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Immune responses of bovine leukemia virus-infected guinea pigs to betulinic acid amide administration

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ABSTRACT

Introduction. Betulinic acid is a naturally occurring compound with significant potential for the development of anti-leukemia therapeutics. It demonstrates antiviral activity alongside a marked immunomodulatory effect, properties that can be potentiated through the introduction of a novel pharmacophore group into its molecular framework.

Objective. The study was aimed at evaluating the therapeutic antiviral activity of betulinic acid amide, as well as the immune response of bovine leukemia virus (BLV)-infected guinea pigs to its administration.

Materials and methods. Fifteen Agouti guinea pigs, 4–5 months of age, were used for the study. Ten animals received a single intraperitoneal inoculation of lymphocyte suspension from leukemic cows; the remaining five animals served as intact controls. On day 21 after infection, five of the infected animals were subcutaneously injected with betulinic acid amide at a dose of 40 μg/kg (group 1), whereas the other five infected animals were left untreated (group 2). Blood samples were collected prior to the treatment, and then at 14 and 28 days after treatment to assess cellular and humoral immune responses and for molecular biological studies.

Results. The study showed that the immune response of the guinea pigs to inoculation with the virus-containing suspension was characterized by a significant increase in lymphoid cell counts by day 49 of the experiment. This increase was caused by a 1.85-fold boost in B-lymphocyte and a 2.7-fold boost in cytotoxic T-lymphocyte proliferation. Administration of betulinic acid amide on day 21 post-infection resulted in suppression of B-cell and cytotoxic T-lymphocyte proliferation, 2.1-fold increase in myeloperoxidase and cationic protein activity in neutrophils, and stimulation of antibody production. However, the experimental drug failed to confer protection against the infection: specific fluorescence was detected with direct immunofluorescence assay and proviral DNA was detected in blood samples from 100 and 60% of the animals, respectively.

Conclusion. Administration of the betulinic acid derivative prevents immune dysregulation in BLV-infected guinea pigs as evidenced by normalized lymphoid cell counts and enhanced neutrophil functional and metabolic activity. These findings indicate that betulinic acid amide is a promising veterinary candidate for preventing the development of clinical and hematological leukosis in infected animals.

Keywords: guinea pigs, bovine leukemia virus, betulinic acid amide, antiviral activity, immune status

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Иммунные реакции у морских свинок, инфицированных вирусом лейкоза крупного рогатого скота, на введение амида бетулиновой кислоты

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РЕЗЮМЕ

Введение. Перспективным природным химическим соединением для разработки противолейкозных препаратов является бетулиновая кислота, обладающая противовирусной активностью в сочетании с выраженным иммуномодулирующим эффектом, которые можно усилить путем введения в ее структуру новой фармакофорной группы.

Цель исследования. Оценка терапевтической противовирусной активности и эффективности иммунных реакций на введение амида бетулиновой кислоты морским свинкам, инфицированным вирусом лейкоза крупного рогатого скота.

Материалы и методы. Исследование провели на 15 морских свинках линии агути 4–5-месячного возраста, из которых 5 интактных служили

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в качестве контроля, а остальные 10 особей были инфицированы взвесью лимфоцитов, выделенных из крови больных лейкозом коров, путем однократного внутрибрюшинного введения. Затем на 21-е сут после инокуляции патогена 5 животным подкожно инъецировали амид бетулиновой кислоты в дозе 40 мкг/кг (1-я группа), другим 5 особям препарат не вводили (2-я группа). До введения, а также на 14-е и 28-е сут после инъецирования экспериментального препарата производили отбор проб крови для оценки клеточных и гуморальных реакций иммунитета, а также молекулярно-биологического исследования.

Результаты. Установлено, что иммунный ответ на инокуляцию морским свинкам вируссодержащей суспензии сопровождался достоверным увеличением к 49-м сут от начала эксперимента числа лимфоидных клеток, происходящим за счет пролиферации В-лимфоцитов в 1,85 раза и цитотоксических Т-лимфоцитов в 2,7 раза. Введение на 21-е сут после инфицирования амида бетулиновой кислоты индуцировало подавление клеточного размножения В-клеток и цитотоксических Т-лимфоцитов, усиливало активность внутриклеточных компонентов нейтрофилов миелопероксидазы и катионных белков в 2,1 раза, а также стимулировало выработку антител. Однако экспериментальный препарат не предотвращал заражения животных, на что указывало наличие специфического свечения в прямой реакции иммунофлуоресценции, а также наличие провирусной ДНК в пробах крови соответственно у 100 и 60% особей.

Заключение. Применение производного бетулиновой кислоты сдерживает развитие нарушений баланса в иммунной системе у морских свинок, инфицированных вирусом лейкоза крупного рогатого скота, оптимизируя численность лимфоидных клеток и усиливая функционально-метаболическую активность нейтрофилов. Полученные результаты позволяют предположить возможное использование амида бетулиновой кислоты в ветеринарии с целью профилактики развития клинической и гематологической формы лейкозной инфекции у животных.

Ключевые слова: морские свинки, вирус лейкоза крупного рогатого скота, амид бетулиновой кислоты, противовирусная активность, иммунный статус

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INTRODUCTION

Bovine leukemia virus (BLV) is an oncogenic retrovirus that exhibits tropism for B-lymphocytes. The resulting chronic infection manifests in one of three clinical outcomes: an asymptomatic carrier state, which occurs in the vast majority of animals; persistent lymphocytosis, observed in approximately one third of infected animals; or, in rare cases, the development of lymphoid tumours [1, 2, 3].

Even in the most common subclinical disease form, where the pathogen is detectable based only on antibody and/or proviral DNA presence, distinct immune system imbalance is observed. This results in substantial economic losses in animal production due to reduced host resistance and increased susceptibility to opportunistic infections. As a result, the risk of udder infections increases, particularly during the critical period of a cow's life [4, 5], while rumen and large intestine microbiota [6] undergo significant changes. At the same time, the effectiveness of antibacterial and anti-parasitic treatments – including those for chronic diseases – as well as vaccines against other animal diseases is reduced [7, 8].

Despite the widespread occurrence of leukemia infection and the substantial economic losses it causes, there are currently no effective vaccines or treatment methods capable of eliminating the virus [9, 10, 11]. Nevertheless, some researchers are actively investigating natural chemical compounds that may confer effective resistance to BLV infection [12, 13, 14]. The data obtained by them convincingly show that certain metabolites have the potential to serve as the basis for developing new anti-leukemic drugs.

During long-term studies aimed at discovering new natural products with high antiretroviral activity, betulinic acid and its precursor, betulin, were identified. Due to their unique chemical structures, these compounds are promising natural metabolites and their synthetic transformation and modification can enhance biological activity and help to overcome microbial resistance [15]. It has been shown that, depending on its chemical structure, betulinic acid can act either as an inhibitor of human immunodeficiency virus type 1 (HIV-1) entry or as an inhibitor of HIV-1 protease or reverse transcriptase [16, 17].

Structural modifications of betulin and betulinic acid have yielded antiviral compounds with improved pharmacological properties. In particular, betulinic acid ionic salts conjugated with glycine slowed down cellular metabolism and reduced the production of oncogenic murine leukemia virus in mouse embryonic fibroblast cells [18]. Furthermore, several compounds based on betulinic acid and its synthetic derivative, bevirimat, has been successfully synthesized. These compounds represent a promising class of agents for the development of next-generation HIV-1 maturation inhibitors [19, 20, 21].

Our recent study [22] showed that a new amide derivative of betulinic acid incorporating a pharmacophoric 1-(1-adamantyl)ethylamine group

demonstrated a pronounced inhibitory effect against BLV *in vitro*. Its antiviral activity was subsequently confirmed in guinea pigs experimentally infected with BLV.

The study was aimed at evaluating the therapeutic antiviral activity of betulinic acid amide, as well as the immune response of guinea pigs experimentally infected with BLV to its administration.

MATERIALS AND METHODS

Agouti guinea pigs (4–5 months of age, body weight 400–500 g) were used for the experiments in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the experiments were approved by the local independent Ethics Committee of the Omsk Agrarian Scientific Centre.

Fifteen clinically healthy guinea pigs were selected. The guinea pigs were confirmed free from BLV antigen in their blood with direct immunofluorescence reaction (DIF), and free from proviral DNA with polymerase chain reaction (PCR). The animals were divided into 3 groups, 5 animals per group. The first group (n = 5) contained intact animals (control group). The remaining guinea pigs (n = 10) were inoculated once intraperitoneally with 1 mL of lymphocyte suspension from the cow with haematological leukosis manifestations (lymphocyte concentration in 1 mL of suspension was 10.0×10^9, the DIF virus titre – 1:2048).

When the animals were tested positive with DIF and PCR on day 21 of the experiment, 5 animals (group 1) were subcutaneously injected with betulinic acid amide at a dose of 40 μg/kg, the other 5 animals (group 2) remained non-injected and were used as positive controls.

The amide derivative of betulinic acid was generated by I. V. Kulakov, Professor, Doctor of Science (Chemistry), at the Department of Organic and Environmental Chemistry of the University of Tyumen [22].

Blood samples were taken from the retroorbital venous plexus before the experimental drug injection as well as on day 14 and 28 after the experimental drug injection to assess cellular and humoral immune responses, as well as for PCR testing.

Leukocyte counts, differentials, and lymphocyte subsets (T-lymphocytes, B-lymphocytes, cytotoxic T-lymphocytes) were evaluated with spontaneous, complement-dependent, and indirect immunoglobulin rosette formation tests. Neutrophil activity, both spontaneous and stimulated, was measured with nitroblue tetrazolium (NBT) assay using photometer. The functional reserve was subsequently calculated as the ratio of stimulated tetrazolium activity to spontaneous tetrazolium activity [23].

Myeloperoxidase enzymatic activity and neutrophil cationic protein content were determined cytochemically using the Graham – Knoll method with benzidine [24] and the M. G. Shubich method with bromophenol blue, respectively [25]. Smears were examined microscopically, and the percentage of positively reacted cells was calculated. The average cytochemical coefficient (ACC) was then determined using standard methods.

Antibodies in guinea pig sera were detected with indirect immunofluorescence reaction (IIF). Sera

Table 1
Immunological parameters in guinea pigs before the experimental drug administration (day 21 after infection with BLV), $M \pm m$

Parameter, measuring units	Control ($n = 5$)	Group 1 ($n = 5$)	Group 2 ($n = 5$)
White blood cells, thousands of cells/μL	9.72 ± 0.70	12.74 ± 0.94	12.94 ± 1.07
Lymphocytes, thousands of cells/μL	5.34 ± 0.37	7.28 ± 0.51	7.99 ± 0.61[1]
T-lymphocytes, thousands of cells/μL	0.81 ± 0.12	0.82 ± 0.07	0.81 ± 0.09
B-lymphocytes, thousands of cells/μL	1.14 ± 0.17	1.87 ± 0.25	1.74 ± 0.17
Cytotoxic T-lymphocytes, thousands of cells/μL	0.59 ± 0.06	1.62 ± 0.30[1]	2.01 ± 0.04[1]
ACC CP, CU	1.11 ± 0.03	2.01 ± 0.18[1]	2.02 ± 0.08[1]
ACC MPO, CU	0.97 ± 0.09	2.16 ± 0.16[1]	1.89 ± 0.08[1]
Spontaneous NBT test, OD	0.46 ± 0.03	0.44 ± 0.02	0.40 ± 0.04
Stimulated NBT test, OD	0.43 ± 0.02	0.45 ± 0.02	0.42 ± 0.03
Functional neutrophil reserve	0.94 ± 0.02	1.03 ± 0.06	1.11 ± 0.13
IIF antibody titre, $\log_2$	not detected	8.60 ± 0.40	8.80 ± 0.37
PCR reactors, %	not detected	100.0	100.0

[1] statistically significant difference ($p_1 < 0.05$) confirmed by Tukey's test as compared to the control group;
ACC CP – average cytochemical coefficient of cationic proteins; ACC MPO – average cytochemical coefficient of myeloperoxidase; OD – optical density; CU – conventional units.

Table 2
Immunological parameters in guinea pigs on day 14 after the experimental drug administration (day 35 after infection with BLV), $M \pm m$

Parameter, measuring units	Control ($n = 5$)	Group 1 ($n = 5$)	Group 2 ($n = 5$)
White blood cells, thousands of cells/mL	10.00 ± 0.35	10.40 ± 1.58	12.54 ± 1.00
Lymphocytes, thousands of cells/mL	5.52 ± 0.41	4.10 ± 0.74	7.57 ± 0.76[2]
T-lymphocytes, thousands of cells/mL	0.88 ± 0.12	0.75 ± 0.09	0.73 ± 0.29
B-lymphocytes, thousands of cells/mL	1.11 ± 0.14	0.46 ± 0.09	1.90 ± 0.29[2]
Cytotoxic T-lymphocytes, thousands of cells/mL	0.80 ± 0.11	0.53 ± 0.12	2.00 ± 0.42[1, 2]
ACC CP, CU	1.21 ± 0.10	2.30 ± 0.09[1]	1.29 ± 0.05[2]
ACC MPO, CU	1.13 ± 0.08	1.92 ± 0.13[1]	1.24 ± 0.09[2]
Spontaneous NBT test, OD	0.51 ± 0.009	0.49 ± 0.02	0.47 ± 0.03
Stimulated NBT test, OD	0.50 ± 0.02	0.55 ± 0.09	0.41 ± 0.04
Functional neutrophil reserve	0.97 ± 0.03	1.11 ± 0.13	0.87 ± 0.07
IIF antibody titre, $\log_2$	not detected	9.60 ± 0.24	7.80 ± 0.37*
PCR reactors, %	not detected	40.0	100.0

[1] statistically significant difference ($p_1 < 0.05$) confirmed by Tukey's test as compared to the control group;
[2] statistically significant difference ($p_2 < 0.05$) confirmed by Tukey's test as compared to group 1;
* statistically significant difference ($p < 0.01$) confirmed by Student's test as compared to group 1;
ACC CP – average cytochemical coefficient of cationic proteins; ACC MPO – average cytochemical coefficient of myeloperoxidase; OD – optical density; CU – conventional units.

were titrated in 96-well immunological plates (from a dilution of 1:2 to 1:4096) using reagents of the kit for leukemia diagnosis with immunodiffusion assay (BLV antigen, positive bovine serum at a titre of 1:2, negative bovine serum) manufactured by the Kursk Biofactory – BIOK Company (Russia) and luminescent rabbit anti-guinea pig Ig serum (Federal Research Centre for Epidemiology and Microbiology named after the honorary academician N. F. Gamaleya, Russia). Antibody titres were converted to $\log_2$ values to calculate the geometric mean titre.

Bovine leukemia virus antigen was detected by DIF. Lymphocytes were isolated from guinea pig blood by density gradient centrifugation (1.077 g/mL) using a radiopaque contrast medium (Trazograph). Smears prepared from the isolated lymphocytes were then stained with in-house designed diagnostic fluorescent anti-BLV immunoglobulins [26]. Direct and indirect assay results were scored using four-cross system using under Axiostar plus microscope (Carl Zeiss, Germany). Specific fluorescence of the antigen – antibody complex scored as 3–4 crosses was considered a positive result.

Provirus was detected in guinea pig blood samples with real-time PCR using DNA-sorb-B reagent kit and FRT50 F LEUKOSIS variant kit (Central Research Institute for Epidemiology, Russia) for DNA isolation.

Numerical data were processed using one-way analysis of variance (ANOVA), with the significance of differences between groups assessed using Fisher's F-test, followed by post-hoc analysis using Tukey's test for multiple comparisons. Student's *t*-test was used to evaluate the reliability of differences between the geometric mean antibody titres. The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

One-way ANOVA performed on day 21 after intraperitoneal inoculation with the virus-containing suspension showed significant heterogeneity among the three guinea pig groups in mean lymphocyte counts ($F = 6.3$; $p < 0.01$), cytotoxic T-lymphocyte counts ($F = 13.9$; $p < 0.001$), ACCs for cationic proteins ($F = 16.0$; $p < 0.001$), and ACCs for myeloperoxidase ($F = 23.4$; $p < 0.001$).

By day 21 of the experiment, immunological changes were accompanied by a 1.3-fold increase in white blood cell counts in groups 1 and 2 compared to the control group (Table 1). The increase in white blood cells was caused by 1.4- and 1.5-boost in lymphocytes ($p_1 < 0.05$) in groups of guinea pigs inoculated with the pathogen compared to intact animals.

Cytotoxic T-lymphocyte subpopulation exhibited the most significant transformation in both test groups (group 1 and group 2), its level in blood of the animals were 2.7-fold ($p_1<0.01$) and 3.4-fold ($p_1<0.001$) higher, respectively than that one in the control group. B-lymphocytes also demonstrated 1.50–1.65-fold increase, but these changes did not reach statistical significance by Tukey's test, while absolute T-cell counts remained unchanged.

Bovine leukemia virus infection of the guinea pigs also enhanced neutrophil intracellular bactericidal components, as indicated by increased ACC of cationic proteins and ACC of myeloperoxidase. These parameters demonstrated 1.8-fold ($p_1 < 0.01$) and 2.2-fold ($p_1 < 0.001$)

increase in group 1, and 1.8-fold ($p_1 < 0.001$) and 1.95-fold ($p_1 < 0.001$) increase in group 2, respectively, compared to control group.

Neutrophil functional status parameters assessed by the NBT-test showed no significant changes, although a tendency toward increased neutrophil functional reserve was observed in BLV-infected guinea pigs.

It should be noted that geometric mean antibody titres were approximately similar in sera from animals of groups 1 and 2 tested by IIF and all animals without exception were tested positive by both PCR and DIF.

One-way ANOVA of immunological parameters on day 35 of the experiment revealed statistically significant differences between the groups in lymphocyte counts ($F = 6.9$; $p < 0.05$), B-lymphocyte counts ($F = 11.35$; $p < 0.01$), cytotoxic T-lymphocyte counts ($F = 7.3$; $p < 0.05$), ACCs of cationic proteins ($F = 55.6$; $p < 0.001$) and ACCs of myeloperoxidase ($F = 17.6$; $p < 0.001$).

Guinea pigs that had received the experimental drug (group 1) showed a slight increase in leukocyte counts compared to intact animals (Table 2), whereas BLV-infected animals (group 2) showed a more pronounced increase (1.25-fold increase) in leukocyte counts compared to intact animals. The boost in white blood cell counts in group 2 was due to lymphocytes, the lymphocyte counts in animals of group 2 were 1.4 times higher than that ones in control animals, as well as 1.85 times ($p_2 < 0.05$) higher than that ones in guinea pigs, which had been treated with the betulin acid derivative after infection. Moreover, animals of group 1 exhibited a 1.3-fold decrease in lymphocyte counts compared to controls, though this difference was not statistically significant.

T-cell counts tended to decrease slightly in both test groups. More pronounced changes were seen in B-lymphocyte and cytotoxic T-lymphocyte counts. In BLV-infected guinea pigs of group 2 B-cell counts boosted 1.7-fold and 4.1-fold ($p_2 < 0.01$), respectively, and cytotoxic T-lymphocyte counts demonstrated 2.5-fold ($p_1 < 0.05$) and 3.8-fold ($p_2 < 0.05$) increase, respectively, compared to intact animals and to betulinic acid derivative-treated animals (group 1). Whereas, B-cell counts and cytotoxic T-lymphocyte counts in animals of group 1 showed non-significant 2.4-fold and 1.5-fold decrease, respectively, as compared to control group.

Administration of the betulinic acid derivative to the animals on day 35 after their infection induced a significant increase in intracellular metabolism of neutrophils. Thus, guinea pigs of group 1 demonstrated 1.8-fold boost ($p_2 < 0.001$) and 1.9-fold boost ($p_1 < 0.001$) in cationic proteins and 1.55-fold boost ($p_2 < 0.01$) and 1.7-fold boost ($p_1 < 0.001$) in myeloperoxidase enzymatic activity as compared to the guinea pigs infected with BLV (group 2) and guinea pigs of the control group, respectively.

Parameters of phagocyte functional status, assessed based on spontaneous and stimulated tetrazolium activity, did not undergo significant changes. It should be noted that the functional reserve was higher in animals injected with the experimental drug and lower in infected animals.

Guinea pigs treated with the experimental drug (group 1) demonstrated a 1.2-fold increase in antibody titre ($p < 0.01$) as compared to animals of group 2, which did not receive the betulinic acid derivative. DIF revealed

Table 3
Immunological parameters in guinea pigs on day 28 after the experimental drug administration (day 49 after infection with BLV), $M \pm m$

Parameter, measuring units	Control ($n = 5$)	Group 1 ($n = 5$)	Group 2 ($n = 5$)
White blood cells, thousands of cells/mL	10.20 ± 0.34	11.77 ± 1.52	13.22 ± 0.9
Lymphocytes, thousands of cells/mL	5.74 ± 0.30	5.99 ± 0.77	$8.20 \pm 0.46^{1,2}$
T-lymphocytes, thousands of cells/mL	0.86 ± 0.13	1.29 ± 0.27	1.03 ± 0.23
B-lymphocytes, thousands of cells/mL	1.05 ± 0.05	0.81 ± 0.14	$1.94 \pm 0.23^{1,2}$
Cytotoxic T-lymphocytes, thousands of cells/mL	0.87 ± 0.13	1.00 ± 0.21	$2.34 \pm 0.11^{1,2}$
ACC CP, CU	1.14 ± 0.07	2.38 ± 0.17^{1}	1.29 ± 0.08^{2}
ACC MPO, CU	1.04 ± 0.06	2.19 ± 0.20^{1}	1.23 ± 0.09^{2}
Spontaneous NBT test, OD	0.50 ± 0.03	0.48 ± 0.05	0.41 ± 0.01
Stimulated NBT test, OD	0.48 ± 0.02	0.44 ± 0.01	0.41 ± 0.02
Functional neutrophil reserve	0.97 ± 0.04	0.95 ± 0.10	0.99 ± 0.04
IIF antibody titre, $\log_2$	not detected	8.80 ± 0.37	7.40 ± 0.24*
PCR reactors, %	not detected	60.0	100.0

[1] statistically significant difference ($p_1 < 0.05$) confirmed by Tukey's test as compared to the control group;
[2] statistically significant difference ($p_2 < 0.05$) confirmed by Tukey's test as compared to group 1;
* statistically significant difference ($p < 0.01$) confirmed by Student's test as compared to group 1;
ACC CP – average cytochemical coefficient of cationic proteins; ACC MPO – average cytochemical coefficient of myeloperoxidase; OD – optical density; CU – conventional units.

specific fluorescence (scored as 3–4 crosses) in 80% of animals in group 1 and 100% of animals in group 2, while proviral DNA was detected in 40 and 100% of animals, respectively. Guinea pigs of control group were tested with both DIF and PCR with negative results.

One-way analysis of variance of immunological parameters in guinea pigs on day 49 of the experiment, similar to that on day 35, showed significant heterogeneity between groups in mean lymphocyte counts ($F = 6.85$; $p < 0.05$), B-lymphocyte counts ($F = 12.3$; $p < 0.01$), cytotoxic T-lymphocyte counts ($F = 30.4$; $p < 0.001$), ACCs of cationic proteins ($F = 32.9$; $p < 0.001$) and ACCs of myeloperoxidase ($F = 23.8$; $p < 0.001$).

Leukocyte counts exhibited 1.15-fold increase in group 1 and 1.3-fold increase in group 2 compared to that one in control animals. However, in BLV-infected guinea pigs (group 2), unlike the experimental drug-treated animals, this increase was accompanied by a rise in lymphocyte counts (Table 3). Lymphoid cell counts were 1.4-fold higher than that ones in control animals ($p_1 < 0.05$) and 1.4-fold higher than that ones in animals of group 1 ($p_2 < 0.05$).

T-lymphocyte counts tended to increase in both test groups, but the increase was more apparent in guinea pigs that received the experimental drug injection (group 1) where T-cell levels were 1.5-fold higher than that ones in control animals.

In BLV-infected animals (group 2), an increase in B-lymphocyte and cytotoxic T-lymphocyte counts was observed on day 49, similar to that on day 35. Thus, their counts exhibited 1.85-fold ($p_1 < 0.05$) and 2.7-fold ($p_1 < 0.001$) increase, respectively, compared to that ones in intact animals and 2.4-fold ($p_2 < 0.01$) and 2.3-fold ($p_2 < 0.001$) increase, respectively, compared to that ones in the guinea pigs treated with the betulinic acid derivative (group 1). It should be noted that the average statistical parameters in group 1 were consistent with those in the control group, despite a slight tendency toward a 1.3-fold decrease in B-cell count and a 1.15-fold increase in effector cell count.

Neutrophil intracellular bactericidal component activity was higher in group 1 animals, similar to that observed on day 14 after the experimental drug administration. In particular, ACC of cationic proteins and ACC of myeloperoxidase were 2.1 times higher than those in the control group ($p_1 < 0.001$), and 1.8 times higher than those in group 2 ($p_{1,2} < 0.001$). In contrast, following an initial peak in neutrophil metabolic function early after infection, BLV-infected animals exhibited a decline in antimicrobial activity to levels comparable to those in intact guinea pigs. Neutrophil functional status parameters, as determined by the NBT test, did not show statistically significant changes; however, it should be noted that animals in group 2 exhibited a tendency toward reduced reactive oxygen species generation.

IIF antibody titres were 1.2-fold higher ($p < 0.05$) in the experimental drug-treated guinea pigs compared to infected animals. DIF revealed antigen – antibody complex fluorescence in 100% of animals in both group 1 and 2, while proviral DNA was detected in 60 and 100% of animals of group 1 and 2, respectively. All samples from control animals were tested negative. It is worth noting that negative PCR results were observed in animals previously identified as BLV provirus carriers, whereas positive results were obtained in those that had been previously tested negative.

In conclusion, BLV infection in guinea pigs induced changes characterized by cytotoxic T-lymphocyte and B-lymphocyte proliferation, as well as neutrophil bactericidal system malfunction that were consistent with previous reports on leukemic virus infection in sheep and cattle [27, 28, 29] and earlier studies in laboratory animals [30, 31]. Administration of the betulinic acid derivative enhanced immune responses, as demonstrated by normalization of cytotoxic T-lymphocyte and B-lymphocyte counts, increased intracellular neutrophil antimicrobial activity, and elevated antibody titres. Unlike our previous study aimed at preventive antiviral activity testing [22], the experimental drug did not prevent primary infection of guinea pigs. Negative PCR results demonstrated by some animals in the presence of positive specific fluorescence may be accounted for decrease in proviral DNA levels at this stage of viral infection. Intermittent provirus detection in the same animal has also been reported by other researchers in rabbit infection model [32].

CONCLUSION

The study findings indicate that BLV infection in guinea pigs induces an immunological reorganization characterized by sustained increase in lymphoid cell counts throughout the observation period, neutrophil bactericidal hyperreactivity lasting up to 21 days, and a subsequent decline in neutrophil metabolic activity to levels comparable to those in intact animals. Administration of the amide derivative of betulinic acid on day 21 after infection normalizes total lymphocyte, B-lymphocyte and cytotoxic T-lymphocyte counts, enhances neutrophil functional and metabolic activity, and also stimulates antibody production, but does not prevent BLV carrier state. These results suggest that the experimental drug may be used in animals to prevent clinical and hematological leukosis in animals.

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