependent reduction caused by cytarabine (0.1, 1.0 and 10.0 μM) but not by cyclocytidine in general.
The response of γIFN-producing $CD3^+CD4^+$ T helper lymphocytes and $CD3^+CD8^+$ cytotoxic T cells on the exposure to cytarabine or cyclocytidine differed. The percentage of proliferative $CD3^+CD4^+$ T cells in total fraction of PBMCs reduced to 16.5% of control after exposure to 10.0 μM cytarabine whereas the number of γIFN-producing $CD3^+CD4^+$ T cells showed a high reduction to 61.2% of control after exposure to the lowest 0.1 μM concentration of cytarabine. Next, the intracellular γIFN production by stimulated $CD3^+CD4^+$ T cells after exposure to 1.0-10.0 μM cytarabine is altered not so dramatically: to 49.8 and 43.6% of control respectively (Figure 5). Thus the $CD3^+CD4^+\gamma IFN^+$: $CD3^+CD4^+$ ratio was significantly increased by cytarabine from 0.7 to 2.6 in dose-dependent manner.

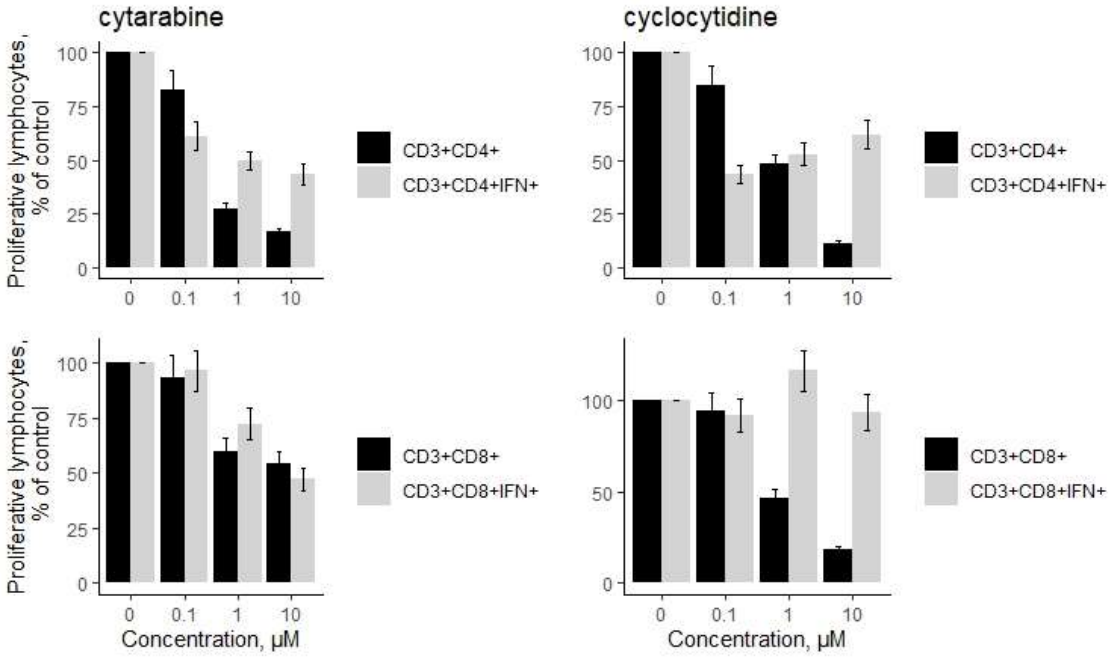

Fig. 5. Intracellular γIFN production of activated subpopulations of T lymphocytes after exposure to cytarabine or cyclocytidine

Cyclocytidine had a similar increasing effect on the $CD3^+CD4^+\gamma IFN^+$: $CD3^+CD4^+$ ratio but only when testing the highest concentration (10.0 μM).
Contrariwise the percentage of γIFN-producing $CD3^+CD8^+$ cytotoxic T cells after the exposure to cytarabine is altered as much as the whole population of cytotoxic T cells and the $CD3^+CD8^+\gamma IFN^+$: $CD3^+CD8^+$ ratio is not changed. In contrast, cyclocytidine altered the percentage of γIFN-producing proliferative $CD3^+CD8^+$ cytotoxic T cells for any concentration tested (0.1, 1.0, 1 and 10.0 μM) meanwhile highly suppressed the number of the whole amount of $CD3^+CD8^+$ cells. Therefore, the $CD3^+CD8^+\gamma IFN^+$: $CD3^+CD8^+$ ratio was significantly increased by cyclocytidine from 0.97 to 5.1 in dose-dependent manner.

The cytotoxicity of non-activated human T cells under cultivation with cytarabine

For evaluating the effects of cytarabine and cyclocytidine on spontaneous and IL-2-stimulated cytotoxicity of lymphoid cells in relation to the K562 tumor cell line, the percentage of dead K562 cells detected as CFSE+PI+K562 cells was used.

Cytarabine inhibits the unstimulated cytotoxicity of PBMCs by 2.1 times ($p < 0.05$), and its combination weakens this inhibitory effect by 57.6%. Cyclocytidine, as well as its combination, does not affect cytotoxicity under experimental conditions. Evaluation of the effects of cytarabine and cyclocytidine and their combinations on the IL-2 stimulated cytotoxicity of PBMCs did not reveal statistically significant differences, but there was also a trend in the inhibitory effect of the compounds (Table 1).

Table 1. Number of non-viable CFSE+PI+K562 (%) in co-cultures with unstimulated and IL-2-stimulated peripheral blood mononuclear cells after exposure to cytarabine or cyclocytidine data presented as median, 25%-75% quantile)

Compound	Spontaneous cytotoxicity	IL-2 stimulated cytotoxicity
Control	19,7 (15,6÷22,0)	41,1 (40,0÷43,2)
Cytarabine	9,2 * (7,0÷10,7)	38,8 (36,2÷42,4)
Cyclocytidine	18,8 (15,2÷21,7)	39,9 (38,7÷41,1)

*Significant from normal control, * P < 0.05*

Co-cultured cells were incubated for 4 hours with 10^{-6} M cyclocytidine or cytarabine separately or together with 10^{-6} M. Each value represents the median, 25th, and 75th percentile of dead K562 cells, calculated as a % of the total cell population.

Cytarabine significantly increased IL-2-stimulated CD107a expression for $CD3^+$ T-lymphocytes (by 2.1 times) and cytotoxic $CD3^+CD8^+$ T-lymphocytes (by 47.5%), but not for natural killer cells ($CD56^+$). The observed effect increased after was added to the culture mixture.

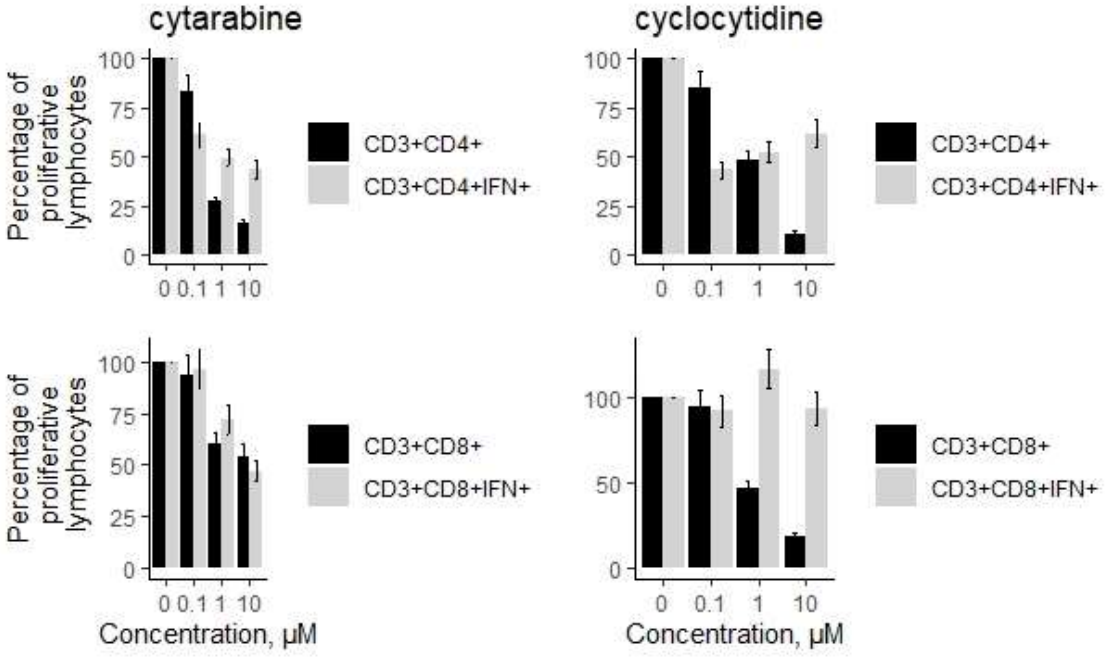

Fig. 6. Percentage (compared to control: PBMC + K562) of $CD107a^+$ lymphoid cells in co-culture with K562 cell line when cultured after exposure to cytarabine or cyclocytidine

*Significant from normal control, * P < 0.05*

Thus, when co-culturing cells with 10^{-6} M cytarabine and 10-6 M, the specific weight of $CD3^+CD107a^+$ cells increased by 3.5 times compared to the control (MPC+K562), and in the case of $CD3^+CD8^+CD107a^+$ cells-by 2.0 times (Figure 6).

4. DISCUSSION

Antimetabolites were the first class of cytotoxic drugs systematically tested in clinical trials that elicited complete clinical responses as monotherapies, albeit with inevitable relapse.[14] Clinical studies have previously proved that cytotoxic agents can affect immune reactions by increasing the antigenic properties of tumor cells, facilitating their recognition of immune system, by stimulation of functional activation effector immune cells, elimination of immunosuppressive factors as well as systemic effects of antitumor therapy. The mechanisms behind these effects are under discussion.

Both cyclocytidine and cytarabine is commonly used in AML therapy. Cytarabine is used at single doses ranging from 10 mg/m^2 up to 3000 mg/m^2 [25] what corresponds to cyclocytidine's doses ranging from 300 mg/m^2 up to 600 mg/m^2 [26]. In our present study we therefore investigated the concentrations 10.0 μM that corresponds to peak levels during high-dose treatment; 1.0 μM that is reached when using the conventional doses of 100-200 mg/m^2; and 0.1 μM that correspond to levels reached early after steady state of low-dose treatment.

Cyclocytidine didn't effect on normal peripheral blood mononuclear cells viability. Cytarabine effects on normal PBMC viability were concentration-dependent; decreased viability was only seen for the higher concentrations and this is in accordance with previous studies suggesting that cytarabine has cytotoxic effects only at concentrations above 100 nM [27]. However, several of our present observations suggest that both cytarabine and cyclocytidine have immunoregulatory effects even at lower level. The cytosine-based nucleoside analogs effects on activated T cells at least partly differ between T cell subsets. This is supported both by (i) the dose-dependent suppressive effect on stimulated T cell proliferation, (ii) the differences between $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells with regard to cytarabine and cyclocytidine effects on γIFN producing during T cell activation; and (iii) previous our studies describing that arabinofuranosylcytosine-5`-monophosphate causes increased in both IFNγ-secreting T-lymphocytes (five times) and IFNγ-secreting other lymphocytes (three times) [19].

Cytarabine decreased AML cell viability only when tested at 0.5 and 0.05 μM [21], in contrast, our results show that T cell proliferation was inhibited with both cytarabine and cyclocytidine only at 10.0 μM concentrations. Thus, based on the proliferation studies we conclude that primary AML cells are more susceptible to cytosine-based nucleoside analogs cytarabine and cyclocytidine than normal T cells; suggesting that there is a therapeutic window for cytarabine and cyclocytidine treatment that makes it possible to achieve antileukemic effects *in vivo* before severe T cell toxicity occurs.

It is known that antitumor therapy is accompanied by significant side effects, which significantly affects the course, prognosis and effectiveness of treatment of diseases [28, 79]. Many of them are directly related to the processes of free radical oxidation, which significantly increase during tumor chemotherapy. Thus, a violation of the balance between free radicals and the antioxidant system can increase intoxication and even lead to toxic damage to healthy organs and tissues, which is a limiting factor when deciding whether to continue therapy [30, 31]. It should be considered that for cancer patients with a malignant tumor, the processes of lipid peroxidation are already activated [32].

However, the widespread use of antioxidant drugs in clinical practice is hindered by their insufficiently studied interaction with elements of the modern generally accepted scheme of complex chemotherapeutic treatment of cancer patients [33-35]. And although a number of studies reflect the possibility of increasing the antitumor effectiveness of individual cytostatics when they are combined with antioxidants, the results obtained cannot be transferred to all components of complex chemotherapeutic treatment and need further detailed studies concerning specific schemes of combined use [36, 37]. In our work, a strong antioxidant emoxipin was used to protect healthy non-malignated cells under the influence of antimetabolites on the tumor culture. As a model of healthy cells, peripheral blood lymphocytes were used, which, along with this, were also a source for subsequent modeling of the immune response to the tumor. At the same time, the results

were obtained indicating the possibility of using antioxidants both to protect healthy cells from death under conditions of oxidative stress caused by antitumor chemotherapy, and as a modulator of the antitumor activity of cytotoxic T-lymphocytes.

5. CONCLUSION

In the present study, neither modified nucleosides nor their combination caused changes in the IL-2-stimulated cytotoxicity of lymphoid cells in relation to the K562 tumor cell line, which does not contradict the known data. However, by the expression of the CD107a marker, we were able to prove a pronounced effect of cytarabine on the activation of a subpopulation of T-lymphocytes ($CD3^+$) and cytotoxic T-lymphocytes ($CD3^+CD8^+$). The obtained results indicate the possibility of direct protection of cytotoxic lymphocytes from death under conditions of oxidative stress caused by antitumor chemotherapy with the antioxidant. Cyclocytidine did not have a pronounced effect, there was a tendency to increase the expression of CD107a on $CD3^+$ T-lymphocytes (by 69.6%) and cytotoxic $CD3^+CD8^+$ T-lymphocytes (by 43.4%).

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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