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Immunomodulatory properties of Cumaside

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Abstract

The medical lead, so-called Cumaside, was created on the basis of triterpene oligoglycosides from the Far-Eastern edible sea cucumber (holothurian) *Cucumaria japonica* and its immunomodulatory properties were studied. The haemolytic activity of Cumaside was significantly reduced in comparison with original glycosides due to the glycoside-cholesterol complex formation. The influence of Cumaside on mouse macrophages in low doses was accompanied by more then two-fold stimulation of lysosomal activity. This preparation was found to increase significantly the animal resistance against bacterial infections elicited by various pathogens. It stimulated phagocytosis, ROS formation, IL6 and TNF-α production in lymphocytes, increased the number of antibody producing cells and amplified the expression of several cell surface molecules (CD3, CD4, CD8) preliminary cultured with hydrocortisone. At the same time the preparation did not affect the delayed-type hypersensitivity, proliferative activity of lymphocytes, cytotoxic activity of NK-cells and cytokine IFNγ and IL12p70 release. The mechanism of Cumaside action is discussed.

Keywords: Cumaside; Cucumariosides; Holothurians; Immunomodulatory activity

1. Introduction

Essential growth of acute and chronic infectious diseases of the bacterial, fungal, protozoan and viral nature is observed at present. The pathogenic or opportunist microbes with atypical biological properties possessing multiple resistances to antibiotics are the etiological factor of these diseases in many cases. It is aggravated by a decrease of immunological reactivity practically in all human populations. Against the background of the decreased human immunity, the application even a new generation of high-performance antibiotics not only does not give good clinical effects often, but also may call into play the further reduction of immunity. Therefore, the creation and development of new highly effective immunostimulators is a relevant scientific and practical problem of the modern immunology and medicine on the whole.

The Far-Eastern edible sea cucumber (holothurian), *Cucumaria japonica*, is a source of at the least 13 different biologically active triterpene oligoglycosides cucumariosides [1–3]. All these compounds contain

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carbohydrate chains with five monosaccharide units linked to C-3 of aglycons, which are represented by the lanostane 18(20)-lactones (so-called holostane derivatives). These glycosides contain one, two or three sulfate groups bounded with sugars (Fig. 1).

A fraction of triterpene glycosides from the holothurian *C. japonica* is known as the active substance of a veterinary immunostimulatory preparation (so-called "CD") approved for the treatment of mink, pigs, dogs and other animals in Russia [4]. Cucumariosides were shown to increase significantly the animal resistance against bacterial infections elicited by various pathogens [5–8]. This effect of cucumariosides was mediated in-part by the increase in macrophage phagocytic activity, which was at least doubled during 7–14 days after the *i.p.* injection of the mixture of cucumariosides at the super low dose of 0.03 μg/mouse. Cucumariosides were also shown to possess strong adjuvant properties potentiating the effect of some bacterial vaccines [6,8] and demonstrated some antiviral activity, possibly through the activation of T- and B-cell cooperation [4]. The cucumarioside CD preparation blocked the mitotic activity of rat liver cells at a concentration of 0.05 μg/kg during first 28–32 h of treatment, but increased the hepatocyte proliferation 12 h later [9]. Recently the influence of different cucumariosides on phagocytosis, lysosomal activity, course and amplitude of intracellular calcium signals and TNF-α release by mouse peritoneal macrophages and human blood immune cells was studied using in vivo and in vitro models [10,11]. Monosulfated cucumariosides were found to be highly cytotoxic in a sea urchin embryo development test due to their membranolytic activity, but in subtoxic concentrations showed more then two-fold stimulation of lysosomal activity and induced a rapid short-term increase in cytosolic Ca^{2+} content in mouse macrophages. Intraperitoneal injection of picogram to nanogram doses of monosulfated cucumariosides significantly induced the macrophage lysosomal activity in a dose-dependent manner.

In the present work the complex of monosulfated cucumariosides with cholesterol named as Cumaside was studied and showed to reduce the glycoside membranolytic activity and retain some immunomodulatory properties in comparison with cucumariosides.

2. Material and methods

2.1. Compounds

Monosulfated cucumariosides from the sea cucumber *C. japonica* (Fig. 1) were obtained as described earlier [1]. Chemical structures and purities of the obtained compounds were confirmed by the determination of their physical constants, mobility on silicagel TLC plates and by ^{1}H and ^{13}C NMR spectra.

Cumaside was obtained as a colorless precipitation using the method described in the Russian patent [2]. To that end butanol solution of cholesterol was added to butanol soluble

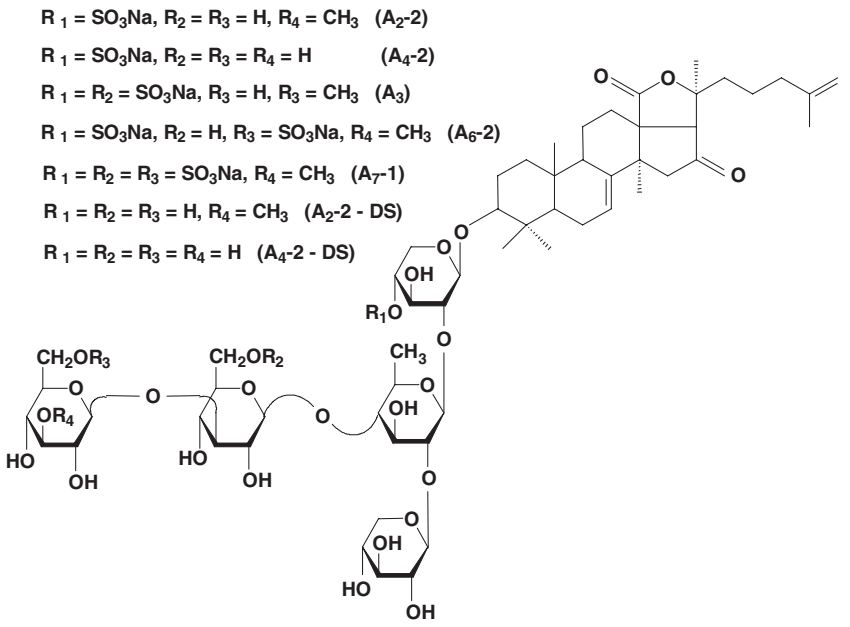

Fig. 1. Chemical structures of some cucumariosides.

materials from alcoholic extract of the sea cucumber *C. japonica* and after 12 h the particles was separated using centrifugation at 2000 rpm during 15 min. The precipitation was rinsed out with ether, ethanol, water and again with ethanol followed by the desiccation in vacuum. The precise disintegration of the residue in water gave a suspension that was used for experiments. The molar ratio cholesterol–glycoside in the preparation as 2 : 1 was determined by ^{1}H NMR spectroscopy on the basis of computation of squares of methyl group signals of cholesterol with these of glycoside in the spectrum.

^{1}H NMR spectrum of the solution of Cumaside in C_5D_5N was recorded on Bruker DRX-500 spectrometer (Bruker Bio-Spin) with tetramethylsilane as a internal standard.

2.2. Mouse resistance to bacterial infection

The investigation was performed using $(CBA \times C57BL_6)F_1$ mouse line. Five groups of animals were formed: 12 mice injected with physiological saline (negative control group); 12 mice injected with *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine in dosage of 100 μg/mouse (positive control); and 12 mice in each of 3 groups injected hypodermically with Cumaside in dosages of 0.1, 1.0 and 10.0 μg/mouse, correspondingly (experimental groups). On the next day after Cumaside injection all mice were infected with lethal dose of *Y. pseudotuberculosis* (strain No. 538/1, LD_{100-} 250×10^3 cell/mouse) or *S. aureus* (Wood-46 strain, LD_{100-} 1×10^{10} cell/mouse). During the experiment the number of survived mice was calculated.

2.3. Mouse spleen antibody plaque-forming cell (pfc) determination

Spleen PFC levels of compound-treated and control mice were determined by the Jerne-Nordin antibody plaque-forming cell assay for response to sheep erythrocytes [12]. Each mouse of treated groups was injected hypodermically with Cumaside saline solution at doses of 0.1, 1.0, 10.0 and 100.0 μg/mouse. Control mice were injected with 0.9% NaCl solution. 3 h after treatment all mice from each of the treated and control groups were injected intraperitoneally with sheep red blood cell (SRBC) suspension in saline in the dosage of 5×10^6 cells/mouse.

On 4th day post immunization mice of all the groups were sacrificed and the spleen were aseptically removed and weighed. Single cell suspensions were prepared by rubbing the spleens through nylon mesh. Spleen cells were suspended in Hank's Balanced Salt Solution (HBSS) (5×10^6/ml). SRBCs (10 μl of 100%) and 50 μl of spleen cell suspensions were added to 0.5 ml of 1% agarose (Merck) in HBSS incubated in water bath at 45 °C. Then 0.5 ml of this mixture was poured over plastic Petri dishes (40 mm in diameter) heated up to 37 °C. The dishes were allowed to get solidified and then incubated during 1 h at 37 °C. Then the 0.5 ml of solution with fresh guinea pig serum diluted in PBS (1 : 10) was added to the surface of each dish and dishes were incubated again at 37 °C for 1 h. Then the plaques (number of hemolysis zones in each Petri dish) were counted. Simultaneously the number of nuclei-containing cell number was calculated. Ability to produce plaque-forming cells by splenocytes of mice treated with Cumaside at different concentrations was compared to that of control mice and expressed as number of PFC per spleen, number of PFC per 1×10^6 nuclei-containing cell and as modulation index.

2.4. Determination of delayed-type hypersensitivity in mice

Mice were immunized with SRBC suspension in saline in the dosage of 2×10^8 cells/mouse 3 h after injection of Cumaside. Each mouse of treated groups was injected hypodermically with Cumaside saline solution at doses of 0.1, 1.0, 10.0 and 100.0 μg/mouse. Control mice were injected with 200 μl of 0.9% NaCl solution. On 5th day post immunization mice of all the groups the right mice foots were injected with 50 μl of SRBS in dosage of 1×10^8 (experimental foots). Left foots were injected with same volume of physiological solution (control foots). After 24 h the weight of experimental and control foots were determined. The reaction index was expressed as a percent of a foot weight increment in relation to control foots.

2.5. Hemolytic activity

Erythrocytes were isolated from mouse blood, washed three times with PBS (pH 7.4) using centrifugation (450 ×*g*, 10 min), and the residue of erythrocytes was resuspended in PBS to a final concentration of 1.0% and kept on ice.

For the hemolytic assay, 100 μl of aqueous solution of the tested substance, having different concentrations was mixed with 900 μl of erythrocyte suspension and incubated at 37 °C for 1 h. The residual cells were sedimented by centrifugation, aliquots of supernatant (200 μl) were transferred to the wells of 96-well microplates and the hemoglobin concentration in the supernatant was evaluated spectroscopically at $\lambda_{ex} = 541$ nm with "μQuant" plate reader (Bio-Tek Instruments, Inc). The results were expressed as percent of hemolysis and plotted.

2.6. Lysosomal activity

BALB/c mice were used for in vivo assay. The investigated compound was injected intraperitoneally (0.5 ml of Cumaside suspension in distilled water). Distilled water was injected in control mice. Four days after treatment, the mice were killed by perivisceral dislocation and peritoneal macrophages were isolated using standard procedure.

An estimation of intracellular lysosomal activity was conducted by staining and the localization of lysosomes in live macrophages with a fluorescent dye acridine orange followed by fluorescence image analysis. For this purpose, 250 μl of a BALB/c mice peritoneal fluid was applied on a microscope cover glass and left at 37 °C in an incubator for 1 h. After

adhesion of macrophages, the cover glasses were washed (3×) with phosphate-buffered saline (PBS, pH 7.5). 250 μl of acridine orange solution ("Calbiochem", 100 μg/ml in PBS) was added dropwise to the cell monolayer and glasses were incubated at 37 °C for 30 min. The cell monolayers were then washed (3×) in PBS. Cover glasses were mounted on a cell chamber of fluorescent imaging system based on inverted microscope Axiovert 200 (Zeiss, Germany). The 75 W Optosource xenon arc lamp and DAC-controlled Optoscan monochromator (Cairn Research Ltd., UK) were used as a light source to excite fluorescence at $\lambda = 489$ nm; HQ FITC filter-block (Chroma Technology Corp., USA) and Fluar 40×/1.30 Oil objective (Zeiss, Germany) were set for visualisation of acridine orange fluorescence in lysosomes. The images of red-orange fluorescent cells were acquired using digital CCD videocamera Hamamatsu Orca-ER C4742-95 (Hamamatsu Photonics K.K., Japan), captured and transferred to a IBM-compatible computer P-IV with Firewire data interface card. The fluorescence intensity of randomly selected 100 cell images was measured with AQM Advance 6 software (Kinetic Imaging Ltd., UK) and expressed as an average pixel intensity of grey level for each cell determined.

Six mice were used for each dose treatment. All experiments were repeated in triplicate. The means and standard errors for each treatment were calculated and plotted using SigmaPlot 3.02 software (Jandel Scientific, San Rafeal, CA).

2.7. Determination of expression of CD3, CD4, CD8, CD25, HLA-DR

Mononuclear cells of healthy volunteer blood were separated by centrifugation in Ficoll-Verografin gradient ($d = 1.077$). Finally cells were washed twice, resuspended in RPMI-1640 (supplemented with 5% of FCS) at concentration of 2×10^6/ml and placed in centrifuge tubes. Cumaside solution was added to the cell suspension in the final concentration of 0.1, 1.0 and 10.0 μg/ml, and tubes were incubated at 37 °C for 24 h. The expression of membrane-associated molecules was determined by the method of nondirect immunofluorescence using monoclonal antibody against CD3, CD4, CD8, CD25, HLA-DR conjugated with FITC (Medbiospectr Ltd, Moscow, Russia).

2.8. The human leucocyte chemiluminescence determination

2 ml of 3% gelatin in PBS (0.15 M NaCl, 1 M K_2HPO_4, 1 M KH_2PO_4, pH 7,4) were added to 4 ml of heparinized blood of the healthy volunteer, mixed and then incubated for 10–15 min at 37 °C for erythrocyte sedimentation. Leucocyte fraction was separated from supernatant and washed twice with PBS by centrifugation and resuspended in PBS to final concentration of 2×10^6 cells/ml.

The following compounds were added to the polysterol tubes: 200 μl of PBS, 100 μl of luminol (Sigma) at concentration of 0.1 mg/ml, 100 μl of leucocyte suspension and Cumaside solution at final concentrations of 0.1, 1.0 and 10 μg/ml. After preliminary incubation of leucocytes with Cumaside the chemiluminescence was recorded during 10 min using LUCY 1 chemiluminometer (Austria). Then zymosan solution (Sigma) opsonized by mixed donor serum was added to the final concentration of 0.5 mg/ml and stimulated chemiluminescence was recorded during 10–15 min.

2.9. Determination of phagocytic activity of human blood phagocytes

Bacteria *S. aureus* were inactivated by heating for 40 min at 100 °C, disintegrated in ultrasonic bath at 51 kHz, 90 W for 2h and then incubated at a concentration of 1×10^9 bacteria/ml with 0.1 mg/ml fluorescein isothiocyanate (FITC, "Sigma", dissolved in DMSO) in 50 mM $NaHCO_3$ solution in 100 mM NaCl (pH 9.5) at +4 °C for 12–16 h in the dark. The bacteria were then washed twice with PBS (centrifugation, 1000 ×*g*, 25 min) to remove free FITC and resuspended to a final concentration of 5×10^8 bacteria/ml in PBS supplemented with 5% mix donor serum and 5% DMSO. The labeled bacteria suspension aliquots were kept at −70°C until experiments.

Leucocytes were isolated from human blood as described above and incubated with Cumaside solution at final concentrations of 0.1, 1.0 and 10 μg/ml in PBS during 1 or 3 h at 37 °C. Then 90 μl of cells were mixed with 90 μl of FITC-labeled *S. aureus* (1×10^8 cell/ml) and incubated during 30 min at 37 °C. After incubation period 2 ml of cold lysing buffered solution was added for 10–15 min to lyse erythrocytes, and leucocytes were sedimented by centrifugation at 200 ×*g* during 3 min, washed once with cold PBS and 0.02% EDTA, and resuspended in the same solution with 1% formaldehyde.

Then samples were analysed with fluorescent flow cytometer FACSCalibur (Becton Dickinson) with argon laser (480 nm) using CellQuest software. The DotPlot graph was plotted using FSC and SSC parameters, neutrophils and monocyte gates were distinguished and fluorescence of channel FL1 (535 nm) was analyzed. The percent of cell with high green fluorescence (cells occluding the labeled bacteria) were calculated.

2.10. Determination of bactericidal activity of human blood phagocytes

Live bacteria *St. aureus* were labeled with FITC as described above. Leucocytes were preliminary incubated with Cumaside solution at final concentrations of 0.1, 1.0 and 10 μg/ml in PBS during 1 or 3 h at 37 °C. Then 90 μl of cells were mixed with 90 μl of live FITC-labeled *St. aureus* (1×10^7 cell/ml) and 20 μl of mix donor serum and incubated during 20 min at 37 °C. After incubation leucocytes were sedimented by centrifugation at 200 ×g during 1 min at +4 °C and washed twice with PBS. The obtained leukocytes were resuspended in PBS and incubated again during 1h at 37 °C. Cells were sedimented by centrifugation at 200 ×*g* for 1 min at +4 °C and

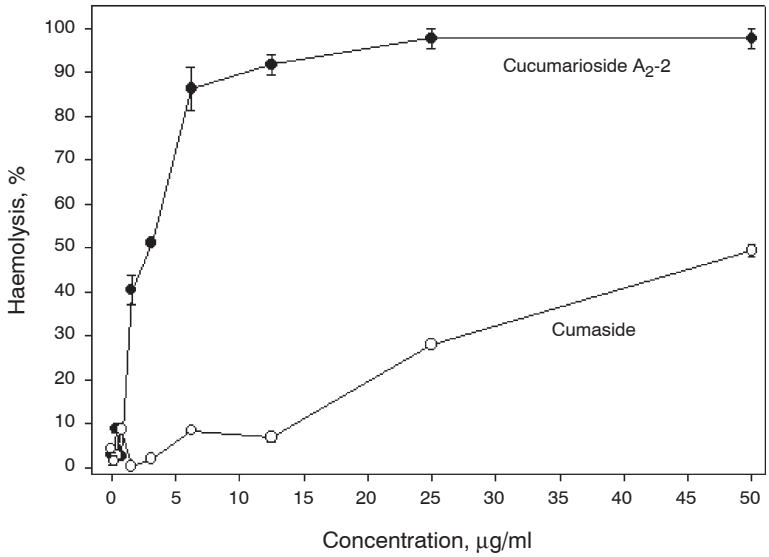

Fig. 2. Hemolytic activities of Cucumarioside A_2-2 and Cumaside.

disintegrated with 0.2% saponin solution in 0.01 M carbonate–bicarbonate buffered solution (pH 9.5) during 5 min. Released bacteria cells were sedimented by centrifugation at 2000 ×*g* for 10 min. To separate dead and alive bacteria cells, the propidium iodide fluorescent probe solution was added (PI, "Sigma", 2.5 μg/ml), and cells were analysed with fluorescent flow cytometer FACSCalibur (Becton Dickinson) using CellQuest software. The DotPlot graph was plotted using FSC and SSC parameters, *Staphylococcus* zone was distinguished and fluorescence of channels FL1 (535 nm) and FL3 (585 nm) was analyzed. The percent of bacteria with green and red fluorescence were calculated. The percent of dead bacteria to total number of cells was calculated.

2.11. Determination of human lymphocyte proliferative activity (reaction of blast-transformation)

Heparinized blood was diluted by culture medium 199 in 2 times and added to 2 ml of Ficoll solution (Sigma). After centrifuging (400 ×*g*, 40 min) at room temperature a ring of cells was collected and washed twice in culture medium 199. Then cells were diluted to the final concentration of 2×10^6 cells/ml in fully completed culture medium: RPMI-1640 (Sigma), included 10% inactivated fetal bovine serum (HyClon), 10 mM HEPES, 2 mM L-glutamine and 40 μg/ml gentamicine (full culture medium).

Cells were cultivated in a volume of 150 μl in full culture medium in 96-well microplates (Nunc) at 37 °C in atmosphere of

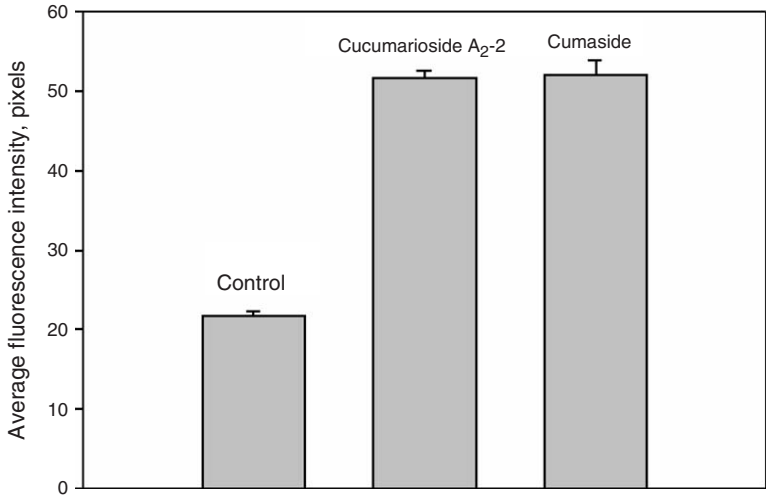

Fig. 3. Influence of Cucumarioside A_2-2 and Cumaside (intraperitoneal administration) upon lysosomal activity of mouse peritoneal macrophages. The concentration of compounds is 1 μg/mouse. Registration on the 8th day of experiment. Results are expressed as $m \pm SE$ ($n = 100$).

Table 1
Expression of membranoassociated immunoregulatory structures (CD3, CD4, CD8) of human blood lymphocytes incubated with Cumaside

Group	CD3	CD4	CD8
Control (non-treated cells)	56.5±7.9	36.3±5.7	26.7±4.5
Hydrocortisone (10^{-5}M)	24.3±5.7	20.5±3.4	18.4±3.7
Cumaside, 0.1 μg/ml	40.2±7.4	26.0±4.3	22.6±4.8
Cumaside, 1 μg/ml	42.3±5.8*	30.6±2.4*	24.4±3.9

The preincubation time with Hydrocortisone is 1 h.
*$p<0.05$, significance in relation to hydrocortisone, $n=8$.

5% CO_2 during 72 h with or without stimulators: iphytohemagglutinin (Sigma) 5 μg/ml, Cumaside 0,1, 1 and 10 μg/ml. ^{3}H-Thd was added in to each well at 6 h prior the end of reaction in final concentration of 1 μCi/well. Upon termination of reaction cell suspension was transferred to glass-fiber filters. The filters were dried overnight and transferred into scintillation flasks with 3 ml of scintillation liquid, and counting of a radioactivity was made using scintillation counter "B-Trac"(USA). The results were expressed as counts per minute.

2.12. Determination of cytotoxic activity of human NK-cells

Isolation of mononuclear cells was made as described previously. The NK-sensitive erythromyeloid cell line K-562 was used as a target cells (2×10^6 cells/ml). Cells were incubated at 37 °C in atmosphere of 5% CO_2 during 1 h in the presence of ^{3}H-Urd (10 μCi/well) in 24-well microplates (Costar). Then the cells were collected, washed in culture medium 199, resuspended in 5 ml of culture medium 199 and left at 37 °C in atmosphere of 5% CO_2 for 1–3 h. Upon termination of the incubation cells were washed twice with culture medium 199 and diluted in full culture medium up to concentration of 1×10^5 cells/ml.

Mononuclear cells (5×10^6 cells/ml) were previously incubated with Cumaside applied in concentrations of 0.1, 1 and 10 μg/ml at 37 °C in atmosphere of 5% CO_2 during 24 h in 24-well microplates (Costar). To eliminate direct effect of Cumaside on tumor cells, the mononuclear cells were washed with culture medium 199 and mixed with target cells (K-562) in the ratio of 50 : 1 in a volume of 200 μl in 96-well microplates (Costar) in triplets.

The spontaneous radioactive label release from target cells incubated without effectors was used as control. After 16 h of incubation at 37 °C in atmosphere of 5% CO_2 the cells were transferred in to millipore filters "Titertek" (Flow). Filters were dried during a night and transferred in to scintillation flasks with 5 ml scintillation liquid, and counting of a radioactivity was made using scintillation counter "B-Trac"(USA). The results were expressed as counts per minute.

Determination of specific lysis of target cells was done using the formula:

$$\text{Lysis } (\%) = ((A-B)/A) \times 100,$$

Where A—radioactivity in control cells; B—radioactivity in cells after incubation with effectors.

2.13. Determination of cytokine production in mononuclear cells

Isolation of mononuclear cells was made as described previously. Mononuclear cells (2×10^5 cells/well) were incubated in flat-bottom 96-well microplates (Costar) in full culture medium in presence or without stimulators: phytohemagglutinin 5 μg/ml, lipopolysaccharide *E.coli* (LPS, Sigma) 200 ng/ml, Cumaside in concentrations of 0.1, 1 and 10 μg/ml at 37 °C in atmosphere of 5% CO_2. After 72 h the supernatants were collected and stored at −20 °C.

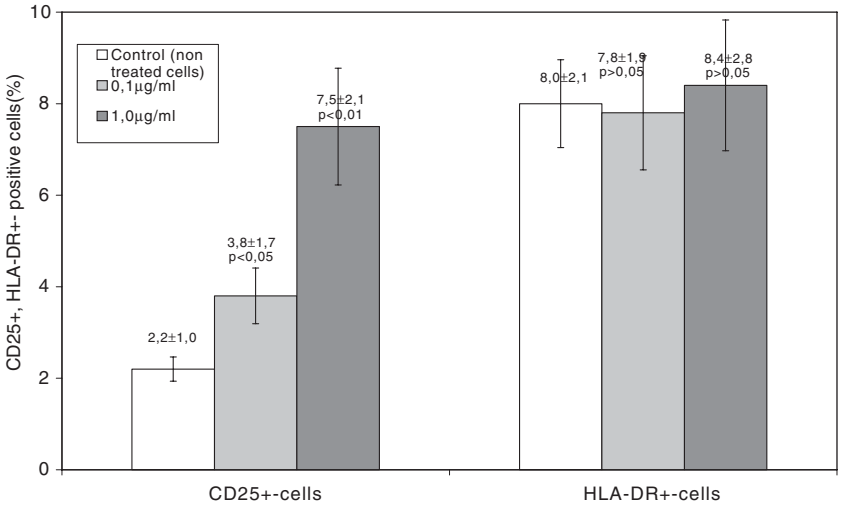

Fig. 4. Expression of activation antigens of human blood lymphocytes incubated with Cumaside.

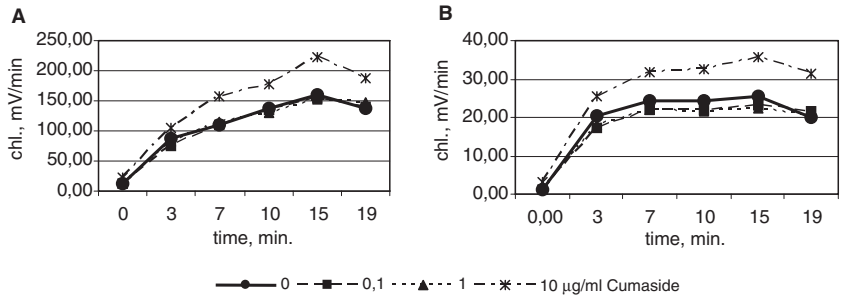

Fig. 5. Influence of Cumaside on zimozan induced luminol-depended chemoluminescence (A) and lucigenin-depended chemoluminescence (B) of human neutrophils after preincubation with Cumaside during 30 min, chl.—chemiluminiscence.

Cytokine concentration in supernatants were determined by solid-phase immunoenzymatic analysis with commercial kits: an interferon γ-OptEIA human IFN-gamma Set (Pharmingen), interleukin 12 p70-OptEIA human IL12p70 Set (Pharmingen), interleukin 6 and tumor necrosis factor (TNF-α)-«ProCon» (St.-Petersburg).

2.14. Viability of the mononuclear cells (MNC) following treatment with Cumaside

Leukocytes were isolated from heparinized blood by gelatin sedimentation. Cells were incubated at a cell density of 1×10^6/ml for 3 h in a full culture medium with Cumaside at a final concentration of 10 μg/ml. To separate dead and alive cells, the propidium iodide fluorescent probe solution was added (PI, "Sigma", 2.5 μg/ml), and leukocytes were analyzed with fluorescent flow cytometer FACSCalibur (Becton Dickinson) using CellQuest software. The fluorescence of channels FL3 (585 nm) was analyzed for lymphocytes, monocytes and neutrophils gates. The percent of cells with high red fluorescence was calculated.

3. Results

3.1. Hemolytic and cytotoxic activity

The hemolytic activities of triterpene glycoside, Cucumarioside A_2-2, and Cumaside, were studied and results were compared with each other. There was not any hemolytic activity in the case of Cumaside application in the concentration range of 0.1–15 μg/ml. EC_{50} for Cumaside hemolytic activity was estimated as 50 μg/ml. Cucumarioside A_2-2 alone demonstrated more pronounced hemolytic activity with EC_{50} of 5 μg/ml (Fig. 2).

Also the viability of the human mononuclear cells (MNC) following treatment with Cumaside (10 μg/ml) was determined. Cumaside haven't significant influence on cell viability. The percent of dead cells from lymphocytes, monocytes and neutrophils gates determined by fluorescent flow cytometry method was less then 10% (data not shown).

3.2. Lysosomal activity

Using the technique of lysosome staining with fluorochromes followed by image analysis of cells, it was found that Cucumarioside A_2-2 and complex of oligoglycosides with sterol (Cumaside) in super-low concentration authentically stimulated lysosomal activity of mouse peritoneal macrophages in in vivo experiments at intraperitoneal administrations (Fig. 3). The Cucumarioside A_2-2 and Cumaside action was dose depended and accompanied by almost two-fold stimulation of lysosomal activity compare to control level expressing in increase of lysosome amount, size and acidity. The immunostimulatory effect was observed on 4–8 day after compound introduction and the maximal stimulatory concentration was found to be 1 μg/mouse.

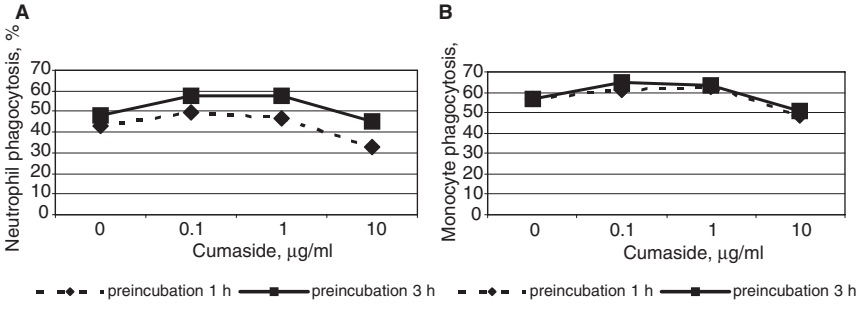

Fig. 6. Influence of Cumaside on human neutrophil phagocytosis (A) and monocyte phagocytosis (B) after preliminary incubation during 1 and 3 h.

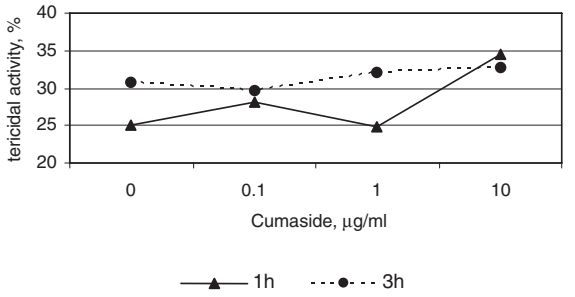

Fig. 7. Influence of Cumaside on human leucocyte bactericidal activity after preliminary incubation during 1 and 3 h.

3.3. CD-marker determination

It was shown that Cumaside did not significantly influence on the basal expression level of CD3, CD4 and CD8-antigens in human blood lymphocytes (data not shown).

In the next sets of experiments the effect of Cumaside on expression level of CD3, CD4 and CD8-antigens in human blood lymphocytes preincubated with immunodepressive agent, hydrocortisone at the concentration of 10^{-5} M was studied. According to the obtained results the lymphocyte incubation with hydrocortisone leads to the decreasing number of cells expressing CD3, CD4 and CD8 markers in 2.2, 1.3 and 1.2 times, correspondingly. The addition of Cumaside at concentration of 1 μg/ml to the cell suspension preincubated with hydrocortisone resulted in partial restoration of CD-antigens on lymphocyte surface (Table 1).

The amount of lymphocytes of the healthy donor blood expressing the early activation markers (CD25) did not exceed 2.2±1.0% of total lymphocytes, and contents of lymphocytes expressing the late activation markers (DR) were 8±2.1%. Thus, activated T-lymphocytes in blood of the healthy donors are presented in the extremely insignificant quantities. The entering of Cumaside in final concentration of 1.0 mg/ml into culture of lymphocytes increased the expression of CD25 (7.5±1.8%, $p<0.05$) and did not change the expression of HLA-DR (Fig. 4).

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3.4. The human leucocyte chemiluminescence determination

The influence of Cumaside on reactive oxygen species (ROS) production detected with luminol- and lucigenin-depended chemiluminescence reaction was studied in in vitro experiments. It was found that the doses of 0.1 and 1 μg/ml at the co-incubation with human neutrophils depressed a little the luminol-depended chemiluminescence and did not affect lucigenin-depended chemiluminescence. However, Cumaside applied at dose of 10 μg/ml significantly increased both types of chemiluminescence on 10, 15 and 17 min of registration ($p<0.05$) (data not shown). After additional cell stimulation with zimozan the smallest doses also did not influence on ROS production, but at 10 μg/ml the increased luminol-depended (on 30–40%) on 10, 15 and 10 min ($p<0.05$) (Fig. 5A) and lucigenin-depended chemiluminescences (on 50%) on 19 min of registration ($p<0.05$) (Fig. 5B) were found.

3.5. Determination of phagocytic and bactericidal activities of human blood phagocytes

The results of experiments with human neutrophils and monocytes are summarized in the Fig. 6. It was shown that Cumaside did not significantly influence on phagocytic activity of neutrophils after 1 h of co-incubation at doses of 0.1 and 1 μg/ml, but the tendency to stimulatory effect was established. The dose of 10 μg/ml caused the inhibition of phagocytosis on 24% ($p=0.001$).

After preliminary incubation during 3 h Cumaside at doses of 0.1 and 1 μg/ml significantly increased phagocytosis (on 20%), while the dose of 10 μg/ml returned the parameters of phagocytic activity of neutrophils to initial values (Fig. 6A).

Table 2
Influence of different concentration of Cumaside on human lymphocyte proliferative activity, cpm

No. of donor	Cumaside, μg/ml				PHA, 5 μg/ml
	0	0.1	1	10	
1	576	975	964	538	15731
2	482	569	647	541	12156
3	458	417	435	346	10558
4	357	275	652	383	6506
5	971	1582	1716	967	27383
6	1921	4166	2770	691	61174
7	3584	4255	3318	524	69513
m±SD	1192.7±1184.73	1748.4±1736.29	1500.3±1142.58	570.0±208.72	29003.0±1736.29*

* $p<0.05$.

Table 3
Influence of Cumaside on cytotoxic activity of human NK-cells of healthy volunteers

No. of donor	Cumaside, μg/ml			
	0	0.1	1	10
1	44	37	40	45
2	44	40	35.5	39.3
3	41.5	36	27.8	29
4	59.2	40.6	64.8	42.5
5	66.3	64	61.8	40
6	70.1	56	28	35.6
M±*m*	54.2±12.60	45.6±11.57	43.0±1643	38.6±5.65*

The percent of dead K-562 target cells is specified.
*$p<0.05$.

Very similar results were obtained in human monocyte experiments (Fig. 6B). The smallest doses of Cumaside did not influence on phagocytosis, while the dose of 10 μg/ml inhibited monocyte phagocytosis on 15% ($p=0.015$) after 1 h of cell preincubation with the preparation. After preliminary incubation during 3 h the smallest Cumaside doses of 0.1 and 1 μg/ml increased phagocytosis on 12–14% ($p=0.037$ for 0.1 μg/ml), but the dose of 10 μg/ml decreased the phagocytic activity of neutrophils on 11% ($p=0.021$).

The results of the studies on human blood leucocyte bactericidal activities are summarized in Fig. 7. It was shown that Cumaside in small doses did not render a significant influence on parameters of bactericidal activity independently on duration of preliminary incubation of the preparation with cells. However, the dose of 10 μg/ml after 1 h of preliminary incubation raised bactericidal activity on 38% ($p=0.016$) and shown a tendency to increase one after 3 h of preincubation.

Thus, the preparation Cumaside renders the moderate stimulatory action on phagocytic activity of human leucocytes at so small doses as of 0.1 and 1 μg/ml and suppresses this cellular function at concentration of 10 μg/ml. It significantly increases the bactericidal activity of leucocytes at the highest of the studied concentrations.

3.6. Determination of human lymphocyte proliferative activity

The results of a rating of lymphocyte proliferative activity under the action of Cumaside are given in Table 2. The classical T-cell mitogen phytohemagglutinin (PHA) was used

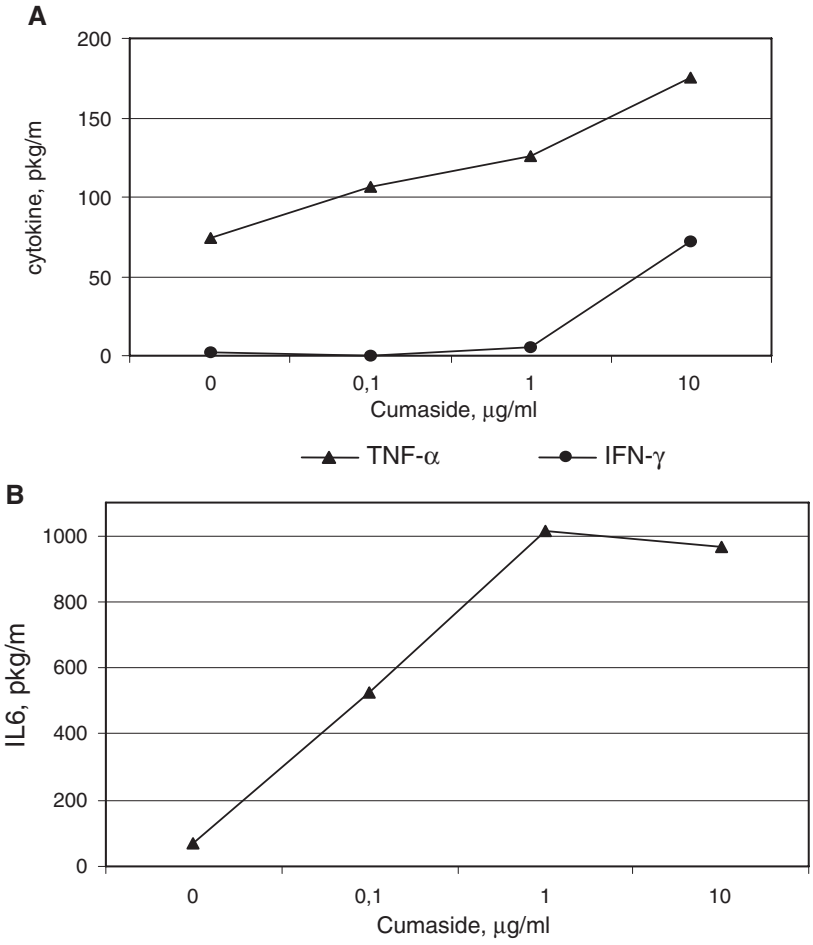

Fig. 8. Effect of Cumaside on TNF-α and IFNγ production (A) and IL6 release (B) in human mononuclear cells.

Table 4
Influence of Cumaside on mouse resistance to different experimental infections

Infection	Dose, μg/mouse	Percentage of survival	Average life time
S. aureus	Control	16.0	1.4
	0.1	33.3	2.6
	1.0	66.6	5.2
	10.0	40.9	2.8
Y. pseudotuberculosis	Control	0	8.7
	0.1	50	13.5
	1.0	50	13.5
	10	50	13.8

as the positive control which has caused 20–30-multiple increases lymphocyte proliferative activity of all donors. Cumaside in the investigated concentrations has not rendered a significant influence on proliferative activity of donor's cells. The preparation in concentration of 10 μg/ml did not either increase or suppress this parameter. Cumaside in concentrations of 0.1 and 1 μg/ml has shown the tendency to stimulate lymphocyte proliferative activity of 5 donors, however the increase of a parameter was not so big—in 1.2–2 times only.

3.7. Determination of cytotoxic activity of human NK-cells

Cumaside applied at low concentrations did not influence on cytotoxic activity of human NK-cells to K-562 target cell line. But at the concentration of 10 μg/ml the preparation affected this parameter and statistically authentic decreased NK-cell activity up to 29% (Table 3).

3.8. Determination of cytokine production in mononuclear cells

The effect of Cumaside on some cytokine production was studied. The summarized results are presented on Fig. 8. The most significant effect of Cumaside was observed in relation to TNF-α. The preparation at concentration of 1 and 10 μg/ml effectively increased TNF-α production in mononuclear cell of donors on 43.7 and 136%, correspondingly (Fig. 8A). Even the lowest Cumaside concentration of 0.1 μg/ml caused this cytokine production at the same level as the classical TNF-α inductor, LPS does it (112.1±26.41 pg/ml TNF-α) in comparison with spontaneous production.

In a lesser degree the preparation Cumaside has influenced on production of IFNγ. Only maximal of the investigated concentrations, 10 μg/ml, caused significant increase of this cytokine production (Fig. 8A).

Cumaside has also shown a stimulatory effect on IL6 release, but the differences between spontaneous and induced production were doubtful (Fig. 8B). The maximal induction was observed at the concentration of Cumaside of 1 μg/ml. However, mononuclear cells of volunteers have shown individual sensitivity to Cumaside: in 3 cases the maximal stimulating concentration has appeared to be of 1 μg/ml, in others—10 μg/ml.

The cytokine IL12p70 did not detected in all cellular supernatants after stimulation by Cumaside applied at all the studied concentrations (data not shown).

3.9. Influence of Cumaside on mouse resistance to different experimental infections

The influence of Cumaside upon mouse resistance against experimental infections elicited by various pathogens was studied. Several different pathogenic microorganisms, namely *St. aureus* and Y. pseudotuberculosis were selected for these experiments. It was found that Cumaside applied at all the studied doses effectively protects mice against *Staphylococcus* infection when applied 1 day before infection. The most effective dose was proved to be of 1 μg/mouse (67% of mice were survived, while in the control animal group the survival was 16% only, Table 4).

Also it was shown that Cumaside applied 3 days before experimental contamination significantly increases mouse resistance against Y. pseudotuberculosis. The mouse survival was increased up to 50% (control—0%) and average life time was increased from 8.7 days in control group up to 13.8 days in the group, in which Cumaside at the dose of 10 μg/ml was injected into each mouse (Table 4).

3.10. Mouse spleen antibody plaque-forming cell (pfc) determination

It was established that Cumaside causes the dose-dependent stimulation of humoral immunoresponse in in

Table 5
Influence of Cumaside upon humoral immunoresponse of mice

Dose, μg/mouse	PFC/spleen (m±SD)		PFC/10^6 NCC (m±SD)		Amount of animals
	Total cell number	Stimulation index	Total cell number	Stimulation index	
0	308±37	1.00	1.67±0.20	1.00	12
0.1	300±38	0.97	1.42±0.18	0.85	10
1	504±75*	1.64	2.42±0.36*	1.45	10
10	654±110*	2.12	3.27±0.55*	1.96	10
100	2270±278*	7.37	11.58±1.42*	6.94	10

*$p<0.05$.

Table 6
Influence of Cumaside on mouse reaction of delayed-type hypersensitivity to sheep red blood cell (SRBC)

Dose, μg/mouse	Experimental mouse foot weight	Control mouse foot weight	Reaction index	Amount of animals
0 (SRBC−)	143±3.30	132±2.99	8.6±0.83	10
0 (SRBC+)	171±2.51	134±2.23	27.9±1.96	10
0,1	174±3.41	134±2.48	30.3±1.84	10
1	176±3.48	135±3.19	31.1±1.86	10
10	177±3.14	137±2.42	29.8±1.59	10
100	180±6.39	134±4.32	34.1±2.58*	10

*$p<0.05$.

vivo experiments. The number of antibody plaque-forming cells (PFC) against sheep erythrocytes after injections of the preparation at doses of 1, 10 and 100 μg/mouse was significantly increased (in 1.5, 2 and 7 times, correspondingly, Table 5).

3.11. Determination of delayed-type hypersensitivity in mice

The obtained results are shown in Table 6. It is clear that Cumaside applied at two studied doses practically does not affect the mouse reaction of delayed-type hypersensitivity to sheep red blood cells. Only at maximal used dose of 100 μg/mouse the small, but significant increasing the reaction was observed and the reaction index was calculated as 34.1.

4. Discussion

Triterpene glycosides are well known as a group of secondary metabolites typically found in higher plants and some marine invertebrates (sea cucumbers, sponges). Triterpene glycosides from sea cucumbers (holothurians) have a wide spectrum of biological effects, including cytotoxic, hemolytic, antifungal, ichthyotoxic, and some other activities [13], but do not exhibit antibacterial properties [14]. Stimulatory effects of certain glycosides on the growth and development of sprouts of some plants [15], human blood leucocyte phagocytosis [16], human macrophage TNF-α production [17], human fibroblast proliferation and Ca^{2+} transport [18, 19] were also reported.

These compounds from sea cucumbers have been studied for over 50 years. The majority of the biological activities were suggested to be related to the interaction of glycosides with sterol-containing cellular membranes. This interaction results in the formation of pores, changes in membrane ion permeability and viscosity that can finally lead to cell death [13,20]. The membranolytic properties of cucumariosides from *C. japonica* were recently reviewed and the mechanism underlying haemolysis was described [21].

At the same time, nanomolar concentrations of marine triterpene glycosides may result in cellular activation and potentiation of cellular functions. For instance, the crude extract from the tropical holothurian *Actinopyga agassizi,* which contains toxic glycosides, were shown to stimulate human blood leucocyte phagocytosis in vitro [16]. Preliminary studies on cucumariosides have shown the potentiation of immunity by these compounds and in vivo host defence against bacterial or viral infections [4–9]. However, these studies did not explain the mechanism(s) leading to a potentiation of immune response, and many peculiarities of immunostimulatory properties of the cucumariosides remain to be elucidated in details.

With purposes of the search for ways of the reduction of cucumarioside cytotoxicity and hemolytic activity with the reservation of the efficiency of their immunomodulatory action, especially at intraperitoneal application, we studied biological activities of a complex of monosulphated cucumariosides with sterols. It was established that indeed the application of the complex with cholesterol (Cumaside) results in sharp reduction of membranolytic properties when this preparation was compared with the most active glycoside Cucumarioside A_2-2. But in spite of Cumaside membranolytic effects reduction, the stimulation of macrophage lysosomal activity remained the similar with that caused by Cucumarioside A_2-2.

It was shown that the preparation Cumaside causes a dose-dependent stimulation of humoral immune response in in vivo experiments and increases the number of antibody plaque-forming cells. The given effect of Cumaside was not realized at the expense of increase of nucleus containing cell (NCC) number in spleen, but at the expense of increase of quantity of active antibody producers.

It was established in in vitro experiments that the preparation Cumaside causes a significant dose-dependent increase of TNF-α production in blood mononuclear cells of healthy volunteers and also shows the tendency to stimulate IL6 release. On the other hand, the preparation does not practically influence on production of main Th1-cytokines, IFNγ and IL12, and did not affect cytotoxic activity of human NK-cells. Cumaside does not affect the mouse reaction of delayed-type hypersensitivity to sheep red blood cell. The latter property can be in a good agreement with the fact that preparation induces proinflammatory factor TNF-α, which may suppress the delayed-type hypersensitivity reaction.

The main mechanism of the first line of defense against pathogenic agents is the absorption and phagocytosis of a microorganism by phagocytes and effective destruction of these agents. The expression of phagocytosis receptors such as receptors for Fc-fragment of

immunoglobulins, lectin-like receptors, etc plays an important role in the absorption. The destruction of pathogenic organisms occurs by the oxygen-depended way (production of the reactive forms of oxygen and nitrogen), as well as by the oxygen-independed way. In low doses Cumaside renders stimulatory action on absorption of *S. aureus* both by monocytes and neutrophils of human peripheral blood. This research was carried out in serum-free medium, where the microbial opsonic factor was excluded. Probably, there is an increase of expression of lectin-like or other phagocytosis receptor having ligands on a *S. aureus* surface under the action of the preparation.

High-grade activation of resting lymphocytes requires, as a minimum, two consecutive processes: stimulation of the trigger processes promoting transition of lymphocytes from a phase of rest—G0-phases in a presynthetic G1-phase of a cellular cycle. Activation of lymphocytes is provided by binding of MHC-associated peptides with TCR, by transduction of CD3-complex and by expression of some molecules, obligatory for the given cell's functional condition, so-called markers of activation. Processes of activation in T-lymphocytes in a G1-phase of a cellular cycle can be detected by expression of receptors to IL-2 (CD25) on T-lymphocytes, which participate in an exit of cells in S-phase of a cellular cycle with the subsequent cellular replication, and also by expression HLA-DR which expressed on surface T-lymphocytes during a S-phase of a cellular cycle or after S-phase. The amplification of expression of HLA-DR and receptors to IL-2 (CD25) on a membrane of lymphocytes of peripheral blood, treated with Cumaside, shows participation of this compound in the process of activation.

Ability of a Cumaside to restore CD-moleculas on lymphocytes with an inhibited metabolism can be beneficial in correction of depressed cell immunity.

Bactericidal activity of leucocytes of human peripheral blood was amplified under action of Cumaside in the dose of 10 μg/ml. It is possible that preparation activates mainly the oxygen-depended mechanisms of killing since the increase of reactive oxygen species (H_2O_2 and O_2^-) production is revealed under the action of the same preparation dose in experiments with neutrophil chemiluminescence detection.

The results of in vitro studies on bactericidal activity of macrophages stimulated by Cumaside were confirmed by in vivo experiments on mice. All applied Cumaside doses significantly raised the mouse resistance against some microbial infections (*S. aureus* and *Y. pseudotuberculosis*), but the maximal effect against *S. aureus* infection was indicated at the dose of 1 μg/mouse (50 μg/kg).

Analyzing the presented results, it is possible to conclude that the preparation Cumaside has expressed evident immunostimulatory properties at low doses, especially the ability to stimulate phagocyte part of immunity. The assumption may be done that immunostimulatory effects of the preparation in vivo are at least partly connected with the action of TNF-α, whose production was induced by Cumaside and it was confirmed in in vitro experiments. Probably, not only TNF-α, but also IL6 cytokine induction are the key processes in the molecular mechanism of Cumaside action.

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