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IMPACT OF N-ACETYLCYSTEINE ON ANTITUMOR ACTIVITY OF DOXORUBICIN AND LANDOMYCIN A IN NK/Ly LYMPHOMA-BEARING MICE

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N-acetylcysteine (NAC) is a dietary supplement demonstrating antioxidant and liver protecting effects that is widely used in clinics. NAC is considered to possess potential therapeutic activity for health disorders characterized by generation of free oxygen radicals, as well as potential for decreasing negative side effects of various drugs. However, the mechanisms of such tissue-protective actions of NAC remain poorly understood. The main aim of this work was to study therapeutic effects of NAC applied together with the "gold standard" of chemotherapy doxorubicin (Dx) or the novel experimental drug landomycin A (LA) to mice bearing NK/Ly lymphoma. It was revealed that NAC significantly decreased the nephrotoxicity of Dx (measured as creatinine level), possessed moderate immunomodulating activity (as revealed by an increase in number of cytotoxic T-lymphocytes), and partially increased survival of NK/Ly lymphoma-bearing animals treated with Dx. On the contrary, there was little tissue-protective effect of NAC towards LA due to the weak side effects of this anticancer drug, however, the combined use of NAC and LA significantly increased survival (60+ days) of LA-treated animals with NK/Ly lymphoma. Summarizing, NAC possesses a moderate tissue-protective activity towards Dx action but lacks a major therapeutic effect. However, in the case of LA action, NAC significantly increases its anticancer activity with no impact on its negative side effects. Further studies of the molecular mechanisms underlying that activity of NAC towards the action of LA are in progress.

K e y w o r d s: N-acetylcysteine, doxorubicin, landomycin A, NK/Ly lymphoma.

Use of antioxidants for enhancement of the therapeutic activity of conventional anticancer drugs and reduction of their negative side effects remains the most controversial topic in modern pharmacology and medicine. Numerous clinical trials of the combined use of various antioxidants and anticancer drugs have demonstrated the opposite results, with a general conclusion that in several cases antioxidants can enhance the quality of life of cancer patients, but have little effect on their survival [1, 2]. In previous studies [3, 4], we have shown that the dietary compounds selenomethionine and D-panthetine significantly decreased nephro- and myelotoxicity of the well-known anticancer drug doxorubicin (Dx) towards mice bearing experimental tumor models. Moreover, these antioxidants also increased survival of NK/Ly lymphoma-bearing animals under Dx treatment, but showed little effect on B16 melanoma, thus, suggesting their tumor-specific therapeutic action. However, little is known regarding the therapeutic efficiency of well-known antioxidants, such as N-acetylcysteine (NAC), that are widely used in modern medicine.

Being a direct precursor of the glutathione, NAC is considered to be a powerful antioxidant and a potential treatment agent for health disorders characterized by the generation of free oxygen radicals (e.g., obstructive lung disease, contrast-induced nephropathy, articular and neurological diseases, and cancer) [5-7]. NAC was shown to be effective in decreasing negative side effects of several drugs, in particular liver damage that is caused by paracetamol overdose [8] and cyclophosphamide-induced hemorrhagic cystitis [9].

Additionally, NAC decreases cardiotoxicity of another well-known anticancer drug Dx *in vivo*, mainly acting via upregulation of the cellular glutathione system [10]. However, the exact mechanisms underlying these effects of NAC remain poorly understood. Little is known about tissue-protective and therapeutic effects of NAC in animal tumor models in combination with various anticancer drugs. It was found that NAC inhibits gelatinolytic activity of the metalloproteases, thus, blocking the invasive activities of tumor cells and leading to a reduction in the number of melanoma lung metastases in nude mice co-treated with Dx and NAC [11]. However, there are no data about the immunomodulatory and kidney-protecting properties of NAC during Dx treatment.

The main aim of the current study was to evaluate therapeutic effects of NAC applied in combination with the "gold standard" of chemotherapy (Dx) and an experimental anticancer drug (angucycline antibiotic landomycin A) in mice bearing NK/Ly lymphoma. Both compounds share the same tetracyclic quinone core that is responsible for DNA intercalation and superoxide generation in mitochondria by Dx [12]. However, a substitution in the position of one carbon cycle in the molecule of landomycins completely changes their mode of action as compared to Dx, leading to extra-mitochondrial hydrogen peroxide production and further induction of specific caspase-7-mediated early apoptosis in tumor cells [13]. The hydrogen peroxide production by the landomycins is also responsible for significantly lower nephro- and myelotoxicity of these experimental drugs as compared to Dx, thus, suggesting a great potential for their further use in clinical practice. Taking into consideration that LA and Dx induce a production of different types of reactive oxygen species (ROS) in tumor cells, it was of great interest to study if the ROS scavenging activities of NAC can also modulate the negative side effects and therapeutic potential of these anticancer drugs *in vivo*.

Materials and Methods

Materials. LA (99.5% purity, according to HPLC data) was prepared in the laboratory of Prof. J. Rohr (University of Kentucky, Lexington, USA) and dissolved in absolute ethanol to obtain a 4 mg/ml stock solution. Dx hydrochloride was obtained from Pfizer (New York, NY, USA), and NAC (A7250) was purchased from Sigma-Aldrich (St. Louis, MO, USA). NAC was dissolved in sterile 0.9% sodium chloride solution prior to *per os* treatment of animals.

Animal studies. 42 adult male Balb/c mice of 25-28 g weight were purchased from private company "Dali-2001" (Kyiv, Ukraine) and kept under standard vivarium conditions with constant access to full food and drinking water. NK/Ly lymphoma was obtained from the tumor strain collection at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv. The ascite tumor was supported by transferring 0.2-0.3 ml of the ascitic fluid (20-30 x 10^6 cells) from a donor mouse into the abdominal cavity of the recipient mouse. Ascites containing lymphoma cells from the tumor-bearing mice was obtained and transplanted on the 7-8th day after the inoculation. Tumor growth was controlled by everyday weighting of mice. The viability and number of cells in the ascitic fluid were checked by cell counting in a hemocytometric chamber (PJSC "Skloprylad", Ukraine) in the presence of 0.05% trypan blue. The vitality of lymphoma cells in ascites used for transplantation was not less than 98%.

Animals were divided into 7 groups with 6 mice in each (see Fig. 1). Mice in groups 2, 4, and 6 were administered NAC (250 mg/kg, cumulative

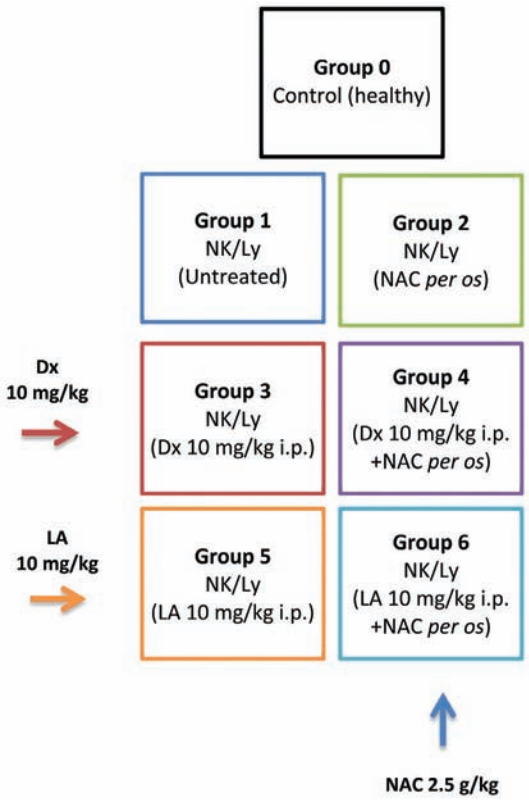

Fig. 1. Animal groups under study. Doxorubicin (Dx); landomycin A (LA); N-acetylcysteine (NAC)

dose 2.5 g/kg) *per os* every second day, from the 2nd until the 10th day of tumor inoculation. Mice in group 0 (healthy controls) and group 1 (NK/Ly lymphoma, untreated) simultaneously received the equivalent volume of 0.9% sodium chloride solution in a similar mode. Dx (2 mg/kg, cumulative dose 10 mg/kg) in groups 3 and 4 and LA (1 mg/kg, cumulative dose 10 mg/kg) in groups 5 and 6 were injected i.p. every second day from the 2nd until the 10th day of tumor inoculation. Treatment of animals with NAC in groups 4 and 6 took place 1 h before Dx or LA injection. Blood sampling for the biochemical and cytomorphological studies was done at the 14th and 21st day after tumor inoculation.

All *in vivo* experiments were conducted in accordance with the international principles of the European Convention for protection of vertebrate animals under control of the Bio-Ethics Committee of the above mentioned Institution (Protocol N4/2017 from 1.06.2017 of the BioEthics Committee at the Institute of Cell Biology, NAS of Ukraine).

Blood analysis. For blood sampling, amputation of the small part of the mouse tail was done and ~50 µl of blood were pumped into a test tube, followed by immediate disinfection of the wound with 70% alcohol. For the counting of red blood cells, 5 µl of blood were dissolved in 5 ml of isotonic NaCl solution (1 : 1,000 dilution), while for leukocytes 5 µl of blood were dissolved in 95 µl of 3% acetic acid solution (1 : 20 dilution). Erythrocytes were counted in 5 big squares (divided into 16 small squares each) of the hemocytometric chamber, while the leukocytes were counted in 100 big squares, divided into 4, under the Evolution 300 Trino microscope (Delta Optical, Mińsk Mazowiecki, Poland). The numbers of erythrocytes and leukocytes were calculated by using standard formulas, as previously described [14].

For blood smear preparation, 3 µl of blood were put at the edge of a slide, and then spread for 1.5 cm using another narrow polished slide, placed at a 45° angle. The obtained smears were dried at room temperature, then fixed with absolute methanol and later rehydrated by subsequent washing in ethanol solutions with decreasing concentrations (96, 75, 50, 25 and 12.5%). Finally, the smears were washed with distilled water, stained with Giemsa dye using standard protocol, and air-dried, after which they were ready for leukogram analysis.

Counting of the leukocytes was performed under the Evolution 300 Trino microscope (Delta Optical, Mińsk Mazowiecki, Poland) using a 90x oil immersion objective. Cell counting was always done using the same system – half of the cells were counted in the upper half part of the smear, and the other 50% of cells were counted on the lower half part of the smear. The percentages of certain types of white blood cells in each smear were determined after counting of at least 300 cells. The obtained values (due to differences in the absolute numbers of cells in each counted smear) were normalized to 100%, and the percentage values of each leukocyte fraction were calculated as previously described [15].

Nephrotoxicity studies. The creatinine level in blood serum of the experimental animals was measured spectrophotometrically by using the Popper method based on the Jaffe reaction [16]. Briefly, blood serum samples were diluted 1 : 20 in working reagent solution (0.75 M NaOH and saturated picric acid, mixed 1 : 1), and their optical density was measured by a Helios UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 510 nm wavelength after 30 (E_1) and 90 sec (E_2) following sample addition to the working reagent solution. The obtained results were compared to standard (creatinine solution, 440 µM) and the final creatinine content in blood serum samples was calculated using the following formula:

$$\text{Creatinine } (\mu M) = 440 \times ((E_2 - E_1)_{sample} / (E_2 - E_1)_{etalon}).$$

All experiments were performed in triplicate and repeated 3 times. Statistical analysis of the data was conducted using GraphPad Prisms Software (GraphPad Software, Inc, La Jolla, CA, USA) using Student's t test. Statistical significance was set at $P \leq 0.05$.

Results and Discussion

We found that both studied anticancer drugs possessed strong therapeutic activity towards NK/Ly lymphoma (Fig. 2). In particular, 33% of animals survived for more than 60 days with Dx treatment (10 mg/kg), while LA used at the same dose led to tumor remission in 66% of NK/Ly lymphoma-bearing animals. NAC alone (2.5 g/kg) did not demonstrate any impact on survival of animals in the control group. However, in combination with Dx, NAC increased the survival index from 33% to 50%. The effect of NAC used in combination with LA was even more profound and led to tumor regression in 100% of animals.

The animal survival indicator was tightly correlated with changes in animal body mass. NK/Ly

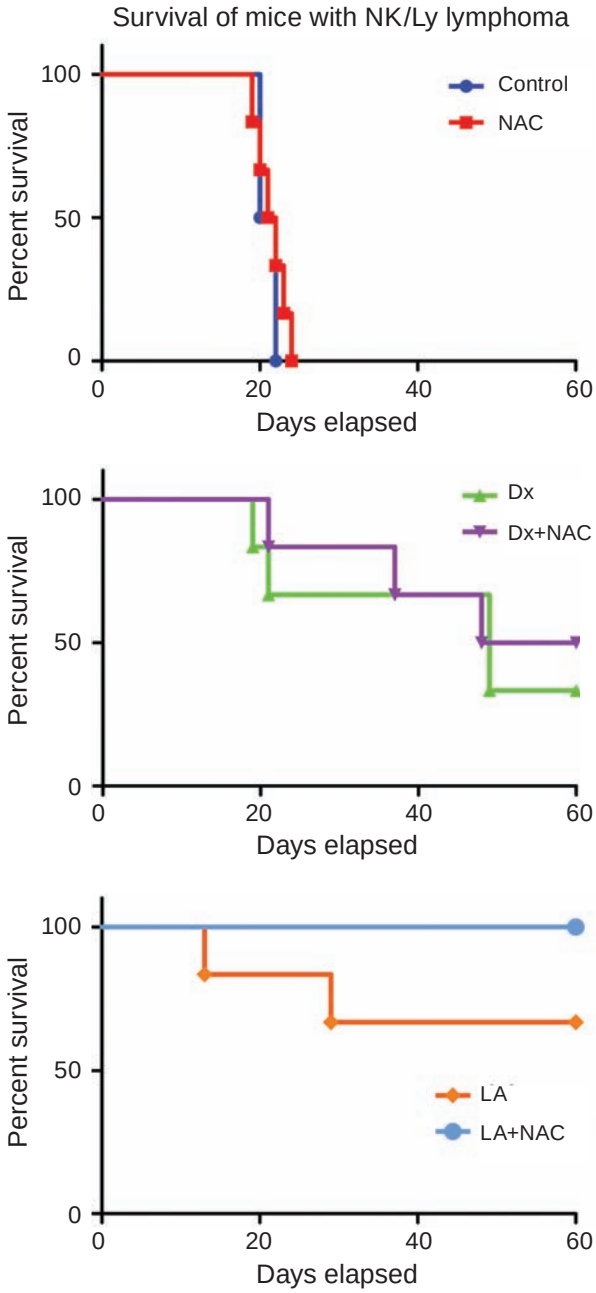

Fig. 2. Percent of survival of animals with NK/Ly lymphoma treated with doxorubicin (Dx), landomycin A (LA) and N-acetylcysteine (NAC)

lymphoma growth was accompanied by development of a large volume of ascites that correlated with an increase in animal body weight [17, 18] – up to 2-fold at terminal stages of tumor growth (20-21 days after inoculation) compared to the control group (Fig. 3). Both anticancer drugs effectively suppressed growth of this ascitic tumor, as revealed by a visual absence of the ascites. However, Dx also produced severe side effects leading to a 15% decrease in animal body weight at the 18th day after tumor inoculation (and 10th day after starting chemotherapy) as compared to controls (healthy animals) (Fig. 3).

In contrast to Dx, LA had no negative impact on the morphophysiological status of animals (body weight loss was less than 5%), assuming that this experimental antitumor compound induced minor side effects. Co-treatment of animals with NAC stabilized animal body weight in both cases, returning it to the control level, though the effect of NAC was more profound for animals in the Dx group (Fig. 3). Weight loss is considered a typical hallmark of Dx toxicity [19]. Thus, NAC treatment enhances the anticancer activity of Dx in two ways – by increasing the therapeutic effect of Dx in a model of NK/Ly lymphoma, as well as by decreasing the negative side effects of the drug. Surprisingly, a combination of LA and NAC was even more effective, leading to 100% survival of NK/Ly lymphoma-bearing animals.

Growth of NK/Ly lymphoma is accompanied by severe cachexia [17, 18], as revealed by a 3-fold decrease in the serum creatinine level as compared to healthy animals due to massive protein degradation (Fig. 4) [20]. However, Dx therapy strongly enhanced this index, indicating massive kidney damage, and that effect was preserved both on the 14th and 21st days after tumor inoculation. Co-treatment of animals with Dx and NAC partially decreased Dx nephrotoxicity, lowering it by 1.5-fold, but it still remained at a high level as compared to the untreated group. LA in the same dose (10 mg/kg) also demonstrated signs of nephrotoxicity (1.75-fold elevation of the creatinine level), while NAC partially decreased it compared to a control. Taking into consideration that nephrotoxicity of Dx is caused mainly by excessive ROS production [21], one can assume that NAC also possesses moderate nephroprotective abilities, hence decreasing the negative side effects of LA and NAC, besides enhancing their therapeutic potential.

Severe leukocytosis and erythropenia are other indicators of NK/Ly lymphoma progression (Fig. 5). Both applied anticancer drugs decreased tumor-induced leukocytosis and partially normalized the amount of erythrocytes in the blood of tumor-bearing animals, while in combination with NAC, Dx possessed even stronger effects (Fig. 5) that suggest the immunomodulatory properties of NAC. In order to study the effect of these agents in more detail, a full-scale analysis of the leukograms of tumor-bearing animals was carried out. It was revealed that

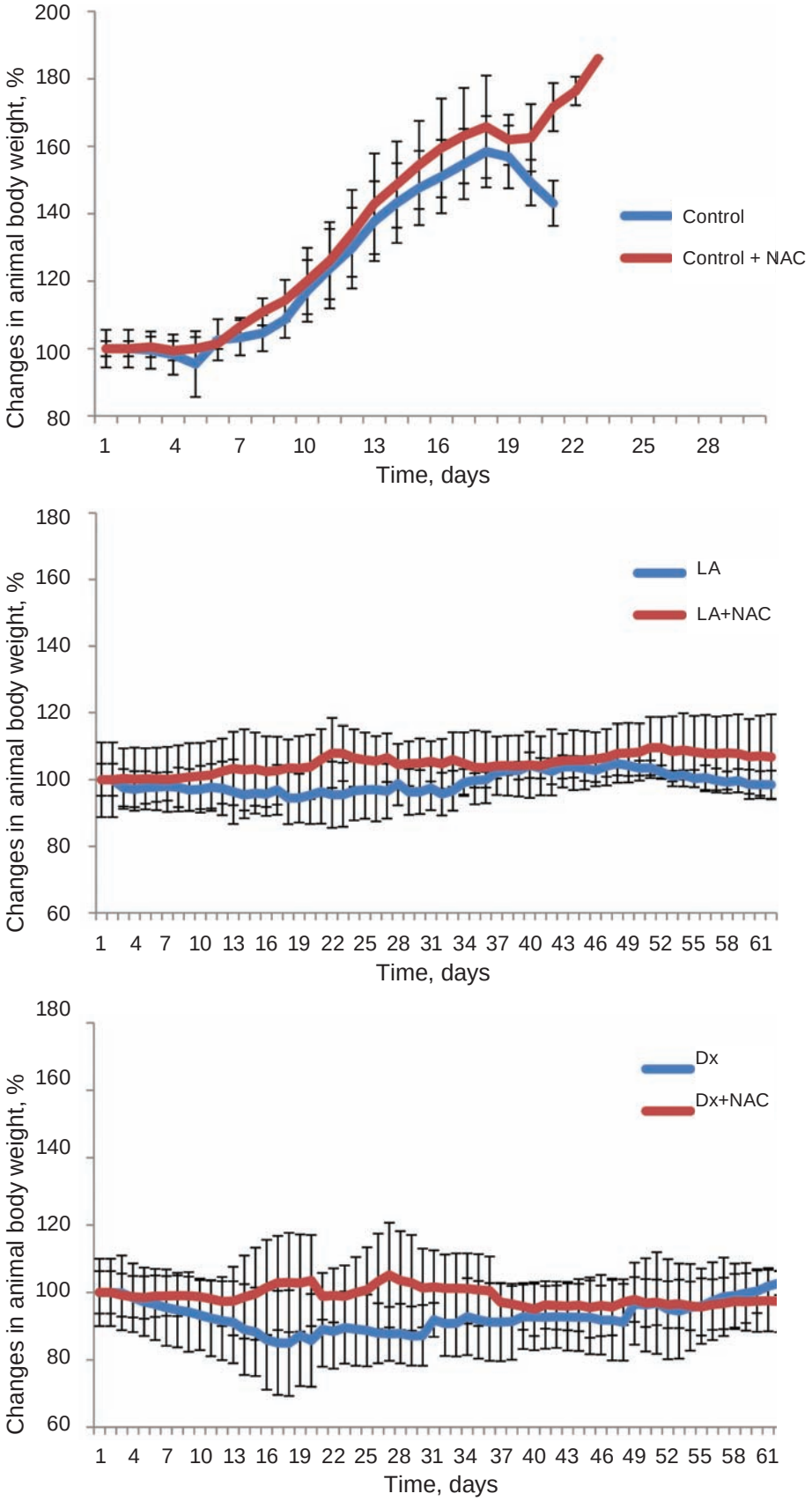

Fig. 3. Dynamics of weight of animals with NK/Ly lymphoma treated with doxorubicin (Dx), landomycin A (LA) and N-acetylcysteine (NAC)

in NK/Ly lymphoma-bearing animals, the leukocytosis was caused mainly by a 3.5-fold increase in the number of segmented neutrophils that was observed already at the 14th day after tumor inoculation, while the number of small lymphocytes (e.g. cytotoxic T-cells) was gradually decreased by 3-fold (Fig. 6). Dx treatment significantly (2-fold) reduced tumor-induced neutrophilia at the 14th day after tumor inoculation, however, it failed to maintain it at the same low level at the 21st day (Fig. 6). A co-treatment with Dx and NAC was more effective at the 21st day after tumor inoculation, as compared to the action of Dx alone decreasing this index by 1.5-fold as compared to the untreated group. An opposite tendency was observed for measuring small (cytotoxic) T-lymphocytes – this index increased by 1.6-fold at the 14th day under Dx treatment (in comparison to the untreated group), however, it dropped at the 21st day, suggesting insufficient therapeutic activity of Dx. A co-treatment with Dx and NAC possessed much stronger immunomodulating properties, increasing that index by 2-fold at the 14th day and by 1.5-fold at the 21st day after tumor inoculation. NAC was also effective in lowering the monocytosis that is another typical hallmark of the negative side effects of Dx (Fig. 6). A similar action of Dx was observed earlier when we used NK/Ly lymphoma and B16F10 melanoma experimental animal models [3, 4]. We did not find a statistically significant impact of Dx on the level of ring-shaped (young) neutrophils, though it restored a number of big lymphocytes (B-lymphocytes and NK cells) almost to control levels, without visible effects of NAC.

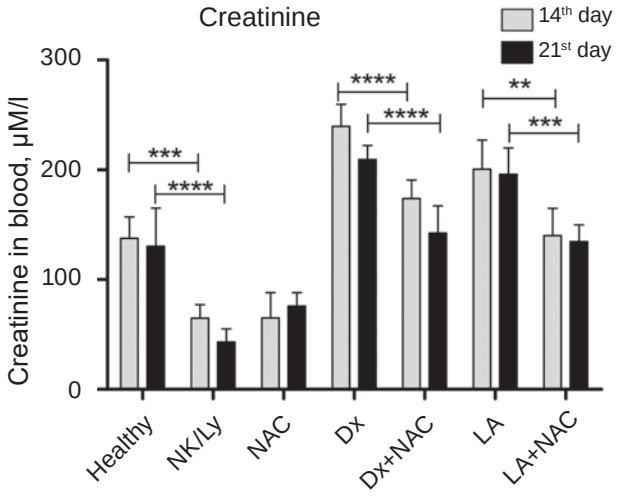

*Fig. 4. Indicators of changes in the level of creatinine in NK/Ly lymphoma-bearing animals treated with landomycin A (LA) and doxorubicin (Dx) alone or in combination with N-acetylcysteine (NAC), at the 14th day and 21st day after tumor inoculation. **P < 0.01; ***P < 0.001; ****P < 0.0001*

In contrast to Dx, LA-based chemotherapy had no myelosuppressive impact on tumor-bearing animals, maintaining all studied blood indices at the level of control (untreated) mice (Fig. 6) both at the 14th and 21st day after tumor inoculation. Thus, due to absence of the myelosuppressive activity of LA,

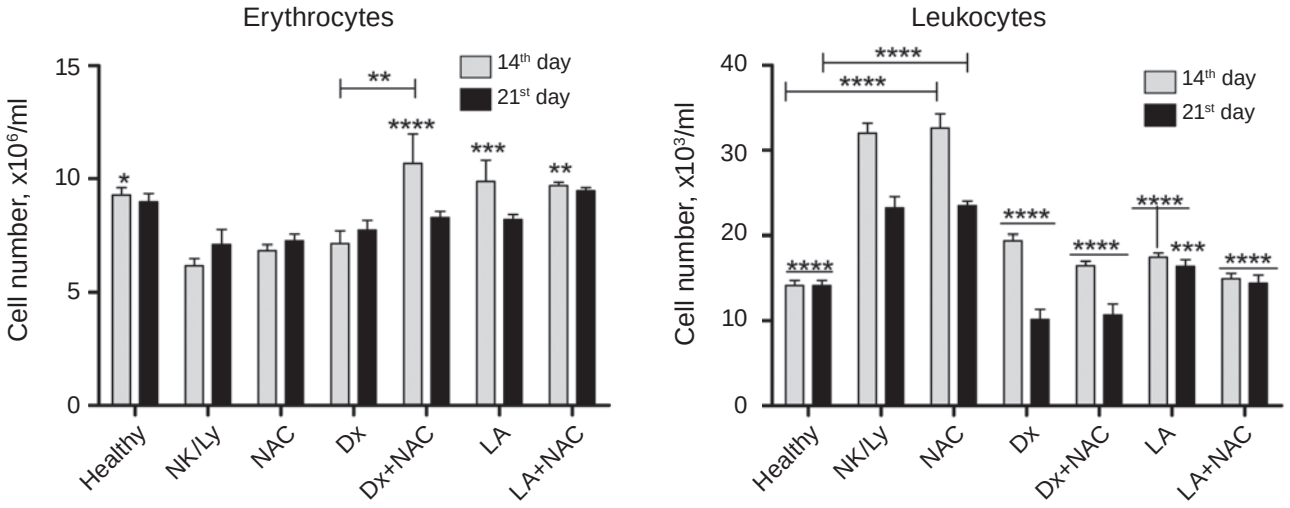

*Fig. 5. Indicators of number of erythrocytes and leukocytes in NK/Ly lymphoma-bearing animals treated with doxorubicin (Dx) or landomycin A (LA) in combination with N-acetylcysteine (NAC), at the 14th day and 21st day after tumor inoculation. *P < 0.05 related to NK/Ly (untreated). **P < 0.01 related to NK/Ly (untreated). ***P < 0.001 related to NK/Ly (untreated). ****P < 0.0001 related to NK/Ly (untreated)*

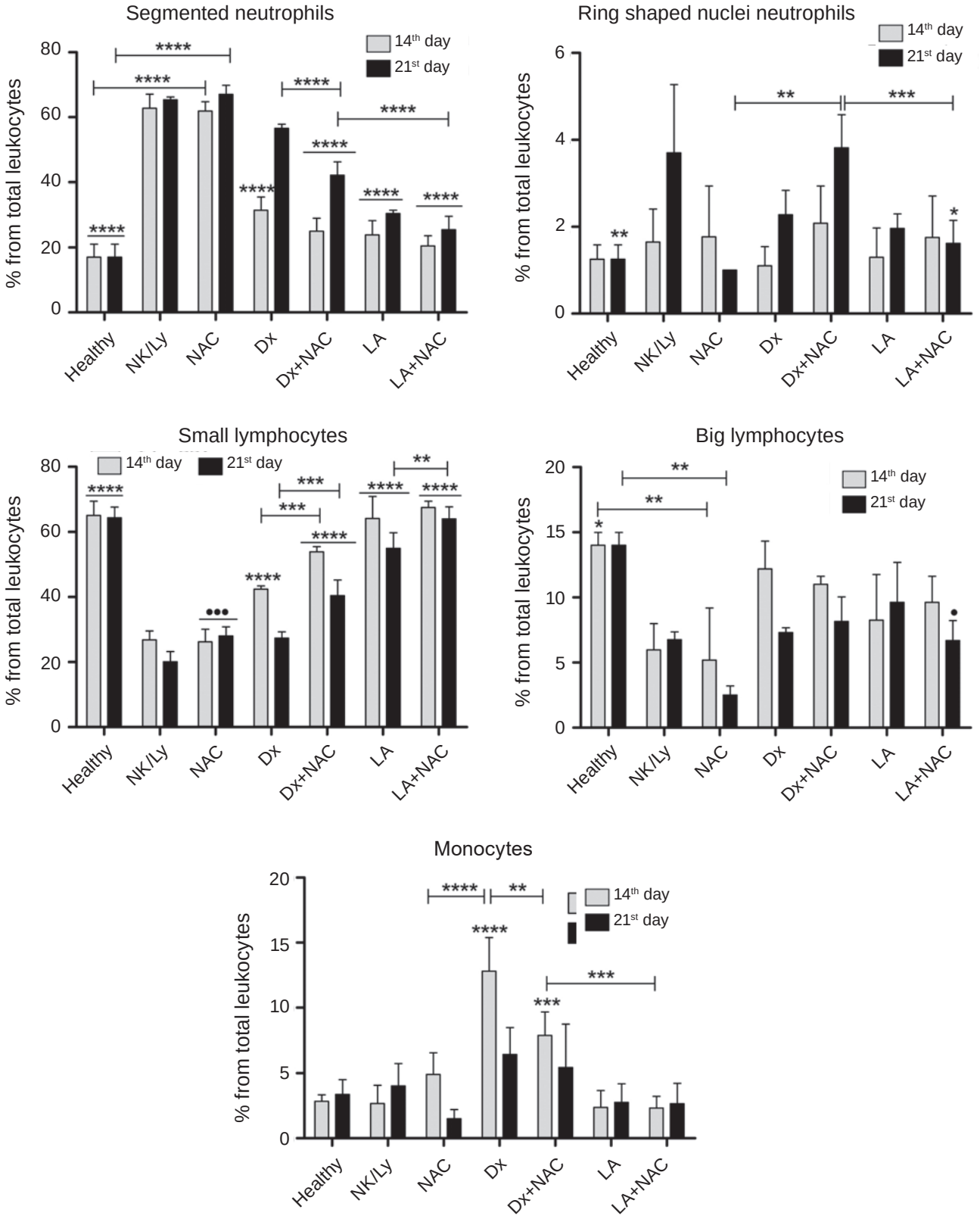

*Fig. 6. The leukogram of NK/Ly lymphoma-bearing animals treated with doxorubicin (Dx) and landomycin A (LA) alone or in combination with N-acetylcysteine (NAC), at the 14th day and 21st day after tumor inoculation. *P < 0.05 related to NK/Ly (untreated). **P < 0.01 related to NK/Ly (untreated). ***P < 0.001 related to NK/Ly (untreated). ****P < 0.0001 related to NK/Ly (untreated). •••P < 0.001 related to control (healthy). •P < 0.05 related to control (healthy)*

we did not observe any immunomodulatory effects of NAC. Nevertheless, a co-treatment of animals with LA and NAC was beneficial for tumor-bearing animals, since it led to a complete remission of NK/Ly lymphoma (60+ day survival), while in the LA group, only 66% of animals survived at the 60th day after tumor inoculation.

Summarizing, NAC reduces NK/Ly lymphoma-induced neutrophilia and lymphopenia and decreases monocytosis caused by Dx treatment in tumor-bearing animals. In addition, this antioxidant partially reverses the nephrotoxicity of Dx, thus, leading to an increased survival of the tumor-bearing animals under Dx treatment. The observed immunomodulating and kidney-protecting properties of NAC might be of great importance for decreasing negative side effects of Dx. On the contrary, NAC did not affect the blood profile of animals treated with LA due to the low toxicity of this experimental drug. At the same time, NAC partially reversed LA-induced nephrotoxicity and caused a complete remission in mice bearing NK/Ly lymphoma. Studies of the molecular mechanisms underlying such features of NAC are in progress.

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