



Heterophilic NeuGcGM3 ganglioside cancer vaccine in advanced melanoma patients: Results of a phase Ib/IIa study

Marta Osorio, Elías Gracia, Edmundo Rodríguez, Giselle Saurez, Maria del Carmen Arango, Elena Noris, Adriana Torriella, Alejandro Joan, Erasmo Gómez, Lorenzo Anasagasti, Jorge Luis González, María de los Angeles Melgares, Imilla Torres, Joel González, Dayamí Alonso, Enrique Rengifo, Adriana Carr, Rolando Pérez, Rolando Pérez & Luis Enrique Fernández

To cite this article: Marta Osorio, Elías Gracia, Edmundo Rodríguez, Giselle Saurez, Maria del Carmen Arango, Elena Noris, Adriana Torriella, Alejandro Joan, Erasmo Gómez, Lorenzo Anasagasti, Jorge Luis González, María de los Angeles Melgares, Imilla Torres, Joel González, Dayamí Alonso, Enrique Rengifo, Adriana Carr, Rolando Pérez, Rolando Pérez & Luis Enrique Fernández (2008) Heterophilic NeuGcGM3 ganglioside cancer vaccine in advanced melanoma patients: Results of a phase Ib/IIa study, *Cancer Biology & Therapy*, 7:4, 488-495, DOI: [10.4161/cbt.7.4.5476](https://doi.org/10.4161/cbt.7.4.5476)

To link to this article: <https://doi.org/10.4161/cbt.7.4.5476>



Published online: 01 May 2008.



Submit your article to this journal [↗](#)



Article views: 383



View related articles [↗](#)



Citing articles: 5 View citing articles [↗](#)

Clinical Study

Heterophilic NeuGcGM3 ganglioside cancer vaccine in advanced melanoma patients

Results of a Phase Ib/IIa study

Marta Osorio,^{1,*} Elías Gracia,¹ Edmundo Rodríguez,¹ Giselle Saurez,² Maria del Carmen Arango,³ Elena Noris,³ Adriana Torriella,³ Alejandro Joan,⁴ Erasmo Gómez,⁵ Lorenzo Anasagasti,⁵ Jorge Luis González,⁵ María de los Angeles Melgares,⁵ Imilla Torres,¹ Joel González,¹ Dayamí Alonso,¹ Enrique Rengifo,⁶ Adriana Carr,⁷ Rolando Pérez⁷ and Luis Enrique Fernández⁷

¹Clinical Trials Unit; ³Immunological Department; ⁴Pathology Department; ⁵Surgery Department; National Institute of Oncology and Radiobiology; Havana City, Cuba; ²Clinical Department; ⁶Quality Division; ⁷Research and Development Division; Center of Molecular Immunology; Havana City, Cuba

Key words: gangliosides, NeuGcGM3, cancer vaccines, clinical trial, melanoma, autoimmunity, vitiligo

NeuGcGM3 ganglioside is especially attractive because it is expressed on melanoma cells but it is minimally or not expressed at all on most normal human tissues.

A Phase Ib/IIa clinical trial was carried out in patients with advanced cutaneous and ocular malignant melanomas, to evaluate immunogenicity and toxicity of an intramuscularly administered cancer vaccine and composed by NeuGcGM3 in a proteoliposome of *Neisseria meningitidis* with Montanide ISA 51 as adjuvant.

Twenty two patients were included, twelve at dose level of 200 µg and 10 at 400 µg. The first five doses were administered every other week and then monthly until 9 doses. 12 patients received additional immunizations.

Vaccination induced specific anti-NeuGcGM3 IgM, IgG and IgA antibodies responses. Titers of IgM were greater for the highest vaccine doses. Vaccination also elicited DTH response in 45.5% of patients in the lower doses and 77.8% in the higher doses. Toxicities were mostly grade 1 or 2, according CTC-NCI criteria. Interestingly, 3 patients developed vitiligo at the lower dose (none in the highest dose) although the nominal antigen NeuGcGM3 is not present in melanocytes. Survival analysis was not the goal of this Phase I trial; nevertheless, the fact that seven patients are alive for more than 2 years after inclusion is noteworthy.

Safety and immunogenicity with NeuGcGM3 vaccine treatment in advanced melanoma patients were established. The prognostic value of autoimmunity and the possibilities of dissociating anti-tumor immunity from autoimmunity deserve further research.

Introduction

Melanoma continues to be “the big trouble” for both, patients and oncologists. Advances in prevention, early diagnosis and treatment have not change the actual situation characterized as “epidemic” worldwide.¹

Decades ago, considerable efforts had been directed to the characterization of antigens and increase knowledge of the biology and immunological processes of the disease, but in spite of the extent of the available literature about immunology and immunotherapeutics, all of this has not been properly translated in clinical benefit for melanoma patients.² Many immunological approaches have been attempted, knowing that the absence of effective treatment after surgery is a major reality.³ Adjuvant high dose interferon, considered as standard treatment in most countries did not change consistently prognosis and survival, and its high toxicity have led patients to refuse it categorically.⁴ Another therapy trend is Biochemotherapy, that combines cytotoxic drugs to one or several biological response modifiers as Interleukin-2 (IL-2) and Alpha interferon (IFNα), but its true relevance is still controversial; large randomized and well designed trials are needed before any conclusion can be drawn.⁵

Active immunotherapy with vaccine formulations that involve host activation of innate immune system is actually an interesting field to consider in melanoma, taking into account treatment feasibility and tolerability.⁶ Changes in the pattern of carbohydrate expression are described in association with malignant transformation in many types of cancer.⁷ According to this, overexpression of certain gangliosides in some tumors like Melanoma might be an attractive therapeutic target for active immunotherapy by vaccines or by monoclonal antibodies as passive immunotherapy.⁸

The presence of the N-glycosylated variants of gangliosides in tumor cells has been considered a controversial subject for a long time. Nevertheless, four interesting observations are available related to this type of ganglioside in melanoma:

- Patients with detectable anti-NeuGcGM3 antibodies prior to treatment with melanoma cell lysates and vaccinia virus showed increased time to progression rates.⁹

*Correspondence to: Marta Osorio; National Institute of Oncology and Radiobiology; Clinical Trials Unit; 29 and E, Vedado; Plaza Havana, Havana City 10400 Cuba; Tel.: 53.7.8369209; Fax: 53.7.2720644; Email: mcosorio@infomed.sld.cu

Submitted: 09/11/07; Revised: 12/29/07; Accepted: 01/02/08

Previously published online as a *Cancer Biology & Therapy* E-publication: <http://www.landesbioscience.com/journals/cbt/article/5476>

- Increased 5 year survival rates were observed¹⁰ in melanoma patients with anti- NeuGcGM3 present at diagnosis.

- Hanganutziu Deicher (HD) Glycoprotein in samples of melanoma tumors.¹¹

- A highly specific murine anti-NeuGcGM3; showed an intense reaction with melanoma cells in primary tumors.¹²

NGcGM3 (NeuGc α 2,3Gal β 1,4Glc-Cer) ganglioside is a sialic acid- containing glycosphingolipid with the neuraminic acid NeuGc. In general, this residue (NeuGc) is not expressed in human and chicken normal tissues, but it is widely present in the plasma membrane of other vertebrates.¹³ The significance of this ganglioside for tumor cells biology was not clear, but recently, de Leon et al., reported for the first time, the capacity of this ganglioside to down-modulate CD4 expression in murine and human T lymphocytes, especially in non-activated T cells.¹⁴ Nevertheless, in spite of the tumor immunosuppression induced by this ganglioside, Carr et al., previously described the immunogenic and toxicity profile of the heterophilic NeuGcGM3 ganglioside vaccine in patients with advanced breast cancer.¹⁵ This vaccine is formulated by the combination of the gangliosides with the outer membrane protein of *Neisseria meningitidis*, to form very small size proteoliposomes (VSSP).¹⁶ This technology allows for an active immunotherapy that involves activation of the potent innate natural immune system, a possible way to improve immunological and therapeutical results.¹⁷

We performed a Phase Ib/IIa study with 22 advanced melanoma patients with this molecular ganglioside vaccine, NeuGcGM3/VSSP/ Montanide ISA 51 in order to evaluate safety, immunological and any evidence of anti-tumoral response. NeuGcGM3 expression was confirmed in primary tumors and loco regional metastases in these patients. An important outcome (skin depigmentation and/or vitiligo) were observed in the first dose level during the immunization. Additionally, anti-NeuAcGM3 and anti-NeuAcGM2 gangliosides antibodies were induced during the vaccination. These events might characterize the presence of antigen spreading or immunorestitution after stimulating the immune system by NeuGcGM3/VSSP/ Montanide ISA 51 immunization.

Results

Patient population. Twenty two patients were included in the study; Twelve in the 0.2 mg level and 10 in the 0.4 mg dose level. All of them provided written informed consent. Demographic characteristics, previous therapies, site of metastases and total vaccination doses are shown in Table 1.

Eighteen patients were assessable for the purposes of the study and four were not because they did not complete the induction phase of the treatment due to quick progressive disease.

Most patients in both levels had tumor burden and were in progressive disease status.

Expression of NeuGcGM3 in primary melanoma tumor, metastases and normal skin.

Normal skin from both healthy individuals and the adjacent region of the subcutaneous melanoma evidenced no reactivity with 14F7 MAb (Fig. 1A), while primary tumors showed intense membrane

Table 1 **Patient characteristics**

	Dose level 0.2 mg	Dose level 0.4 mg
Eligible patients	12	10
Evaluable patients	10	8
Gender		
Female	4	7
Male	8	3
Age (median - range)	45(24–62)	56.7(34–74)
Performance status (PS) 0–1 (WHO)	12	10
Site of primary tumor		
Head and neck	2	2
Trunk	4	2
Ocular	-	4
Arms - legs	4	2
Unknown	2	-
Metastatic site		
Skin + node + subcutaneous tissue	6	7
Lung + skin + nodes + subcutaneous tissue	3	1
Visceral + bone	3	2
Total resection of metastases	0	2
Prior treatment		
Surgery	8	4
Surgery + immunostimulants	2	3
Surgery + interferon	1	1
Surgery + radiotherapy + chemotherapy	1	2
Disease status		
Stable disease	1	3
Progressive disease	11	7
Treatment doses		
<5 doses	2	2
Between 5 and 9 doses	3	3
>9 doses	7	5

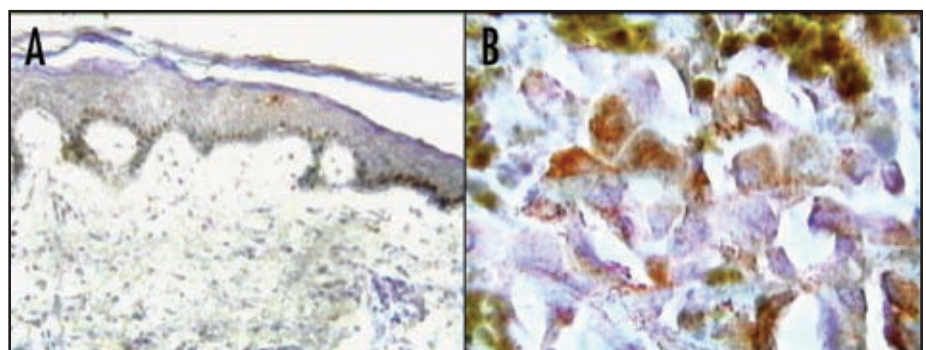


Figure 1. (A) Normal skin showing no reaction with 14F7 Mab 10x. (B) Subcutaneous metastases of a melanoma H/E 6.3x, cytoplasm and membrane of metastatic cells are intensively stained by 14F7 Mab.

and cytoplasmic immunostaining. On the other hand, uveal primary melanomas were the exception so they exhibited moderate to intense heterogeneous reactivity. Five subcutaneous metastases presented

Table 2 Adverse events

Type of events	Dose level 0.2 mg (n:12)			Dose level 0.4 mg (n:10)			
	Grade patients (No. of events)			Grade patients (No. of events)			
	I	II	Total	I	II	III	Total
Pain in the injection site	10(50)	0	10	7(44)	2(11)	-	9
Local inflammatory changes (Erythema, Induration)	-	-	-	3(6)	2(11)	1(6)	6
Fever	3(26)	5(5)	8	2(42)	6(13)	-	8
Myalgia	2(3)	-	2	2(10)	2(6)	3(7)	7
Chills	3(4)	2(4)	5	5(14)	5(1)	-	10
Asthenia	2(7)	-	2	3(7)	1(5)	1(3)	5
Anorexia	3(3)	-	3	3(4)	-	-	3
Nausea and vomiting	1(1)	-	-	6(15)	1(2)	1(2)	8
Increase of Liver Enzymes	-	-	-	1(5)	1(1)	-	2
Increase of Bilirubin	1(1)	-	1	2(2)	-	-	2
Leukopenia	-	-	-	1(1)	-	-	1
Headache	1(1)	-	-	6(10)	1(2)	-	7
Abdominal pain	1(1)	-	-	1(2)	-	-	1
Vitiligo and skin depigmentation	2	1	3	-	-	-	-
Disgeusia	-	-	-	1(3)	-	-	1

intense membrane and cytoplasmic immunostaining (Fig. 1B) except for a case showing moderate to intense reaction. The 14F7 MAb intensely stained cells of metastatic lymph nodes in eleven out of twelve cases studied. NeuGcGM3 expression was observed in primary melanoma tumor and metastatic melanoma cells but not in normal skin.

Safety. All patients developed vaccine-related adverse events grade I–II. Toxicity profile is described in Table 2.

The main adverse events observed were of three types: the local adverse events consisted of: pain, induration and erythema; systemic adverse events as flu-like symptoms consisted of: (fever, myalgia, chills, headache and skin changes: like halo depigmentation and vitiligo.

The toxicity profile was wider in 0.4 mg dose level. Constitutional symptoms and local reactions were more frequent and intense, especially asthenia and myalgia but were successfully controlled with Acetaminophen 500 mg (po) and Difenhidramine (20 mg, IM). Local skin reactions were common and intense at this dose level.

Only 6 patients experienced adverse events classified as grade III. All were successfully controlled without patient's damage.

As this trial allowed additional reimmunizations for patients having good performance status independently of clinical response, 12 patients were reimmunized with more than 9 doses, and 6 of them received further doses up to 15 to 26 vaccinations (patients 2, 3, 5, 8, 18 and 22.) There was no evidence of cumulative toxicity. Despite continuous immunizations for 2 years, all these patients tolerated vaccinations very well.

Immunological response. Serological response. Pre and post-immune antibody titers against NeuGcGM3 ganglioside of all 18 assesable patients were evaluated (Fig. 2). Either IgM or IgG antibodies (titers 1:80 to 1:320) were present in 7/18 sera before vaccine administration. In addition, anti- NeuGcGM3 IgA pre-immune titer was detected in a single patient vaccinated with 0.2 mg. Although the

presence of the three Igs isotypes was tested before the immunization, it was observed just in patient no.1 (1/160 titer). The results of the three classes of antibodies in sera from 14/18 patients after the vaccination are shown. Anti-NeuGcGM3 IgM (Fig.2A) and IgG (Fig. 2B) antibodies responses were induced in all of vaccinated patients with 0.2 mg dose, and in 67% of them, the antibody titers were greater than 1:1280 (maximum titer range, 1:1280 to 1:5120) whereas only a third of these patients developed maximum IgA titers above 1:1280 (Fig. 2C).

Nine patients who received doses of 0.4 mg generated anti-NeuGcGM3 IgM (Fig. 2A) and IgG (Fig. 2B) antibodies responses. High IgM titers and IgG-specific antibodies were also induced in 78% of the second dose level of assesable patients with more than 1:2560 titers (maximum titer range, 1:2560 to 1:20 480). Additionally, IgA specific antibodies (titer greater 1: 1280) were detected in 33% of these patients (Fig. 2C).

No statistical differences were found in anti-NeuGcGM3 ganglioside antibodies titers among dose levels for the maximum value of IgG ($p = 0.401$) and IgA ($p = 0.462$), but it was observed in IgM values ($p = 0.044$) while analyzing IgM, IgG and IgA class during the immunization period.

The IgM absorbance value (405 nm) against NeuAcGM3 and NeuAcGM2 was lower in the first level of dose (Fig. 3A and B). However, high standard deviations in both groups were observed and statistical differences ($p < 0.05$) were established only in week 4 for NeuAcGM3 and week 8 for NeuAcGM2. The onset of IgM maximal titers response to NeuAcGM3 began early in comparison with those against NeuAcGM2.

Cellular response. Induction of cellular response by NGcGM3/VSSP/Montanide ISA 51 was evaluated by DTH response and tested with NGcGM3/VSSP. Seventeen of the 18 patients had no DTH response prior to treatment. DTH responses were observed in 45.5% of patients (5/11) at the lower dose level, and in 77.8% of patients (7/9) at the higher one (Table 3). Interestingly, one patient with

bulky lung metastases showed a long period of disease stabilization (22 months) with positive DTH reaction on days 168 and 336 of 10 mm of induration.

Clinical response and vitiligo development. Despite the advanced stage of the disease at inclusion, 7 patients (out of 22) who received more than 15 doses of the vaccine, survived more than 2 years with PS: 1. Two of these patients had lymph nodes and subcutaneous metastases removed prior to inclusion, (Patient 11 and 16). These patients are still alive after 64 and 55 months respectively.

One patient had partial response for bulky lung metastases (patient 22) lasting 12 months and five patients had stable disease lasting between 6 to 34 months (Patients 2, 3, 14, 15 and 18). Three patients with stable disease had visceral metastases in lung and bone and countless skin and subcutaneous metastases in both legs. Although it was not the main objective of this study, these results are worthy of consideration. Interestingly, 3 patients in the first dose level (0.2 mg) showed evidences of autoimmunity as skin depigmentation (vitiligo-like halo) in areas of mixed tumor response (patients 1, 5 and 22) and during immunizations; two developed generalized vitiligo (patients 5 and 22). Patient 22 showed vitiligo in the face area (Fig. 4A), and also developed halo depigmentation around previous melanoma scar and vitiligo-like halo in melanoma lesions (Fig. 4B).

Discussion

After our finding that N-Glycolyl-GM3 ganglioside is a tumor-specific antigen in human tumors, as breast cancer and melanoma,¹² our staff has been evaluating N-glycolylated ganglioside based vaccine in clinical trials with advanced breast cancer patients.¹⁵ In the present study we demonstrated the NeuGcGM3 ganglioside expression in subcutaneous and lymph nodes metastasis of melanoma tumors. Gangliosides have been reported in plasma membrane essentially,⁸ but some studies have shown the presence of gangliosides as GD3 in the cytoplasm of the mature neuronal cells.²⁵ NeuGcGM3 ganglioside in human tumor cells has been associated with two different cytoplasm mechanisms: (i) dietary origin²⁶ and (ii) endogenous metabolic origin, starting from glycolyl-CoA as precursor.¹³ The expression of NeuGcGM3 ganglioside in melanoma tumor strengthens the position of this molecule as an immunological target. Knowing the immunogenic nature of this disease, active specific immunotherapy with NeuGcGM3/VSSP/Montanide ISA 51 vaccine results in a field to explore in this set of patients.

Several studies have demonstrated humoral responses after immunization with melanoma associated gangliosides. Association of humoral response with improved survival in patients with resected stage IIB an III disease suggests possible benefits but it might be an indicator of more relevant mechanisms.⁶

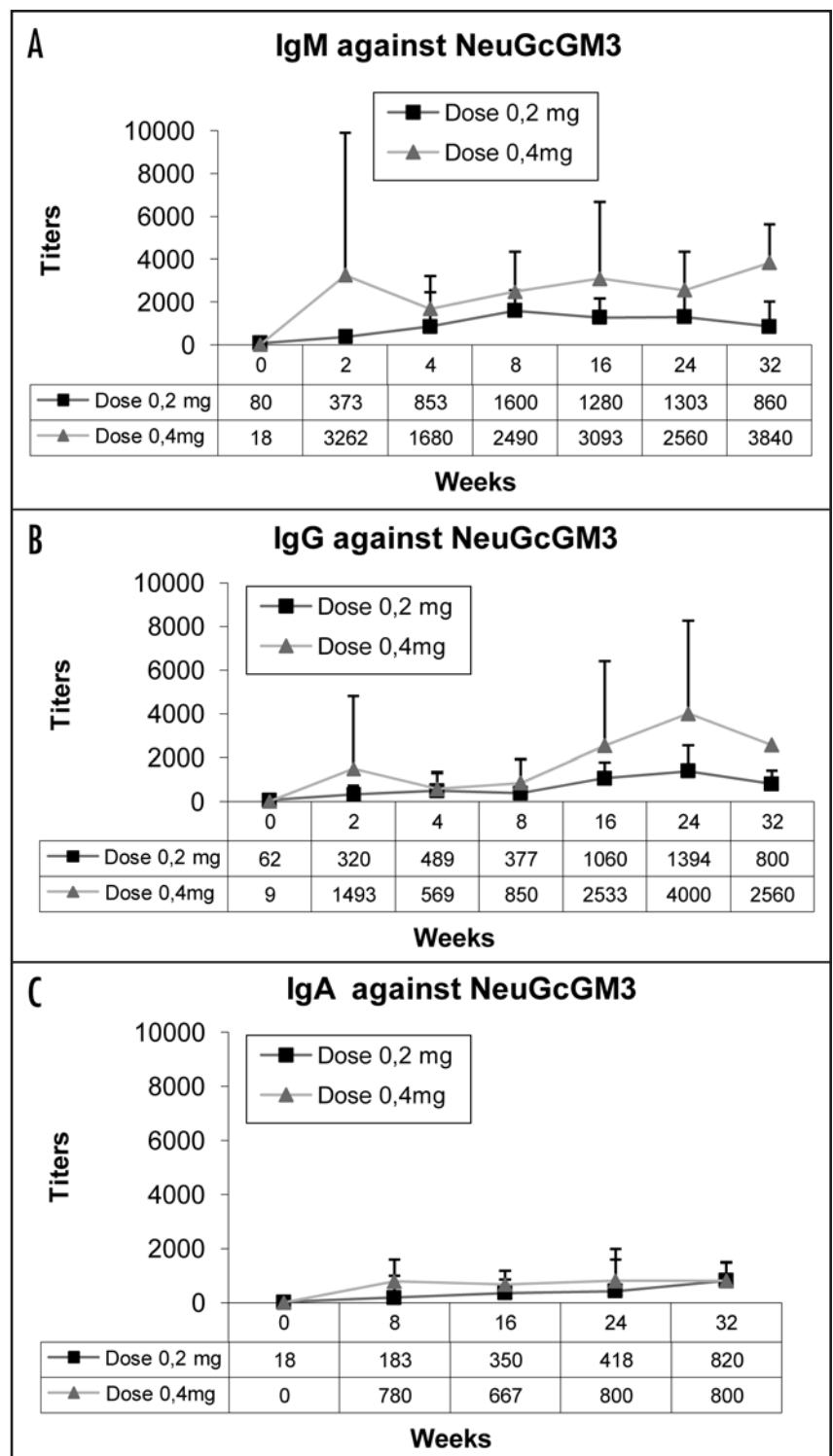


Figure 2. Mean values $\pm$ standard deviation of anti NeuGcGM3 IgG (A), IgM (B) and IgA (C) titers during the immunization schedule were plotted. These values are significantly higher than the pre-vaccine values in all evaluated patients, ($n = 18$) ($p < 0.05$) by Wilcoxon test, but no statistical significance was observed between both dose levels. ELISA titers 1:80 and above are considered positive.

According to its physicochemical properties the NeuGcGM3/VSSP vaccine formulation is a nanoparticle proteosome. However, Lowell et al.²⁷ described a particle with similar size but associated with antigens (peptides) and without adjuvants. The gangliosides

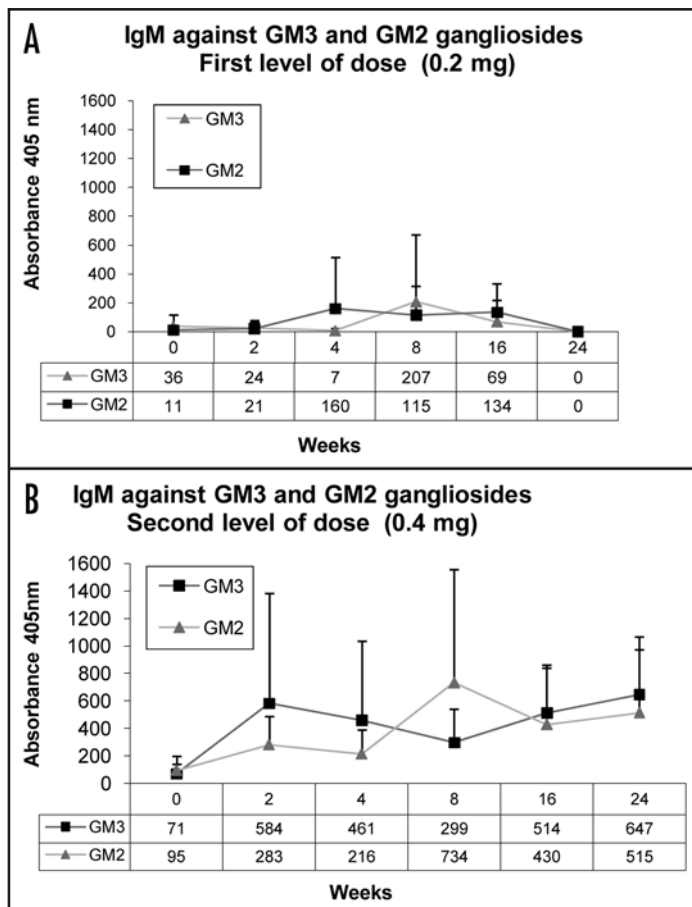


Figure 3. Mean values $\pm$ standard deviation from the absorbance to 405 nm of IgM anti NeuAcGM3 and NeuAcGM2 ganglioside antibodies at the lower (0.2 mg) (A) and higher (0.4 mg) vaccine doses (B). Differences in absorbance value among two groups were analyzed by Mann-Whitney U-test.

are less immunogenic than peptides. For this reason we incorporated oil adjuvants in our vaccine. Despite of the known tolerability of NeuGcGM3 and NeuAcGM3 in mice, our preclinical studies demonstrated that it was generated IgG and IgM isotype anti-ganglioside antibodies when they were injected with Montanide ISA 51 or Complete Freund's Adjuvant (CFA) as adjuvants by intramuscular route.¹⁶ Additionally, when our vaccine formulation with NeuAcGM3 or NeuGcGM3 were administered without oil adjuvants intramuscularly did not induce immunogenic nor antitumoral response in mice.²⁸ In the other hand, the proteoliposome of *Neisseria* with GD3 ganglioside (the most immunogenic in mice) used by Livingston,²⁹ induced only lower IgM titers against GD3 molecule.

In this phase Ib/IIa study, we showed that immunization of metastatic melanoma patients with NeuGcGM3/VSSP/Montanide ISA 51 vaccine is safe and feasible. All patients in both dose levels experienced adverse events, but the 0.2 mg dose level was better tolerated. Local adverse events were most probably related to the adjuvant used and the amount of adipose tissue at the injection site, as these types of events have been reported for other vaccines adjuvanted with Montanide ISA 51.³⁰ Tissue hardening and swelling at the immunization site had been previously observed in the preclinical studies in rats and monkeys with NAcGM3/VSSP/Montanide

Table 3 **DTH value (mm) against NeuGcGM3/VSSP (50 μ g) intradermally administered in melanoma patients from booth doses level**

Patients	Day 0	Day 56	Day 168
0.2 mg			
1	0 mm	11 mm	-
2	5 mm	0 mm	8 mm
3	0 mm	0 mm	0 mm
4	0 mm	9 mm	8 mm
5	0 mm	6 mm	-
6	0 mm	0 mm	0 mm
7	0 mm	-	-
8	0 mm	0 mm	0 mm
9	0 mm	0 mm	-
10	0 mm	-	-
11	0 mm	15 mm	-
14	2 mm	3 mm	5 mm
0.4 mg			
12	0 mm	5 mm	-
13	0 mm	-	-
15	0 mm	0 mm	6 mm
16	0 mm	5 mm	8 mm
17	0 mm	0 mm	8 mm
18	0 mm	20 mm	5 mm
19	0 mm	10 mm	-
20	0 mm	-	-
21	0 mm	6 mm	FF



Figure 4. Vitiligo in patient 22. (A) Vitiligo in the face area and (B) Halo depigmentation around previous melanoma scar and vitiligo like halo in melanoma lesion.

ISA 51 as vaccine or with the emulsion of Montanide ISA 51 as control.^{31,32} However, it is difficult to clarify in this small clinical trial if the skin toxicity observed is only related with the oil adjuvant, or was increased with the NeuGcGM3/VSSP vaccine.

The NeuGcGM3/VSSP/Montanide ISA 51 vaccine was immunogenic, inducing both, humoral (IgG, IgM, IgA specific antibodies) and cellular responses. Antibody titers were lower than those obtained in breast cancer patients stage III, but very similar to those

in breast cancer patients stage IV.¹⁵ No relationship was observed between immunological response (serological and DTH) and the clinical outcome. The onset of DTH occurs on week 8, and at this time an increase of titers becomes evident. However, Portoukalian et al.,³³ observed, in advanced melanoma patients vaccinated with total ganglioside fractions, a significantly longer disease free intervals in those with antibody responses after the treatment as compared with the non responding group. Only two of 21 patients included in our trial were characterized as disease free of advanced melanoma although that tumor burden is often difficult to estimate. The relevance of tumor burden in the immune response after vaccination has been described by several authors.³⁴

One of our most important results was the measurement of IgM response against NeuGcGM3, NeuAcGM3 and NeuGcGM3 gangliosides during the immunization period with NeuGcGM3/VSSP/Montanide ISA 51 vaccine. The anti-ganglioside IgM response significance has been reported previously by Ravindranath.³⁵ This author stated that therapy that actively augments serum levels of IgM against tumor-derived immunosuppressive gangliosides might restore immunocompetence and thereby slow tumor progression.

The maximal IgM and IgG antibody titers against NeuGcGM3 appears relatively late between 4–6 months. However, Portoukalian et al.³³ obtained an increase of antibody titers against melanoma ganglioside between 2–4 months mostly of the IgG isotype. In this clinical trial the evaluation period was only up to 8 months. IgG response against NeuGcGM3 decreased after six months, but IgM titers remained stable during the trial. About IgA anti-NeuGcGM3 ganglioside antibodies response, no correlation was observed with the clinical outcome. Further studies are needed to confirm this parameter. Although high level of total IgA is related to clinical improvement in uveal melanoma.³⁶

An interesting observation was the appearance of vitiligo and depigmentation events, reported here for the first time for a NeuGcGM3 vaccine. In a previous clinical trial with this vaccine in advanced breast cancer patients, no vitiligo events were observed.¹⁵

We did not find a direct association of vitiligo, the presence of anti-NeuGcGM3 ganglioside antibodies and the clinical outcome, but its relation with the vaccination process was evident, since it appeared after the administration of the first 5 vaccine doses and continued increasing after interrupting immunizations. In our case, it should be noticed that vitiligo was induced despite the fact that the vaccine formulation does not contain melanocyte antigens. Spontaneous vitiligo occur in less than 0.1% of stage IV melanoma patients (de Gast GC, unpublished observation), the observed incidence of vitiligo in three (25%) of 12 patients in lower dose vaccine group can be considered as a vaccination effect. Our results showed a higher frequency of vitiligo than data published by Luiten et al.³⁷ who reported 14% of vitiligo with the vaccination with autologous GM-CSF transduced tumor cells in metastatic melanoma patients.

NeuGcGM3 is not present in normal human cells and gangliosides do not participate in the melanin synthesis pathway. We confirm here that NeuGcGM3 ganglioside expression is not present in human normal skin. With the results of this study is difficult to establish if vitiligo is related with an antigen spreading phenomenon or the immunorestitution associated with the NeuGcGM3/VSSP/Montanide ISA 51 vaccination. However, this is the first report of vitiligo connected with a molecular ganglioside vaccine immuniza-

tions. Some authors have reported induction of vitiligo in other trials. This fact has been interpreted as a predictor of better clinical outcome in melanoma patients.³⁸ There are reports of vitiligo related with better survival in melanoma patients treated with biotherapies like interferon alfa -2b³⁹ or IL-2+ GM-CSF.⁴⁰ Rosenberg has previously reported vitiligo during treatment of melanoma patients with IL-2, but it was not observed in metastatic renal cell carcinoma⁴¹ nor events of this type were reported by Kirkwood and colleagues with the GM2-KLH-QS21 (GMK) vaccine.⁴² Vitiligo has also been observed in a clinical trial with an autologous GM-CSF transduced tumor cell vaccine. These melanoma vaccines do contain antigens present in melanocytes.

Although the mechanisms of vitiligo in humans after immunotherapy have never been clearly defined^{43–46} these lesions can be attributed to melanocyte—specific CD8+ cells which causes loss of cutaneous melanocytes.⁴⁷ The activation and increased presentation of other differentiation antigens present in melanocytes (not in the vaccine formulation) elicit anti-melanocyte CD8+ T cells responses or specific antibody responses against them. However, the role of infiltrating T cells in the destruction of melanocytes has never been further evaluated. Several studies suggest that it could be related with melanoma associated depigmentation occurs spontaneously or followed by vaccination strategies.⁴⁸ Using reverse transcriptase polymerase chain reaction, identical clonally expanded cytotoxic T cell lymphocytes were detected in regressing melanoma and vitiligo-like halo surrounding the tumor. The specific pathway of destruction of melanocytes is not known. Apoptosis by Melan A/Mart-1 specific CD8+ T cells is a suggested hypothesis.⁴⁹

Antigen spreading phenomena have been described in other cancer vaccine trials and have been claimed as a predictor of successful anti-tumor response.^{46,50} Association between autoimmune phenomena and prolonged survival has been shown in recent trials of melanoma treatment.³⁷ In these studies, the autoimmunity events appeared to be linked to anti-tumor responses. However, these observations have not been validated in prospective studies.⁴⁴ The prognostic value of this fact and the possibilities to dissociate anti-tumor immunity from autoimmunity with this vaccine deserves further research.

Survival analysis was not the goal of the present trial; however, several of our patients survived more than 1 year (9/22) and even more than 2 years (7/22) which is an uncommon observation for advanced melanoma patients.

The results obtained for the dose levels seems to unquestionably favor this dose due to the following reasons:

- The 0.2 mg dose offers a safer toxicity profile.
- Observation of some evidence of anti-tumor response, longer periods of both disease stability and survival rates than those reported in the literature for this type of patients.
- Presence of vitiligo as autoimmune reaction in some patients.

Our results presented here support the safety and immunogenicity of the vaccine, and are the first clinical connection of vitiligo to a ganglioside molecular vaccine in melanoma patients. Our data strengthens the position of gangliosides as targets for immunotherapy approaches in this disease. At present, the results of a randomized phase II clinical trial in metastatic breast cancer patients with the NeuGcGM3/VSSP/Montanide ISA 51 vaccine is under statistical evaluation. Also, a clinical trial with this vaccine in the adjuvant setting is under evaluation in melanoma patients.

Patients and Methods

Patients. Eligible patients in this study consisted of individuals with advanced cutaneous and ocular melanomas, recruited at the National Institute of Oncology and Radiobiology (INOR), Havana; Cuba, with tumor burden or none totally resected metastases, age between 18 and 70 years, life expectancy >6 months, good performance status according to WHO criteria and appropriate marrow, liver and renal functions by conventional tests. All of the patients signed informed consent forms prior inclusion and all of them had no contraindications such as: pregnancy, autoimmune disease, brain metastases or previous history of encephalopathy, acute and severe allergic events, or active infections. The study was performed according with the principles of the Declaration of Helsinki and after approval by the institutional review board and authorized by the Cuban National Regulatory Authority for Drug Quality Control (CECMED). Pregnancy tests were performed before the inclusion of women in fertile age. Blood samples were collected prior treatment for serological tests and all patients were included after 4 weeks of any oncospecific treatment different from surgery. Adverse events were graded using Common Toxicity Criteria (CTC) 2.0 version¹⁸ and tumor response according RECIST Criteria.¹⁹ Staging criteria used was the AJCC TNM, Melanoma version 2000.²⁰

Immunohistochemical recognition of NeuGcGM3 ganglioside. Tumor samples (primary tumors, subcutaneous (SC) and lymph node metastases) and adjacent normal skin from patients bearing melanoma were taken at surgery. Normal skin samples were obtained from healthy donors. The study was carried out in archive formalin fixed and paraffin embedded tissues. Immunostaining was performed with 14F7 Mab (murine IgG1, highly specific against NeuGcGM3 ganglioside)¹² by the avidin-biotin peroxidase method.²¹ Skin and melanomas immunoreaction was developed with aminoethylcarbazol (AEC).

Vaccine formulation and administration. The vaccine was obtained through the incorporation of NeuGcGM3 (a specific tumor ganglioside) into the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP). NeuGcGM3 ganglioside was obtained from horse erythrocytes by a modification of the Folch method.²² Non carbohydrate or peptide contaminations were identified in NeuGcGM3 purified ganglioside. The ganglioside purification was performed by the combination of modifications of Svennerholm's²³ and Freedman's²⁴ Methods for this glycosphingolipid. Vaccine lots were prepared as described by Estevez et al. in the Good Manufacturing Process (GMP) facilities of the Center of Molecular Immunology, Havana; Cuba.¹⁶

The vaccine formulation was emulsified with Montanide ISA 51 adjuvant (Seppic, Paris; France) immediately before injection. Patients were vaccinated intramuscularly (IM) with 5 doses fortnightly as induction phase, and then one injection every month as reimmunization phase up to 9 doses. Additional immunizations were allowed at the discretion of the physician.

Design of the study. This study was designed as an open-label trial, evaluating two levels of doses of NeuGcGM3/VSSP/Montanide ISA 51 (0.2 mg and 0.4 mg) in 2 cohorts of 12 and 10 patients each. Major objectives were safety, immunogenicity and evidence of antitumoral response. Patients who did not receive all doses at the induction period were considered non eligible for immunologic

and anti-tumoral response. Hematological and Biochemistry tests were performed every other week during the induction period and then monthly. Presence of Vitiligo and other skin related lesions were exhaustively searched in all patients by physical examination prior inclusion and during therapy. Tumor response was evaluated by imagenology in months: 0, 4, 8 and 12. Surgery and/or palliative radiotherapy were allowed in case of progressive disease if necessary.

Immunological procedures and statistics. Blood samples were analyzed as it was previously described.¹⁵ Samples from 18 patients were also assessed for anti-NeuGcGM3 (NGcGM3) antibody titers with an ELISA test. Briefly, gangliosides (200 ng/well) in 50 μ L of methanol were dried in 96-well polystyrene plates (Poly Sorp, Nunc, Denmark) for 90 minutes at 37°C. Then, plates were washed with phosphate-buffered saline (PBS) pH = 7.4 Tween 20 (0.05% vol/vol). Serum samples dilutions were incubated overnight at room temperature; after that, they were washed with PBS containing Tween 20.

Biotin-conjugated goat antihuman immunoglobulins IgM, IgG or IgA (μ , γ or α chain specific respectively, Jackson ImmunoResearch laboratories, Inc. Grove, PA.) were incubated for 90 min at room temperature. The plates were washed again and streptavidin-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories) was used under the same incubation condition. Finally, a solution of p-nitrophenylphosphate (Sigma Chemical, S.Louis; MO) in 1M diethanolamine buffer (pH 9.8) was added. After 30 minutes, absorbance was measured at 405 nm with an ELISA reader (Organon Teknika, Salzburg, Austria). IgM antibodies responses against NeuAcGM3 (GM3) and NeuAcGM2 (GM2) were measured using only 1:80 dilution under the same ELISA test conditions described previously.

Delayed type hypersensitivity responses (DTH) were evaluated with NeuGcGM3/VSSP vaccine (50 μ g) injected intradermally without Montanide ISA 51. Antigens were injected 3 days before 1st, 5th and 9th doses and at day 336. Dermal induration was measured 48 hours after NeuGcGM3/VSSP injection and considered positive if the diameter was higher than 5 mm.

Wilcoxon test was used to compare the maximum of antibody titers. This analysis was performed for the IgM, IgG and IgA anti-NeuGcGM3 response values to NeuGcGM3 ganglioside vaccine at dose levels between 0.2 mg and 0.4 mg. Mann-Whitney U test was used to compare the absorbance value among two groups.

Acknowledgements

The authors thank Milagros Frometa for her histological technical assistance, Yaima Galan for statistical assistance and Dr. Blanca Tormo for valuable manuscript edition. This work was supported by Recombio (Madrid, Spain) and the Cuban Government.

References

1. Meyskens FL., Management of Human Melanoma: What the last decade wrought? The Oncologist 2003; 8:448-50.
2. Sondak VK, Sabel SS. Adjuvant therapy for melanoma: does it really work? Educational Book, proceedings of the 38th Annual Meeting of the American Society of Clinical Oncology, 2002.
3. Morton DL, Essner R, Kirkwood JM, and Wollman RC. Malignant Melanoma in Cancer. Holland JF and Frei E. Cancer medicine review, Section 34; p 1849-69. 5th ed. Hamilton, Ont.; London: BC Decker, 2000.
4. Tsao H, Atkins MB, Sober AJ. Management of Cutaneous Melanoma. N Engl J Med 2004; 351:998-1012.
5. Chung MH, Gupta RK, Hsueh E, Essner R, Ye W, Yee R, Morton DL. Humoral Immune response to a Therapeutic polyvalent Cancer vaccine after Complete Resection of Thick primary Melanoma and sentinel Lymphadenectomy. J Clin Oncol 2003; 21:313-19.

6. Hersey P. Immunotherapy of non-resected melanoma. In: Thompson JF, Morton DL and Kroon BB. Editors. Text Book of Melanoma. First edition. London and New York Martin Dunitz 2004; 602-9.
7. Hakomori S: Aberrant Glycosylation in cancer cell membranes as focused on glycolipids: Overview and perspectives. *Cancer Res* 1985; 45:2405-14.
8. Zhang S, Cordon Cardo C, Zhang HS, Reuter VE, Adluri S, Hamilton WB, O. Lloyd K, Livingston PO. Selection of Carbohydrate Tumor antigen as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. *Int J Cancer* 1997; 73:42-9.
9. Doré JD, Pourtokalian J, Berthier Vergnes O, Jacobovich R, Genève J, Bailly M, Lefthériotis E, Weissbrod A, Mayer M. Réponse des malades atteints de mélanome à l'immunisation par oncolysats de mélanome au virus de la vaccine. *Bull Cancer* 1990; 77:881-91.
10. Nakarai H, Chandler PJ, Kano K, Morton DL, Irie RE. Hanganutziu-Deicher (HD) antigen as a possible target for immunotherapy of melanoma. *Int. Arch Allergy Appl Immunol* 1990; 91:323-28.
11. Kawachi S, Saidá T. Analysis of the expression of Hanganutziu-Deicher (HD) antigen in human malignant melanoma. *J Dermatol* 1992; 19:827-30.
12. Carr A, Mullet A, Mazorra Z, Vázquez AM, Alfonso M, Mesa C, Rengifo E, Pérez R, Fernández LE. A mouse IgG 1 monoclonal antibody specific for N-Glycolyl GM3 ganglioside recognized breast and melanoma tumors. *Hybridoma* 2000; 19:241-47.
13. Malykh YN, Schauer R, Shaw L. N-Glycolylneuraminic acid in human tumors. *Biochimie* 2001; 83:623-34.
14. De Leon J, Fernandez A, Mesa C, Clavel M and Fernandez LE. Role of tumor associated N-Glycolylated variant of GM3 ganglioside in cancer progression effect over CD4 expression on T cells. *Cancer Imm Immunotherapy* 2006; 55:443-50.
15. Carr A, Rodriguez E, Arango MC, Camacho R, Osorio M, Gabrí M, Carrillo G, Valdés Z, Bebelagua Y, Pérez R, Fernández LE. Immunotherapy of Advanced Breast Cancer with a Heterophilic Ganglioside (NeuGcGM3) Cancer Vaccine. *J Clin Oncol* 2003; 21:1015-21.
16. Estevez F, Carr A, Solórzano L, Valiente O, Mesa C, Barroso O, Sierra GV, Fernandez LE. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP) Vaccine 2000; 18:190-7.
17. Mesa C, de León J, Rigley K, Fernández LE. Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for Th1 induction and dendritic cell activation. *Vaccine* 2004; 22:3045-52.
18. NCI Common Toxicity Criteria (CTC) version 2.0 Final 1998.
19. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New Guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000; 92:205-16.
20. Balch C, Buzaid A, Atkins M, Cascinelli N, Coit DG, Fleming ID, Houghton A, Kirkwood JM, Mihm MF, Morton DL, Reintgen D, Ross MI, Sober A, Soong SJ, Thompson JA, Thompson JF, Gershenwald JE, McMasters KM. A new American Joint Committee on cancer staging system for cutaneous melanoma. *Cancer* 2000; 88:1484-91.
21. Hsu SM, Raine L, and Fanger H. Use the avidin-biotin—peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabelled antibody PAP procedures. *J Histochem Cytochem* 1989; 29:577-80.
22. Folch PJ, Arsove S, Meath JA. Isolation of brain stradin, a new type of large molecular tissue component. *J Biol Chem* 1957; 226:497-509.
23. Miller Podraza H, Månsson JE, Svennerholm L. Isolation of complex gangliosides from human brain. *Biochim Biophys Acta* 1992; 1124:45-51.
24. Fredman P, Nilsson O, Tayot JL, Svennerholm L. Separation of gangliosides on a new type of anion-exchange resin. *Biochim Biophys Acta* 1980; 618:42-52.
25. Kawai K, Kuroda S, Watarai S, Takahashi H and Ikuta F. Occurrence of GD3 gangliosides in reactive astrocytes—an immunocytochemical study in the rat brain. *Neuroscience Lett* 1994; 174:119-24.
26. Tangvoranuntakul P, Gagneux P, Diaz S, Bardor M, Varki N, Varki A, Muchmore E. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc Natl Acad Sci USA* 2003; 21:12045-50.
27. Lowell GH, Smith LF, Seid RC, and Zollinger WD. Peptides bound to proteosomes via hydrophobic feet become highly immunogenic without adjuvants. *J Exp Med* 1988; 167:658-63.
28. Carr A, Mazorra Z, Alonso DF, Mesa C, Valiente O, Gomez DE, Perez R and Fernandez LE. A purified GM3 ganglioside conjugated vaccine induces specific, adjuvant-dependent and nontransient antitumor activity against B16 mouse melanoma in vitro and in vivo. *Melanoma Research* 2001; 11:219-27.
29. Livingston PO, Calves MJ, Helling F, Zollinger WD, Blake MS, Lowell GH. GD3/proteosome vaccines induce consistent IgM antibodies against the ganglioside GD3. *Vaccine* 1993; 11:1199-204.
30. Aucouturier J, Dupuis L, Deville S, Ascarateil S, and Ganne V. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev Vaccines* 2002; 1:111-18.
31. Bada AM, Casaco A, Mancebo A, Fuentes D, Gonzalez B, Hernández O, Subirós N, Arteaga M, Hernández J, Carr A and Fernández LE. Acute and Repeated Dose Intramuscular Toxicity of GM3 cancer vaccine in SD rats. *Pakistan Journal of Biological Sciences* 2005; 8:1045-50.
32. Bada A, Casaco A, Arteaga M, Martínez J, León A, Santana E, Hernández O, Orphee R, González A, Mesa C, González C, Montero E, Fernández LE. Toxicity of a GM3 cancer vaccine in Macaca fascicularis monkey: a 12 month study. *Human and Experimental Toxicology* 2002; 21:263-67.
33. Portoukalian J, Carrel S, Doré JF, Rümke P. Humoral immune response in disease-free advanced melanoma patients after vaccination with melanoma-associated gangliosides. EORTC Cooperative Melanoma Group. *Int J Cancer* 1991; 49:893-9.
34. Frey AB, Monu N. Effector-phase tolerance: another mechanism of how cancer escapes antitumor immune response. *J Leukoc Biol* 2006; 79:652-62.
35. Ravindranath MH, Muthugounder S, Hannah MR, Morton DL. Significance of endogenous augmentation of antiganglioside IgM in cancer patients: potential tool for early detection and management of cancer therapy. *Ann N Y Acad Sci* 2007; 1107:212-22.
36. Kotlianskii EO. Immunoglobulins M, G and A as prognostic criteria of the treatment outcome in uveal melanoblastoma. *Allerg Immunol (Leipzig)* 1981; 27:14-21.
37. Luiten RM, Kueter EW, Mooi W, Gallee MP, Rankin EM, Gerritsen WR, Clift SM, Nooijen WJ, Weder P, van de Kastele WF, Sein J, van den Berk PC, Nieweg OE, Berns AM, Spits H, de Gast GC. Immunogenicity, including vitiligo, and feasibility of vaccination with autologous GM-CSF-transduced tumor cells in metastatic melanoma patients. *J Clin Oncol* 2005; 23:8978-91.
38. Yang JC, Sherry RM, Steinberg SM, Topalian SL, Schwartzentruber DJ, Hwu P, Seipp CA, Rogers Freezer L, Morton KE, White DE, Liewehr DJ, Merino MJ, Rosenberg SA. Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer. *J Clin Oncol* 2003; 16:3127-32.
39. Gogas H, Ioannovich J, Dafni U, Stavropoulou Giokas C, Frangia K, Tsoutsos D, Panagiotou P, Polyzos A, Papadopoulos O, Stratigos A, Markopoulos C, Bafaloukos D, Pectasides D, Fountzilas G, Kirkwood JM. Prognostic significance of autoimmunity during treatment of melanoma with interferon. *N Engl J Med* 2006; 354:709-18.
40. Boasberg PD, Hoon DSB, Piro LD, Martin MA, Fujimoto A, Kristedja TS, Bhachu S, Ye X, Deck RR, and O'Day SJ. Enhanced survival associated with vitiligo expression during maintenance biotherapy for metastatic melanoma. *J of Investigative Dermatology* 2006; 126:2658-63.
41. Rosenberg SA, White DE. Vitiligo in Patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy. *J Immunother Emphasis Tumor Immunol* 1996; 19:81-4.
42. Kirkwood JM, Ibrahim JG, Sondak V, Kagarwala SS, Ernstoff MS, Rao U. High-dose interferon alfa-2b significantly prolongs relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIB-III melanoma: results of intergroup trial E1694/S9512/C509801. *J Clin Oncol* 2001; 19:2370-80.
43. Rocha IM, Oliveira LJ, De Castro LC, de Araújo Pereira LI, Chaul A, Guerra JG, Silvestre MC, Batista KM, Pereira FA, Gomide MA, Guillo LA. Recognition of melanoma cell antigens with antibodies present in sera from patients with vitiligo. *Int J Dermatol* 2000; 39:840-3.
44. Das PK, Van der Wijngaard RM, Wankovicz Kalinska A, Le Poole IC. A symbiotic concept of autoimmunity and tumour immunity: lessons from Vitiligo. *Trends Immunol* 2001; 22:1306.
45. Merimsky O, Shoenfeld Y, Baharav E, Zigelman R, Fishman P. Reactivity to tyrosinase: expression in cancer (melanoma) and autoimmunity (vitiligo). *Hum Antibodies Hybridomas* 1996; 7:151-6.
46. Merimsky O, Shoenfeld Y, Fishman P. The clinical significance of antityrosinase antibodies in melanoma and related hypopigmentary lesions. *Clin Rev Allergy Immunol* 1998; 16:227-36.
47. Yee C, Thompson JA, Roche P, Byrd DR, Lee PP, Piepkorn M, Kenyon K, Davis MM, Riddell SR, Greenberg PD. Melanocyte Destruction after Antigen-specific Immunotherapy of Melanoma: Direct Evidence of T Cell-mediated Vitiligo. *J Exp Med* 2000; 192:1637-43.
48. Ongenaes K, Van Geel N, Naeyert JM. Evidence for an Autoimmune Pathogenesis of Vitiligo. *Pigment Cell Res* 2003; 16:90-100.
49. Huang CL, Nordlung JJ, Boissy R. Vitiligo. A manifestation of apoptosis. *Am J Clin Dermatol* 2002; 3:301-8.
50. Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, Duray PH, Steinberg SM, Allison JP, Davis TA, Rosenberg SA. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associate antigen blockade in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 2003; 100:8372-7.