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Acute and Repeated Dose Intramuscular Toxicity of GM3 Cancer Vaccine in SD Rats

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Abstract: GM3 is a ganglioside over-expressed in some tumors, but it is also an autoantigen present in normal mammalian tissues. A novel ganglioside-based cancer vaccine for the treatment of human breast and melanoma tumors was designed. Two studies were carried out to evaluate the toxicity of GM3 cancer vaccine: Acute and repeated dose intramuscular administration in SD rats. The objective of the present study was to determine the toxicity of the GM3 vaccine in rats after intramuscular administration of single and repeated doses after 14 days. All rats were inspected daily for clinical signs. Body weight and rectal temperature were measured during the administration of test article. Gross necropsy was performed on all animals at the end of study and histological examination was performed on tissues from the repeated dose study. Blood samples were collected for hematological and serum biochemical determinations at the repeated dose assay. There were no deaths significant differences in mean body weight and rectal temperature with treatment. A slight treatment-related decrease in hemoglobin and hematocrit was observed and the white blood cell and neutrophils were increased in the GM3 vaccine group. Total proteins and albumin were significantly decreased in the treated group. All treated rats showed tissue hardening and an inflammatory reaction around the administration site. Increases in spleen weights were observed in treated animals. No other tissues in any animal of the treated group showed signs of toxicological lesions. In conclusion, GM3 vaccine was found to have a low toxicity.

Key words: Ganglioside, cancer vaccine, rats, toxicity

INTRODUCTION

The specific activation of the immune system to control cancer growth has been a primordial goal in cancer immunology and medical oncology^[1]. Since most of the currently used anti-cancer therapies are traumatic for patients; a cancer vaccine is one of the most promising and exciting fields in cancer research^[2].

The presence of substantial amounts of GM3 ganglioside and its derivatives in several epithelial tumor types such as malignant melanoma and neuroectodermal human tumors, together with its specific biological properties, make these glycolipids unique targets for cancer immunotherapy^[3-5]. GM3 is an autoantigen present in normal mammalian tissues and because its natural abundance is not immunogenic in mammalian. This ganglioside should remain cryptic in normal tissues, unavailable to circulating antibodies^[5]. Unfortunately, because of this poor immunogenic property, it is necessary the use of a bacterial origin carrier to form Very Small Size Proteoliposomes (VSSP), composed by the noncovalent GM3 conjugation with outer membrane

proteins from *Neisseria meningitidis*^[6]. Pharmacology tests showed that GM3 cancer vaccine possesses antitumor activity and minor side effects^[7-9]. This study was undertaken to assess the intramuscular toxicity of GM3 cancer vaccine in rats by single and repeated administration.

MATERIALS AND METHODS

Test article: GM3 was hydrophobically conjugated with proteins derived from the outer-membrane-protein complex from *Neisseria meningitidis* (Finlay Institute; Havana, Cuba) to form very small size proteoliposomes (GM3/VSSP). Montanide ISA 51 was selected as the immunological adjuvant.

Animals: Seven to nine weeks old Sprague dawley rats (Cenp:SPRD) of both sexes were obtained from The National Center for Laboratory Animals Breeding (CENPALAB; Havana, Cuba). After seven days of acclimation, 15 males (170-200 g) and 15 females (150-170 g) were chosen for the acute toxicity

test (14 days) and 15 males (150-170 g) and 15 females (130-150 g) were used for the repeated dose toxicity test (14 days). The environmental conditions and animal handling routines were in accordance with Good Laboratory Practice (GLP). Rats were housed two or three animals per cage. The room temperature oscillated between 22-26°C and relative humidity 50-70% with 12 h light cycle and air change 12 fold/h. Water and feed (EMO 1002, ALYco®, CENPALAB) were sterilized and available ad libitum during the acclimation and observation periods.

Acute intramuscular toxicity test: Animals of both sexes were divided into three groups (control, vehicle and treated) (Ten animals per group). The control group received saline (0.1 mL); vehicle group was treated with 0.1 mL Montanide ISA 51 (Sepic; Paris, France) and Tris-HCl buffer, pH 8.5 and treated group received GM3 cancer vaccine (concentration: 0.67 mg mL⁻¹, dose: 50 µg kg⁻¹ of body weight). Rats were observed for 14 days after injection, focusing on clinical appearances and death. Body weight was weekly measured. Rectal temperature was measured before and four hours after single administration. At the end of observation period (14 days), all rats were euthanized by cervical dislocation. Gross necropsy was made in all animals and the administration site was microscopically examined.

Repeated intramuscular toxicity test: Three groups of 5 male and 5 female were injected with saline (control group), Montanide ISA 51 and Tris-HCl buffer, pH 8.5 (vehicle group) and GM3 cancer vaccine as treated group (concentration: 0.7 mg mL⁻¹, dose: 50 µg kg⁻¹ b.w). The daily dose of vaccine used was ten-fold the human proposed dose. The health status of the rats was observed daily for 14 days. Body weight was weekly measured and rectal temperature was measured before and four hours after administration. Blood samples were collected from orbital sinus at the end of the experiment for hematological and serum biochemical determinations.

In hematological examinations, Red Blood Cell (RBC), White Blood Cell (WBC), Hemoglobin (HGB), Hematocrit (HTC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Platelet Count (PLT) were determined using a hematological automatic analyzer (Micros ABX, Roche Diagnostic Systems). The differential leukocyte count, Neutrophils (N), Lymphocytes (L), Monocytes (M), Eosinophils (E) and Basophils (B), was determined on the blood smears. Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Glucose (GLU), Blood Urea Nitrogen (BUN), Total Protein

(TP), Total Cholesterol (T-CHO), Total Bilirubin (T-BIL), Creatinine (CREA), Uric Acid (UA) and Triglycerides (TG) were determined in a biochemical analyzer (Hitachi-704; Japan). Organ weights were measured for the thymus, adrenals, gonads, heart, lung, kidney, spleen, liver and brain. Organs, tissues and administration site were taken and fixed in 10% neutral buffered formaldehyde. These tissues were processed by paraffin wax embedding prior to sectioning and stained with hematoxylin-eosin and histopathologically evaluated.

Statistics: All data were entered into a database using the Statistical Package Scientific System, SPSS for Windows, version 10.0. The mean (X) and Standard Deviation (SD) were calculated for all parameters. One-way Analysis of Variance (ANOVA) and Duncan's multiple test were adopted for statistical testing; p < 0.05 is used as the criterion for significance.

RESULTS

Acute toxicity intramuscular test: All rats survived until the end of the study. Tissue hardening and inflammatory reactions were observed at the administration site in the 80% of treated animals. Other vaccine-related changes were not clinically observed. No marked differences between groups were observed in body weight gain (Fig. 1) and rectal temperature (Fig. 2).

Gross necropsy demonstrated swelling of muscular tissue at the administration site. All treated rats showed tissue hardening and inflammatory reaction, characterized by cysts, mononuclear cells and fibroblasts around the administration sites and several spaces of various sizes,

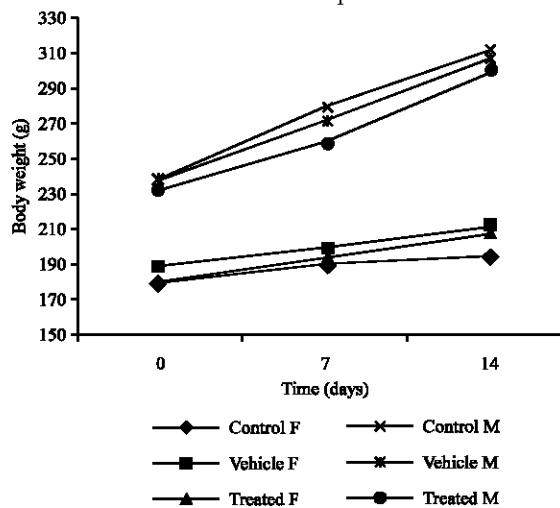


Fig. 1: Changes in body weights of C57BL/6J rats treated with GM3 cancer vaccine in acute toxicity test. F: Female, M: Male

Table 1: Hematological values in Cenp: SPRD rats treated with GM3 cancer vaccine

Groups	Sex	HGB (g dL ⁻¹)	RBC (x10 ⁶ μL ⁻¹)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g dL ⁻¹)	PLT (x10 ⁹ μL ⁻¹)	WBC (x10 ³ μL ⁻¹)	N (%)	L (%)	M (%)	E (%)	B (%)
Control	F	13.4±0.7	7.08±0.45	40.4±2.2	56.8±1.8	18.9±0.7	33.1±0.2	753.4±177.7	4.2±0.8	6.6±2.3	92.8±2.8	0.4±0.5	0.2±0.4	0
	M	14.4±0.6	7.21±0.22	43.6±1.6	60.4±1.1	20.0±0.4	32.2±0.7	629.6±140.4	6.6±0.5	8.4±2.3	90.4±1.8	0.8±0.8	0.4±0.5	0
Vehicle	F	12.8±0.5	6.64±0.36	38.5±1.6	57.8±1.3	19.3±0.9	33.3±0.6	754.0±93.1	7.7±2.6	7.6±3.0	90.2±3.4	1.6±1.1	0.6±0.5	0
	M	13.1±1.0	6.75±0.5	40.2±2.8	59.0±2.2	19.3±0.7	32.7±0.3	785.0±95.0	7.5±1.3	8.8±1.3	89.8±1.6	1.2±0.8	0.2±0.4	0
Treated	F	11.3±0.9*	6.08±0.33*	33.4±2.2*	54.8±1.6	18.6±0.9	33.8±0.8	778.2±44.1	8.8±4.2	10.0±1.6*	88.6±2.6	1.2±0.8	0.2±0.4	0
	M	13.7±0.5	7.21±0.4	41.2±1.5	57.0±1.6	19.0±1.0	33.2±1.0	837.4±50.2	12.8±2.5*	8.4±1.8	89.8±0.8	1.2±0.8	0.6±0.9	0

All values are the mean±SD. Significant differences as compared with the control: * p < 0.05

Table 2: Biochemical serum values in Cenp:SPRD rats treated with GM3 cancer vaccine

Groups	Sex	ALP (U L ⁻¹)	AST (U L ⁻¹)	ALT (U L ⁻¹)	ALB (g dL ⁻¹)	T-BIL (mg dL ⁻¹)	TP (g dL ⁻¹)	GLU (mg dL ⁻¹)	BUN (mg dL ⁻¹)	UA (mg dL ⁻¹)	T-CHO (mg dL ⁻¹)	TG (mg dL ⁻¹)	CREA (mg dL ⁻¹)
Control	F	167.2±24.4	194.6±47.1	44.4±6.1	5.0±0.3	0.23±0.05	7.5±0.5	65.2±16.0	43.4±5.5	3.0±0.7	64.0±12.7	64.0±34.1	0.66±0.04
	M	283.2±44.3	158.4±18.6	44.2±7.8	4.6±0.3	0.20±0.02	7.0±0.4	96.6±23.4	32.4±2.4	3.0±0.5	62.4±10.8	102.0±46.3	0.56±0.03
Vehicle	F	164.4±40.7	298.8±52.2	49.0±11.0	4.3±0.2	0.19±0.04	6.1±0.5	73.6±18.3	60.4±17.1	3.2±0.4	67.8±7.0	88.0±32.1	0.64±0.1
	M	259.2±50.6	269.4±116.1	59.4±27.1	4.2±0.2	0.19±0.02	5.9±0.3	105.6±17.0	41.6±6.2	2.9±1.8	60.0±8.7	83.6±12.8	0.56±0.04
Treated	F	151.6±31.9	243.0±56.2	41.4±4.4	3.9±0.4*	0.17±0.03	6.2±0.3*	116.0±14.6	60.0±8.9	3.3±0.6	64.8±7.3	77.6±40.0	0.68±0.06
	M	264.0±41.8	129.9±8.2	37.2±4.8	3.8±0.4*	0.16±0.03	6.1±0.4*	108.2±14.8	38.8±5.4	3.0±0.7	61.2±9.3	98.8±25.3	0.52±0.01

All values are the mean±SD. Significant differences as compared with the control: * p < 0.05

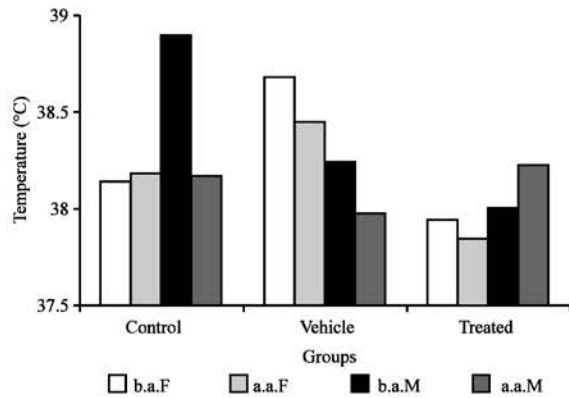


Fig. 2: Changes in rectal temperature of Cenp: SPRD rats treated with GM3 cancer vaccine in acute toxicity test. F: Female, M: Male. b.a.: Before administration, a.a.: After administration (4 h)

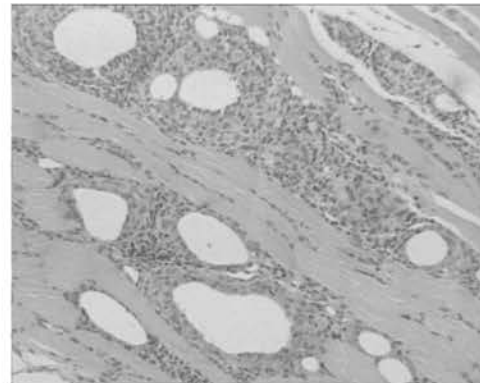


Fig. 3: Reaction in the administration site in animal treated with GM3 cancer vaccine. Note the inflammatory infiltrate surrounding spaces of various sizes occupied with the substance. H-E 100x

occupied with test substance [cysts-like] (Fig. 3). Inflammatory infiltrate consisting of histiocytes, macrophages, some lymphocytes and polymorphonuclear neutrophils leukocytes were present at the injection sites. Cysts and inflammatory reaction with fibrocytes, fibroblasts, histiocytes, macrophages and some lymphocytes in perimysium were also observed. These reactions were also observed in the vehicle group. No other tissues showed signs of macroscopic abnormalities.

Repeated toxicity intramuscular test: All rats survived until the end of the study. Changes at the administration site were similar to the acute toxicity test. Differences in body weight (Fig. 4) and rectal temperature were not observed. Slightly decreases in hemoglobin and hematocrit values were observed in treated females

(Table 1). Albumin was significantly decreased in the treated group (Table 2). All treated rats showed a similar tissue reaction at the injection sites to the ones found in the acute test. Inflammatory infiltrates consisting of histiocytes, macrophages, some lymphocytes and polymorphonuclear neutrophils leukocytes were also found at the injection sites of the repeat dose animals. In perimysium, a severe proliferation of fibrous tissue, including cysts generally surrounded by histiocytes and macrophages was observed, although in some areas there were polymorphonuclear neutrophils leukocytes and eosinophils, as well as cellular detritus present (Fig. 5). An inflammatory reaction with lymphocyte predominance and many plasmatic cells was seen at the administration site of the treated group (Fig. 6).

Table 3: Relative organ weights of Cenp:SPRD rats treated with GM3 cancer vaccine

Groups	Sex	Spleen	Adrenals	Kidneys	Liver	Ovaries/Testis	Thymus	Heart	Lungs	Brain
Control	F	0.20±0.01	0.026±0.005	0.85±0.03	3.19±0.53	0.04±0.00	0.17±0.02	0.38±0.02	0.48±0.01	0.98±0.05
	M	0.22±0.01	0.016±0.005	0.86±0.08	3.29±0.17	0.54±0.02	0.22±0.04	0.38±0.02	0.40±0.01	0.73±0.04
Vehicle	F	0.24±0.02	0.03±0.00	0.83±0.04	3.29±0.25	0.04±0.01	0.20±0.06	0.40±0.01	0.47±0.02	0.95±0.06
	M	0.21±0.02	0.018±0.004	0.87±0.05	3.23±0.10	0.49±0.02	0.15±0.03	0.38±0.02	0.41±0.01	0.69±0.04
Treated	F	0.29±0.06*	0.028±0.004	0.83±0.10	3.46±0.24*	0.04±0.01	0.16±0.04	0.39±0.04	0.56±0.11*	1.07±0.22
	M	0.31±0.06*	0.02±0.00	0.89±0.08	3.40±0.24*	0.53±0.06	0.18±0.04	0.40±0.03	0.56±0.12*	0.73±0.06

All values are the mean±SD. Significant differences as compared with the control: * p < 0.05

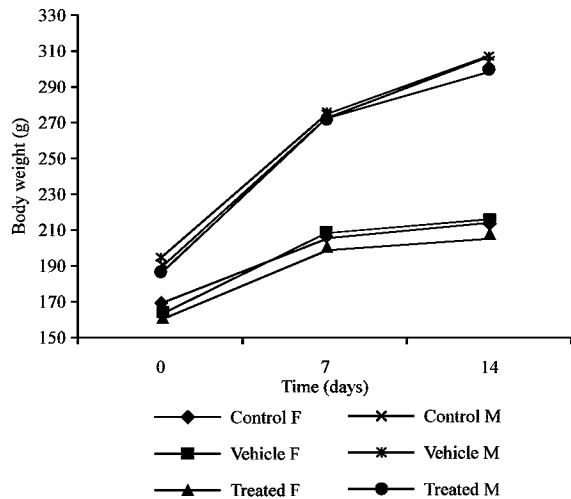


Fig. 4: Changes in body weights of Cenp: SPRD rats treated with GM3 cancer vaccine. Repeated Toxicity Test. F: Female, M: Male

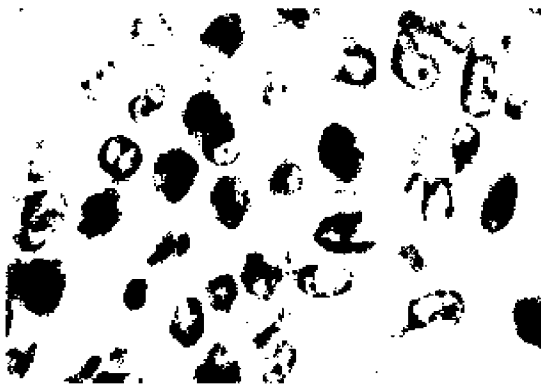


Fig. 5: Administration site: Abundant histiocytes and macrophages, although in some areas there were polymorphonuclear neutrophils leukocytes and eosinophils, as well as cellular detritus around. H-E 100x

Mean relative weight of spleen, liver and lungs were significantly higher in the treated group (Table 3) and the histopathological evaluation of spleen showed a slightly increased size and number of lymphoid follicles in vaccine-treated animals.

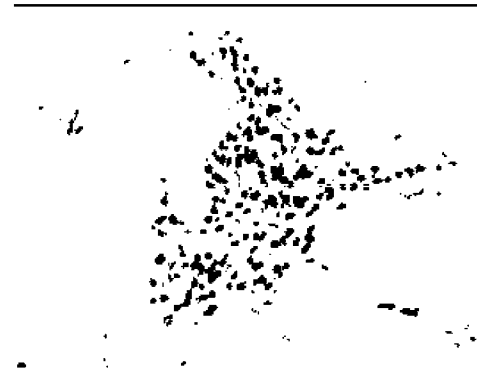


Fig. 6: An inflammatory reaction with lymphocyte predominance and many plasmatic cells in the administration site of animal treated with vaccine. H-E 200x

DISCUSSION

Therapeutic vaccination against cancer-associated antigens represents an attractive option for cancer therapy in view of the comparatively low toxicity and excellent safety profile of this treatment^[10].

Although rectal temperature evaluation is not a typical end-point in toxicology assessment, it is of great importance in this instance. Since during testing vaccines, a temperature increase was expected because of the occurring inflammatory reaction, previously reported in Phase I clinical trials. However, in this experiments, except in occasional animals, this parameter showed a general tendency to decline at 4 h post-treatment. The slightly increase in vaccine-treated males (0.22°C) (Fig. 2) is not a sign of an adverse response to the vaccine, since it has been previously reported in control animals of studied animal strain. In the repeated dose assay, rectal temperature always declined at 4 h post-treatment.

Tissue hardening was the most observed clinical sign in both assays, besides, in some cases, slight swelling of vaccine and adjuvant administration sites. Previously, we have demonstrated that in monkeys, in a 12 month toxicity study, the GM3 vaccine in Montanide ISA 51 produces local damage. In all cases, this reaction disappeared weeks after treatment cessation (animals were not sacrificed)^[9]. In these studies, the observed damage persists until the end

of the assays. Subsequent studies with similar scheduled treatment, but via the subcutaneous route and without adjuvant, did not show the same tissue reaction. Adjuvants are an important component of many vaccine formulations; they are used with the aim of obtaining a higher immune response to antigen^[11]. Sometimes, the adjuvant dose to obtain adequate efficacy, is so high that the principal adverse effects of the vaccine is derived from the adjuvant^[12]. GM3 is a poorly immunogenic molecule, so it needs the use of a carrier (of bacterial origin) and a strong oil-based adjuvant, Montanide ISA 51^[6]. Tissue reactions at the administration site are commonly associated with the use of this type of adjuvant. The vaccines that use this adjuvant are reported as producing local reactions such as abscesses and granulomas^[13,14]. These reports suggest that the localized effect is mainly mediated by the oil-based adjuvant Montanide ISA 51. The adverse effects of adjuvants can be a direct consequence of the inclusion of toxic or non-metabolizable components in the formulation, or use of elements overstimulating the immune or inflammatory response^[15].

The observed slightly decrease of hemoglobin and hematocrit in treated animals (only females) could be related with the reaction at the administration site, mediated, in part, by the combined action of the vaccine and adjuvant. In previous assays, same situation has been observed, but in both sexes. In a previous study with this vaccine in Non Human Primates, one animal showed anemia, recovering from this several weeks after the end of the treatment^[9]. Slightly leucocytosis observed in males, was produced without alteration of the differential formula. The increase of the leukocytes count is associated with the increase in neutrophils^[16]. However, it is thought that this slightly leucocytosis could be associated with the inflammatory reaction observed at the administration site.

The presence of many lymphocytes and plasma cells at the vaccine administration site, suggest that this vaccine is capable of stimulating immune mechanisms. The splenomegaly observed in vaccine treated animals could be result of the presence of a sustained stimulus, causing enlargement of the follicles and subsequent antibody production. With immunostimulatory biopharmaceuticals, immune complex disease, lymphoid aggregates at the injection site, or generalized lymphoid hyperplasia may be observed^[11]. Alonso *et al.*^[7] observed a strong inflammatory reaction, with dense neutrophils leukocyte infiltrates in the alveolar septa, as well as edema and congestion. An activation of extramedular myelopoiesis in liver and spleen was also observed. We did not observe any pathological alterations in the livers and lungs of rats receiving vaccine.

The histopathologic lesions associated with the administration of biopharmaceuticals are generally not as extensive as those that can be observed in toxicological studies with small molecules, due to differences in the doses administered. Histopathologic findings in safety studies of biopharmaceuticals may be limited to local effects at the injection site, as with the present vaccine^[17].

The results of these acute and repeat dose studies with GM3 cancer vaccine have shown that the potential for toxicity is very low.

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