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Cucumarioside A_2-2 causes changes in the morphology and proliferative activity in mouse spleen

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ABSTRACT

The immunomodulatory effect of triterpene glycoside cucumarioside A_2-2 (CA_2-2), isolated from the Far Eastern sea cucumber *Cucumaria japonica*, on the mouse spleen was investigated in comparison with lipopolysaccharide (LPS). It has been shown that the intraperitoneal (*i.p.*) glycoside administration did not influence on splenic weights, while the statistically significant increase in splenic weight was observed after LPS administration. Changes in the ratio of red to white pulp after CA_2-2 or LPS administration were observed. The proportion of splenic white pulp after glycoside or LPS administration increased by up to 34% and 36%, respectively. A detailed study of the distribution of the PCNA (Proliferating Cell Nuclear Antigen) marker showed that the proliferative activity in the white pulp under CA_2-2 and LPS influence increased 2.07 and 2.24 times, respectively. The localization of PCNA-positive nuclei in the white pulp region, as well as their dimensional characteristics, suggests that a large proportion of the proliferating cell population consisted of B cells. The mass spectrometry profiles of spleen peptide/protein homogenate were obtained using the MALDI-TOF-MS (Matrix –Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) approach. It was found that *i.p.* stimulation of animals with CA_2-2 or LPS leads to marked changes in the intensity of revealed characteristic peaks of peptides/proteins after exposure to immunostimulants.

1. Introduction

Triterpene glycoside cucumarioside A_2-2 (CA_2-2) isolated from the Far-Eastern edible sea cucumber *Cucumaria japonica* is a natural bioregulator with a wide range of biological activities, including cytotoxic, hemolytic, antimicrobial, and anticancer functions. However, at non-toxic low concentrations, CA_2-2 has recently been found to have pronounced immunomodulatory effects (Aminin et al., 2001, 2014; Pislyagin et al., 2012).

It is known that one of the target organs for CA_2-2 immunomodulatory action is the spleen. Previously, by MALDI-TOF-MS and MALDI-IMS (MALDI-Imaging Mass Spectrometry), we determined the quantitative content and pharmacokinetics of CA_2-2 in mouse spleen after drug *i.p.* administration (Pislyagin et al., 2013). The cucumarioside A_2-2 was absorbed fairly rapidly: the glycoside maximum concentration (C_{max}) in spleen tissue homogenate was observed in the first 30 min after injection, and minimum values were recorded within 3 h. CA_2-2 moderately cleared from the spleen. The biological half-life (T½) of the compound in the spleen was approximately 80 min, and the mean residence time of the preparation (MRT) was calculated to be approximately 140 min. These results correspond to the radiospectroscopic studies of ^{3}H-cucumarioside A_2-2 dynamics. It was established that CA_2-2 localized in the tissue at high concentrations in the regions surrounding the organ followed by its decline on the surface and by a very slight redistribution to the internal part of the spleen.

However, despite the large number of studies of the physiological activity of sea cucumber triterpene glycosides, the immunomodulatory mechanism of their action on the cellular and organ level has been insufficiently studied.

The aim of this work was to study the immunomodulatory effect of triterpene glycoside cucumarioside A_2-2 in *in vivo* experiments. The influence of CA_2-2 on the weight of mouse spleens, the ratio of splenic red to white pulp, the proliferative activity in the white pulp and mass spectrometric profiles of peptides/proteins in spleen tissue homogenates were investigated after *i.p.* administration of the

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drug. LPS from *Escherichia coli* was used as a comparative immunostimulatory drug.

2. Materials and methods

2.1. Triterpene glycoside isolation

Triterpene glycoside cucumarioside A_2-2 or 3b-*O*-{[3-*O*-methylb-D-glucopyranosyl-(1-3)-b-D-glucopyranosyl-(1-4)]-[b-Dxylopyranosyl-(1-2)]-b-D-quinovopyranosyl-(1-2)-4-*O*-sodium sulfate-b-D-xylopyranosyl}-holosta-7,25-diene-16-one was isolated from an ethanol extract of Far-Eastern holothurian *C. japonica* using hydrophobic chromatography on polytetrafluoroethylene powder Polychrom-1 (Biolar, Latvia) followed by chromatography on a Si gel column and HPLC as described previously (Avilov et al., 1990). The purity of the compound was checked by ^{13}C NMR and compared with published data.

2.2. Animals

Female Balb/c mice weighing 20 g were purchased from the RAMS nursery «Stolbovaya» (Russia) and kept at the animal facility under standard conditions. All experiments were conducted in compliance with all rules and international recommendations of the European Convention for the Protection of Vertebrate Animals used for experimental studies. All procedures were approved by the Animal Ethics Committee at the G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences, according to the Laboratory Animal Welfare guidelines. Three groups (6 mice in each group) were used in the experiments: group 1 (control)—animals were treated with PBS; group 2 (experimental)—animals were administrated with cucumarioside A_2-2, 3 mg/kg; and group 3 (experimental)—animals were administrated with LPS, 1.5 mg/kg.

2.3. Tissue preparation

A solution of cucumarioside A_2-2 or LPS in water was administered *i.p.* once to Balb/c mice at a dose of 3 mg/kg and 1.5 mg/kg, respectively. Negative control mice received injections with PBS alone. After 18 h, mice were sacrificed by cervical dislocation, and spleens were then immediately surgically removed within 10 min and fixed for 8 h at 4 °C in 10% phosphate buffered formalin. After rinsing, spleens were processed and embedded in paraffin according to standard embedding techniques and used for further immunohistochemical staining procedures.

For MALDI-TOF-MS experiments, each isolated fresh spleen was then gently homogenized with a glass homogenizer and immediately used to detect peptide/protein profiles.

2.4. Immunohistochemistry

To evaluate the proliferative activity of the spleen, PCNA-immunoperoxidase reaction was detected and measured using mouse primary monoclonal antibodies for PCNA [PC10] (1:3000, Abcam, ab29, USA). Secondary antibodies (ImmPRESS HRP anti-mouse Ig (Peroxidase) Polymer Detection, Vector Laboratories, USA) were used according to the manufacturer's instructions. PBS was used as a negative control instead of primary antibody in the immunohistochemical reaction. Paraffin sections of spleen (7 μm) after deparaffinization were incubated in 3% hydrogen peroxide for 10 min. After three washes in PBS, sections were incubated for 60 min with 2.5% normal horse blocking serum. Spleen sections were incubated with primary antibodies on glass in a humidified chamber at 4 °C for 24 h. After 3 washes, sections were incubated in a secondary antibody solution for 30 min. After washing, sections were treated for 5–10 min with chromogen (Thermo Scientific, DAB Plus, USA). The slices were washed with PBS, dehydrated and embedded in balsam.

2.5. Histological staining

Spleen paraffin-embedded sections (7 μm) were stained with H&E according to the standard procedure for morphological tissue analysis and calculation of the relationship of white to red pulp. All preparations were examined by light microscopy (Axio Image Z2, Carl Zeiss, Germany), and images were captured using a digital camera (AxioCam HRc, Carl Zeiss, Germany). The area ratio of white and red pulp and number PCNA-immunopositive cells was calculated in every eighth serial sections using 100 randomly taken photographs (10x lens) in each group of animals. The absolute number of PCNA-immunopositive cells was calculated as a share of the total area of the white pulp using the Image J 1.41 software package (Plugin Cell Counter).

2.6. MALDI-TOF-MS

Mass spectra were acquired with an Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Germany) in the linear positive ion mode with mass range from 2000 to 17000 *m/z*. A pulsed smartbeam laser at a wave length of 355 nm was operated at a frequency of 10 Hz with a delayed extraction time of 150 ns. Each mass spectrum was the average of typically 500 laser shots obtained from several positions within a given sample spot.

The tissue homogenate sample was spotted on a stainless-steel MALDI target, then the target was placed into a desiccator for a minimum of 60 min. After drying, the plates were covered with saturated solution of sinapinic acid (SA) matrix.

Prior to each data acquisition, external calibration was conducted using a protein calibration standard I (Bruker Daltonics) Mixed with matrix, it was deposited separately onto a MALDI target to determine optimum MS parameters and verify the results obtained with tissue homogenates. SA was dissolved at 10 mg/mL in water/acetonitrile/TFA (49.95/49.95/0.1, vol/vol/vol). The calibration standard samples were directly mixed with the matrix in a 1/1 (vol/vol) ratio. One μL of the mixture was spotted on a stainless steel plate and allowed to dry under ambient conditions. Data were acquired with the Flex Control 3.0 software and processed with the Flex Analysis 3.0 software.

2.7. Statistical analysis

The data obtained by histological and immunohistochemistry studies were subjected to statistical analysis using one-way ANOVA followed by Tukey's post hoc test. Data were expressed as mean ± SEM and $p < 0.05$ was considered statistically significant. All statistical tests were performed using the GraphPad Prism 4.00 software.

3. Results

3.1. CA_2-2 does not causes changes in the splenic weight

To confirm the immunostimulatory activity of CA_2-2 *in vivo*, a dose of 3 mg/kg of compound was injected intraperitoneally to Balb/c mice. The mice were sacrificed 18 h post-administration, and spleens were removed, blotted to dryness and weighed. Changes in spleen weight in treated and untreated mice were used as a parameter for immunostimulatory activity. LPS was used as a positive control at dosage of 1.5 mg/kg; the negative control mice received injections with PBS alone.