Conjugates of Aberrant Gangliosides in Antiglioma Vaccine: Toxicological Assay

V. Lombardi, R. Shnaar*, B. Minev, R. Misasi***, Kh. Gitstsi***, M. Sorice***, and A. Troncone**

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We studied sterility and toxicity of vaccine LS1 containing aberrant gangliosides isolated from brain bioptates of 48 patients with gliomas of different malignancy (classification of primary brain tumors, World Health Organization [3]) and covalently bound to keyhole limpet hemocyanin. The vaccine was safe. This preparation produced no side effects in experimental animals. Our findings substantiated the necessity of father development of this method of vaccination. The vaccine should undergo clinical tests in patients with malignant gliomas.

Key Words: *aberrant gangliosides; keyhole limpet hemocyanins*

Glioblastomas and malignant oligodendrogliomas are the most malignant brain tumors [3]. Despite advances in surgery, radiation therapy, and chemotherapy, the duration of life in patients with glioblastomas is 30 weeks. The mechanisms underlying malignant transformation of cells and their dissemination to normal brain tissues remain unclear. Despite considerable recent progress in immunobiology, metabolic changes in normal cells leading to the formation of tumors are poorly understood.

Gangliosides (GLS), glycophospholipids containing sialic acid, are the most important information molecules on the cell surface [1,5,14]. Immunobiological assays of cancer evaluated the role of these compounds [5,14]. Cancer cells have specific GLS bound to the membrane and possessing invasive activity. GLS consisting of saccharides, sialic acid, sphingosine, and fatty acids are components of the immunoreactive system. They play a role of antigens in complex substances of the blood and are associated with tumors. GLS enter the molecular structure of tumor antigens and act as anti-antigens. GSL are widely distributed in the central nervous system and brain tumors. However, their immunobiological and metabolic activity in membranes is still unknown.

Nuclear magnetic resonance spectroscopy and high-performance liquid chromatography (HPLC) revealed considerable changes in the molecular structure and conformation of GLS from glioma tissues, presence of various fatty acids, and increased content of sialic acid Neu5Ac. Aberrant GLS from glioblastoma tissues were investigated [12]. These data suggest that the molecular structure of GLS entering the composition of anticancer vaccines differs from the structure of compounds present in normal human brain.

We obtained antiglioma vaccine containing conjugates of aberrant GLS and keyhole limpet hemocyanin (KLH) [6,7,14]. KLH is a copper-containing respiratory protein of marine mollusks *Megathura crenulata* that acts as the carrier of cancer GLS. Here we studied sterility and toxicity of this vaccine.

MATERIALS AND METHODS

Samples of glioma tissues were homogenized in a 10-fold volume of cold methanol and centrifuged. The pellet was resuspended in a chloroform-methanol mixture (2:1, 1:1, 1:2 v/v). Phases containing the solvent were pooled and evaporated to dryness. The total lipid

University of Bari, Institute of Pathological Anatomy, Italy; *Johns Hopkins Medical School, Baltimore, USA; **University of California, La Jolla, USA; ***Institute of Experimental Medicine and Pathology, University of Rome, La Sapienca, Italy. vinlombardi@libero.it. Lombardi V.

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extract was separated by the method of Folch in a chloroform-methanol mixture (2:1) and 2 ml H_2O. GLS were transited into the upper phase. The lower phase and freshly prepared upper phase were separated 2 times. The upper phases were mixed and evaporated to dryness.

Covalent conjugates of GLS and KLH were obtained as described elsewhere [6,7,14]. GLS were specifically oxidized with ozone by the 4th-5th double bond in the sphingosine molecule. Binding of simple aldehyde to carbon in the 4-position in sphingosine resulted in the formation of a long-chain fatty aldehyde. Covalent binding of this GLS aldehyde to KLH was performed in the reaction of reductive amination. The procedure led to the formation of a stable secondary amine bond between GLS and protein. Conjugates of KLH and GLS extracted from glioma tissues were obtained by the method described for cerebral GLS [14]. As an example, we described specificity of the compound to GM1.

GM1 (2 mg) was dissolved in 2 ml methanol and cooled to a temperature of dry ice in a glass vessel. Ozone was obtained from oxygen in a GE60/FM100 tabletop generator (Ozone Services) at power and flow settings of "1" and "$^1/_4$", respectively. At the output of the ozone generator, the product was bubbled for 5 min through GLS solution cooled with dry ice. Dimethylsulfoxide (100 μl) was added to the solution and stirred at dry-ice temperature for 30 min and then at room temperature for 90 min. The solution was placed in a thick-walled screw-cap glass tube (13×100 mm). The solvents were vacuum evaporated. Fatty acid aldehyde was removed by extraction in heptane. Hexane (4 ml) was added to the tube and sonicated in a special bath. The tube was centrifuged at 200*g* for 5 min, and the transparent supernatant was thoroughly removed. Hexane was separated from the pellet by evaporation in the nitrogen flow. The pellet was dissolved in 200 μl solution containing 10 mM phosphate buffer and 100 mM NaCl (pH 7.2). KLH (2 mg in 200 μl phosphate buffer) and freshly prepared recrystallized sodium cyanoborohydride (1.75 mg in 175 μl phosphate buffer) were added. The reaction was performed at 42°C and mixing for 48 h. Sodium cyanoborohydride was added 24 h after the start of this reaction. The conjugate was dialyzed against phosphate buffer. The quantitative analysis was performed by ultrasound spectroscopy. Thin-layer chromatography and specific staining for sialic acid [14] revealed this acid, which indicated that GLS underwent covalent binding. The quantitative analysis of sialic acid bound to the protein was performed by its isolation followed by HPLC and impulse amperometric assay. The efficiency of GLS binding was 60% (as estimated in relation to the total amount of this substance).

Sterility of the vaccine was studied using Viromed Laboratories kits. These tests were performed according to the requirements of USP XXIV. Endotoxin content in the preparation was measured by the limulus test with *Limulus polyphemus* amoebocyte lysate (Associates of Cape Cod, Inc.). Endotoxin concentration in the vaccine was 0.06 U/ml, which is much below the level permissible for medicinal preparations (as recommended by FDA).

Toxicity of the vaccine containing conjugates of GLS and KLH was tested on 6-week-old (BALB/c× C57Bl/6)F1 mice (Jackson Laboratories). The animals were divided into 3 groups. Each group consisted of 10 mice. Phosphate buffer was injected subcutaneously to group 1 mice. Group 2 and 3 animals subcutaneously received the vaccine containing 5 and 10 μg GLS in 200 μl phosphate buffer, respectively.

Body weight was estimated on days 1, 2, 3, 7, and 14. Then the mice were weighted weekly. The survival rate was evaluated over 3 months. Experimental animals did not receive preparations except vaccine with conjugates of GLS and KLH [9].

RESULTS

Body weight did not decrease in mice receiving the vaccine. Moreover, this vaccine did not cause death and changes in behavioral characteristics and external appearance of mice.

Our results indicate that LS1 vaccine is safe. The preparation produced no side effects in experimental animals. This approach to vaccination holds much promise. The vaccine should undergo clinical tests in patients with malignant gliomas.

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