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The Effect of Salinomycin on Ganglioside Production in Lead-Intoxicated Mice. An Immunological Study.

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GM3 gangliosides have been described as biomarkers for oncogenic tissue changes. Anti-GM3 antibodies in mouse sera were tested in a lead intoxication model, followed by treatment with the polyether ionophorous antibiotic salinomycin. The results show abiding high titer of IgG antibodies against the GM3 gangliosides only in sera from salinomycin-treated mice. It could be concluded that the high titer of anti-GM3 antibodies is associated with signs of oncogenity.

Key words: salinomycin, anti-GM3 ganglioside antibodies, lead intoxication

Introduction

Lead (Pb) is a heavy toxic metal and a major environmental pollutant. Pb-poisoning in humans is possible by the consumption of contaminated food and water, inhalation or absorption through the skin [1]. Entering in the body, lead induces damages in the brain and kidneys, in the cardiovascular, nervous and reproductive system [2, 7, 15, 16, 17].

Chelation therapy is used to treat metal ion poisoning. Among the polyether ionophorous antibiotics, salinomycin is the representative with the lowest *in vivo* toxicity [12] and it has a potential application as antitumor agent for treatment of cancer stem cells [13]. In our previous study we demonstrated that the polyether ionophorous antibiotic salinomycin reduced significantly the concentration of Pb in the organs of Pb-exposed mice [14].

Numerous studies have recognized gangliosides as promising biomarkers for different disorders. Gangliosides are acidic glycosphingolipids. They occur not only in cells and tissues but also in tissue fluids. Over the last decades many studies have shown that gangliosides are immunogenic [9, 10] and their potential for cancer immunotherapies has been discussed.

The aim of the present work is to explore the effect of salinomycin on the presence of anti-ganglioside GM3 antibodies in a mouse model of lead intoxication.

Materials and Methods

Experimental design. Mature 60-days old male ICR mice weighting 25-30 g were obtained from Experimental and Breeding Base for Laboratory Animals (EBBLA) – Slivnitza, Bulgaria. The animals were housed at the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences at standard conditions: 12:12 h light/dark cycle, 25°C temperature and constant humidity. Each animal was accommodated in a single cage with polypropylene bottom. The animals were divided into four experimental groups as follow:

– Control group (untreated control animals; n =10) – the mice from this group obtained distilled water – **A** group.

– Toxic control group (n = 10) – the mice from this group were exposed to $Pb(NO_3)_2$ treatment for 14 days. The compound was dissolved in distilled water and administrated *per os* in an average daily dose of 80 mg/kg body weight (b.w.). From the 15th to 28th day the mice obtained distilled water – **B** group.

– Lead and Salinomycin treated group (n = 10) – animals from this group were intoxicated with $Pb(NO_3)_2$ in an average daily dose of 80 mg/kg b.w. Next two weeks animals obtained tetraethylammonium salt of salinomycinic acid dissolved in drinking water in an average daily dose of 20 mg/kg b.w. – **C** group.

– Salinomycin-treated group (n =10) – the mice from this group obtained tetraethylammonium salt of salinomycinic acid dissolved in drinking water in an average daily dose of 20 mg/kg b.w. for 14 days – **D** group.

On the 29th day of the experimental protocol the animals were anesthetized and sacrificed. The samples were collected for analysis. Blood was collected and centrifuged (for 10 min at 1500 rpm). Pool samples were prepared from the separated sera and stored at -20 °C prior to analysis. The protocol was approved by Ethics committee of the Institute of Experimental Morphology, Pathology and Anthropology with Museum of Bulgarian Academy of Sciences.

Titers of antibodies to GM3 gangliosides served as biomarkers. Immunoglobulin G (IgG) is used for chronic disorders. The serum anti-GM3 antibodies were estimated by the enzyme-linked immunosorbent assay (ELISA) with slight modifications of the method [10].

Results

Our observations in salinomycin-treated animals show strongly elevated titer of anti-GM3 antibodies **(Table 1)**. More than a 4-fold increase in the antibody titer was measured in the pool sample of animals treated only with salinomycin, compared to the untreated control mice. In the Pb-intoxicated group elevated titer was not observed. Subsequent treatment with salinomycin though, induced a 4-fold and a 6-fold increase in the anti-GM3 titer compared to the untreated control and the intoxicated group, respectively. The highest antibody titer was measured in the pool sera sample of Pb-intoxicated mice with subsequent administration of the ionophorous antibiotic. The results found confirmation in 5 of 6 repeats, although the capriciousity of the method (relative to pH, temperature and other parameters). High titer of antibodies was not measured in only one of the pools which could be explained by the possibility gangliosides to be linked to each other in micelles.

There are many literature data on the increased levels of GM3 molecules in different pathological conditions with various interpretations of these findings. The high titer of anti-GM3 antibodies may be a marker for common metabolic disorders and/or diabetes.

Table 1. Numeric values of the anti-ganglioside GM3 antibodies titers in sera in lead intoxication model mice treated with the polyether ionophorous antibiotic salinomycin

Probe (n =10)	Numerical value of the IgG titre of anti-GM3 antibodies	Symbol
A group, Control	0.0175 ± 0.01	-
B group, Pb	0.0244 ± 0.02	-
C group, Pb+Sal	0.1105 ± 0.03	++
D group, Ctrl+Sal	0.0825 ± 0.02	+

Legend:
A group – Control group (untreated control animals; Ctrl, n =10); **B** group – Toxic control group (only Pb, n = 10); **C** group – Lead and salinomycin-treated group (Pb+Sal, n = 10); **D** group – Salinomycin-treated group (treated control animals; Ctrl+Sal, n =10)

Numerical value of the titre:
- ≤ 0.047; Normal 0.047; ± 0.062; + 0.077; ++ 0.107

According to literature data, elevated anti-GM3 antibody levels are also reported in malignant cells as a neoplastic marker, including their protective functions. Similarly, our results could be indicative for the relevance of salinomycin to cancer therapy.

Discussion

Gangliosides perform different biological functions. They are involved as membrane regulatory molecules in the cell growth, differentiation and axon-oligodendrocyte interaction formation in myelinogenesis. Ganglioside composition in the serum is very constant and shows no significant variations in healthy individuals associated with age and gender. However, in pathological conditions, the serum ganglioside spectrum undergoes significant alterations. Our long-term research has shown that it is of critical importance to establish the clinical significance of serum IgG anti-GD1a and anti-GM1 ganglioside antibodies as potential biomarkers for neuronal damage in neurodegenerative diseases, immuno-mediated neuropathies and demyelination [9, 10].

The antibiotic salinomycin (E716) is a carboxylic polyether ionophore, produced by *Streptomyces albus*. Salinomycin, due to its lipophilic properties, easily penetrates through the plasma membrane into the cell and through intracellular membranes into various cellular organelles. In 2009 Gupta et al. discovered that salinomycin is able to kill cancer stem cells, as well as to inhibit breast cancer growth and metastasis in mice [4]. Cancer stem-like cells (CSCs) in different types of cancers may account for the failure of treatments because they are resistant to many current anticancer therapies. Therefore Gupta's discovery could be very important for cancer therapy in the future [4, 6]. Salinomycin induces apoptosis of human prostate cancer cells owing to accumulation of reactive oxygen species, DNA damage and mitochondrial membrane depolarization. This drug also inhibits chemoresistant cancer cells and sensitizes DOX- or ETO-treated or irradiated cancer cells by increasing apoptosis causing DNA damage and reducing p21 protein levels. Salinomycin inhibits Wnt-signalling and selectively induces apoptosis in

tumor cells [13]. Therefore, at present salinomycin is considered to be a potential anti-neoplastic drug for cancer therapy. It has been shown that T-lymphocytes with CD4+ phenotype, obtained from patients with leukemia (malignant cells), are significantly more sensitive to the action of this drug than CD4+ T-lymphocytes of healthy people [13].

The ionophore antibiotics can disrupt the intracellular balance of cations and ultimately can lead to cell death. The neurotoxicity of salinomycin appears to be primarily due to disturbances in the cell ionic balance [13, 14]. It has been shown that salinomycin can inhibit mitochondrial respiration and disrupt the transmembrane potential. This can cause the release of cytochrome C from the intermembrane space of the mitochondria, activation of caspase-9 and the development of apoptosis.

There are evidences that a synthetic derivative of salinomycin exhibits a more potent and selective activity against breast CSCs *in vitro* and *in vivo*, by accumulating and sequestering iron in lysosomes. In response to the ensuing cytoplasmic depletion of iron, cells triggered the degradation of ferritin in lysosomes, leading to further iron loading in these organelles. Iron-mediated production of reactive oxygen species (ROS) promoted lysosomal membrane permeabilization, activating a cell death pathway consistent with ferroptosis. These findings reveal the prevalence of iron homeostasis in breast CSCs, pointing towards iron and iron-mediated processes as potential targets against these cells [11]. Salinomycin is able to kill different types of non-stem tumor cells that usually are resistant to common therapeutic approaches, but the mechanism of action of this molecule remains largely unknown [12]. Since salinomycin has been suggested to act as a K(+) ionophore, Managò et al. explored its impact on mitochondrial bioenergetic performance at an early time point following drug application [12]. In addition, mitochondrial matrix acidification and significant decrease of respiration were observed in intact mouse embryonic fibroblasts (MEFs) and in cancer stem cell-like HMLE cells within ten minutes, while increased production of ROS was not detected [12]. Compatible with its direct modulation of mitochondrial function, salinomycin was able to induce cell death also in Bax/Bak-less double-knockout MEF cells [12]. Since at the concentration range used in most studies (around 10 μM) salinomycin exerts its effect at the level of mitochondria and alters bioenergetic performance [12]. The specificity of its action on pathologic B cells isolated from patients with chronic lymphocytic leukemia versus B cells from healthy subjects was investigated [12]. The results indicate that salinomycin, when used above μM concentrations, exerts direct mitochondrial effects, thus compromising cell survival [12]. Jiang et al. present evidence that a dual role of salinomycin involving in autophagy may account for its unique anticancer effects with preference for cancer cells [8]. GM3 regulates cell adhesion, growth and motility by changing molecular organization in glycosynaptic microdomains. GM3 may change the activation levels of co-localized signaling molecules, which are involved in cancer pathogenesis [5]. B16 melanoma cells showed GM3 on the cell surface and GM3-dependent *in vitro* growth. Depletion of sialic acid residues from the cell surface completely abolished antibody response against melanoma cells [3]. These data indicate that the antitumor activity of GM3 is associated with GM3 expression on tumor cell surface and demonstrate a major role of sialic acid in the humoral response [3].

In conclusion, the results of the present study show that salinomycin treatment following lead intoxication increases the titer of anti-GM3 antibodies. Our findings suggest that GM3 gangliosides can serve as cancer biomarker and provide evidence for the potential application of salinomycin in oncotherapy.

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