

A purified GM3 ganglioside conjugated vaccine induces specific, adjuvant-dependent and non-transient antitumour activity against B16 mouse melanoma *in vitro* and *in vivo*

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The presence of substantial amounts of GM3 ganglioside on human melanomas and other tumours, together with its peculiar biological properties, makes this glycolipid a unique target for cancer immunotherapy. B16 mouse melanoma expresses GM3 and constitutes an appropriate model for the development of novel GM3-based vaccines. Recently, we hydrophobically incorporated purified GM3 into the outer membrane protein complex from *Neisseria meningitidis* to form very small size proteoliposomes (GM3/VSSP). We have examined the antitumour properties of GM3/VSSP vaccine and compared it with GM3 incorporated in very low density serum lipoproteins (GM3/VLDL). Immunization with four doses of GM3/VSSP vaccine (120 µg of ganglioside) plus Freund's adjuvant or Montanide ISA 51 significantly increased the overall survival of mice inoculated in the subcutis with 10^3 B16-F1 cells, whereas the GM3/VLDL immunogen was ineffective. The non-transient character of tumour protection was confirmed in animals surviving the first challenge and re-inoculated with 5×10^3 cells. GM3/VSSP vaccine also reduced the subcutaneous growth of highly aggressive B16-F10 cells. The importance of ganglioside structure in the tumour-protective effect of GM3/VSSP vaccine was confirmed using GM3 containing *N*-glycolylneuraminic acid, a ganglioside absent in melanoma cells. Immunostaining and enzyme-linked immunosorbent assay (ELISA) experiments showed a high specificity of immune sera against GM3 and the presence of all four IgG subclasses, with a preponderance of IgG2b and IgG3. In addition, a strong anti-B16 complement-mediated cytotoxicity was induced by vaccination with GM3/VSSP. The present data indicate the molecular specificity of GM3/VSSP vaccine as well as the adjuvant-dependent and non-transient character of tumour protection in the B16 mouse model. These findings suggest that an appropriate GM3 vaccine may be capable of inducing prolonged tumour protection in melanoma patients. © 2001 Lippincott Williams & Wilkins

Key words: B16 melanoma, cancer vaccines, gangliosides, GM3, proteoliposomes

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Introduction

Several gangliosides are differentiation antigens expressed on the cell surface of human melanomas and other cancers. Direct evidence for the importance of gangliosides as potential targets for active specific immunotherapy has been suggested by the observation that human monoclonal antibodies against these glycolipids induce regression of human cutaneous melanoma metastases.¹ Similarly, a randomized trial of adjuvant vaccination with bacillus Calmette-Guérin (BCG) plus the melanoma ganglioside GM2 in stage III melanoma patients showed an increased disease-free interval in subjects with anti-GM2 antibodies.²

The role of antibodies in the antitumour effect of ganglioside vaccines has been considered to be predominant, if not exclusive.³ Among the mechanisms of antibody action against circulating tumour cells and micrometastases, complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity have been highlighted.⁴ However, data on the relative contribution of the mechanisms are not available due to difficulties in designing appropriate experiments in humans.

On the other hand, while several gangliosides expressed on human cancers are identical to those present in murine tumours,⁵ only a few reports of passive^{6,7} or active⁸⁻¹⁰ ganglioside-based immunotherapy in syngeneic mouse models are available, restricting the understanding of their mechanisms of action. B16 mouse melanoma cells express high amounts of GM3 and this ganglioside is not immunogenic in mice due to its natural abundance in normal tissues. B16 melanoma is therefore an excellent

murine model for the study of GM3 vaccines. The presence of substantial amounts of GM3 on human melanomas¹¹ and breast cancer¹² corroborates that this ganglioside is an important potential target for active specific immunotherapy if immunogenicity can be effectively increased.

Recently we hydrophobically incorporated purified GM3 into the outer membrane protein (OMP) complex from *Neisseria meningitidis* to form very small size proteoliposomes (GM3/VSSP). We reported that GM3/VSSP vaccine consistently induced an anti-ganglioside specific response in mice.¹³ Preliminary studies also showed that preimmunization with GM3/VSSP is able to prevent the establishment of B16 melanoma tumours.¹⁴

In the present study, we examined the antitumour properties of GM3/VSSP vaccine using the B16 melanoma model in C57BL/6j mice, and compared it with GM3 incorporated into very low density serum lipoproteins (GM3/VLDL). The importance of ganglioside structure in the tumour-protective effect of GM3/VSSP vaccine was studied using GM3 containing *N*-glycolylneuraminic acid (NGcGM3) as an alternative target. Experiments were performed introducing variations in the time and type of tumour cell inoculation and re-challenging tumour-free mice with increased numbers of melanoma cells. Clear evidence of the molecular specificity of GM3/VSSP vaccine and of the adjuvant-dependent and non-transient character of the tumour protection was obtained. Immunostaining and enzyme-linked immunosorbent assay (ELISA) experiments showed a high specificity of immune sera against GM3 and the presence of all four IgG subclasses, with a preponderance of IgG2b and IgG3. In addition, we explored the serological activity by measuring complement-dependent cytotoxicity (CDC) in the sera of Balb/c mice immunized with the GM3/VSSP vaccine.

Materials and methods

Gangliosides

GM3 and NGcGM3 were prepared from dog and horse erythrocytes, respectively, by a modification of the Folch's extraction procedure.¹⁵ The solvent partition step was replaced by mild base treatment followed by extraction with hexane.¹⁶ The gangliosides were further purified by silica gel 60 (230–400 mesh) column chromatography (Merck, Darmstadt, Germany) in chloroform:methanol:ammonia 2.5 M 65:25:4 (v/v). Their purity was verified using high performance thin layer chromatography (HPTLC)

and high performance liquid chromatography (HPLC). GM1, GD1a and GT1b were isolated from bovine brain¹⁷ and NGcGM2 from Balb/c mouse liver.¹⁸

Mice

Female C57BL/6j and Balb/c mice aged 7–8 weeks were obtained from CENPALAB (Havana, Cuba) and kept with water and food *ad libitum* in the animal house facility at the Center of Molecular Immunology (Havana, Cuba).

Tumour cells and culture conditions

B16 mouse melanoma cells, sublines F1 (low metastatic capacity) and F10 (high metastatic capacity), syngeneic for the C57BL/6j strain, were cultured in RPMI-1640 containing 2 mM glutamine and supplemented with 10% heat-inactivated fetal calf serum. An antibiotic mixture containing penicillin and streptomycin (final concentrations 100 IU/ml and 100 µg/ml, respectively) was added to the culture medium to prevent contamination. Cell viability was assessed using the trypan blue dye exclusion technique.

Vaccines

OMP from *Neisseria meningitidis* strain 383 (B4: P1.15) was obtained and purified by the Finlay Institute (Havana, Cuba). To prepare the GM3/VSSP and NGcGM3/VSSP vaccines, GM3 or NGcGM3 gangliosides were hydrophobically conjugated with OMP as previously described in detail by Estevez *et al.*¹³ To obtain the GM3/VLDL vaccine, GM3 ganglioside was incorporated into VLDL from chicken sera, following the procedure of Dumontet *et al.*¹⁹

On weeks 0, 2, 4 and 6, groups of at least 10 C57BL/6j mice were immunized intramuscularly on the posterior dorsolateral regions of the body with OMP (120 µg), GM3/VSSP (120 µg of GM3) or NGcGM3/VSSP (120 µg of NGcGM3). Vaccines were mixed before inoculation either with complete (priming) plus incomplete (boosting) Freund's adjuvant (CFA and IFA, respectively) or with Montanide ISA 51 (Seppic, Paris, France), and administered in a total volume of 0.1 ml/dose. No toxicity or morbidity was detected as a consequence of vaccine administration, as reported previously.¹⁴ GM3/VLDL immunogen (200 µg of GM3) was administered on

weeks 0, 1, 2, 3, 5 and 7 in CFA (priming) plus IFA (boosting).

Tumour cell challenge

B16 melanoma cells (10^3 viable cells) were inoculated into the subcutis of the right flank of vaccinated mice, 3 or 9 weeks after initiation of the immunization protocol. Animals were monitored for 60–100 days after the tumour challenge. The largest perpendicular diameters of the resulting tumours were measured with a calliper three times per week, and tumour volume was calculated using the formula $\pi/6 \times \text{length} \times \text{width}^2$. For ethical reasons, animals were sacrificed when the tumour exceeded 15,000 mm³ or when the general condition of the animals was affected. In re-challenge experiments, mice in each group remaining free of tumour for more than 60 days after the first tumour cell challenge were re-inoculated with 5×10^3 B16 viable cells and monitored as described above.

Serological assays

ELISA were performed using either goat anti-mouse IgM and IgG linked to alkaline phosphatase or biotinylated goat anti-mouse IgM and IgG (Jackson, Pennsylvania, USA). Microtitre plates (Polysorp, Nunc, Denmark) were coated with 0.16 nmol/well of ganglioside in methanol and dried at 37°C for 90 min. Wells containing antigens were washed in phosphate-buffered saline (PBS) containing 0.1% Tween 20 before the addition of diluted mouse sera. In order to control non-specific binding, immune sera were also tested on plates that were processed identically but without the addition of gangliosides, and this reading was subtracted from the value obtained in the presence of gangliosides. The titre was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater.²⁰

Immunostaining of the gangliosides with mouse sera was performed after separation on HPTLC silica-gel plates (Merck, Darmstadt, Germany) as described previously.²¹ Plates were developed in chloroform:methanol:ammonia 2.5 M (0.25% KCl) 50:40:10 (v/v) and the gangliosides were visualized by staining with resorcinol/HCl reagent.

Determination of IgG subclass

Determination of the IgG subclass was performed by ELISA using subclass-specific secondary biotinylated rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 monoclonal antibodies (Pharmingen, California, USA). Secondary antibody dilutions were established by ELISA with anti-ganglioside monoclonal antibodies 14F7 IgG1 (Center of Molecular Immunology) and R24 IgG3 (kindly provided by Dr Philip O. Livingston, Memorial Sloan Kettering Cancer Center, New York, USA).

Complement-mediated cytotoxicity assays

B16 melanoma cells (3×10^6) were labelled with 100 μCi Na₂⁵¹CrO₄ (Amersham, Aylesbury, UK) in RPMI with 8% fetal calf serum for 2 h at 37°C in a CO₂ incubator. Cells were extensively washed, and 1.5×10^4 labelled cells/well were incubated in 96-well round-bottom plates in the presence of diluted (1:5) control BALB/c mouse serum, immunized serum or medium alone for 1 h at 37°C. Cells were then washed and incubated with rabbit complement at a dilution of 1:5 for 3 h at 37°C. The plates were spun at 500 g for 5 min and aliquots of 150 μl of supernatant from each well were harvested for the determination of released ⁵¹Cr. Assays were performed in triplicate and included control wells for maximum release in 1% sodium dodecyl sulphate (SDS) and for spontaneous release in the absence of complement. The percentage of specific lysis was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Statistical analysis

The significance of differences in tumour volume was assessed using the Student's t- test. Differences in IgG and IgM titres and in the rate of seroconversion induced in the animals by the group of vaccines tested were studied with the non-parametric Mann-Whitney U test and Fisher's exact test, respectively. Survival was analysed using the Kaplan-Meier method and groups were compared using the log-rank test. Findings were considered significant if $P < 0.05$.

Results

GM3/VSSP but not GM3/VLDL vaccine inhibited tumour formation and increased the survival of mice challenged with B16-F1 melanoma cells

To examine the antitumour activity of two different GM3 vaccines *in vivo*, C57BL/6j mice were immunized with GM3/VSSP or GM3/VLDL plus Freund's adjuvant, or were given only the adjuvant mixed with PBS as a control. Syngeneic, poorly metastatic B16-F1 melanoma cells (10^3 viable cells) were inoculated subcutaneously on week 3 after initiation of the immunization protocol, and tumour formation and survival of mice were monitored. As shown in Figure 1, most control animals developed tumours and died 30–50 days after the B16 challenge. On the other hand, 80% of mice immunized with GM3/VSSP vaccine showed no evidence of tumour. The overall survival of GM3/VSSP-vaccinated mice was significantly higher than that of control mice. In contrast, no impact in survival was seen with GM3/VLDL vaccine.

Vaccination with GM3/VSSP reduced tumour growth in mice challenged with highly aggressive B16-F10 melanoma cells

The antitumour effect of vaccination with GM3/VSSP was also investigated in mice challenged with highly

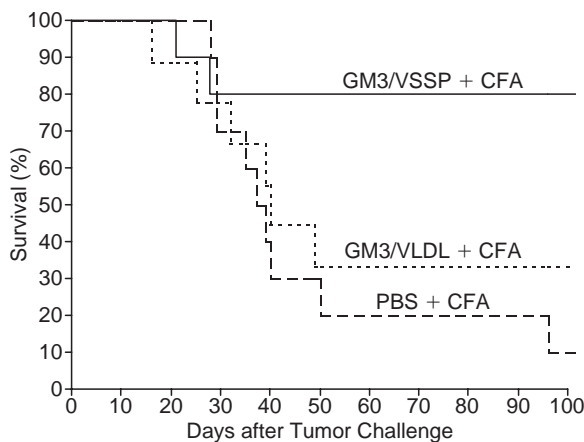


Figure 1. Survival of mice given PBS (control), GM3/VSSP or GM3/VLDL vaccines plus Freund's adjuvant, and challenged with 10^3 B16-F1 melanoma cells on week 3 after initiation of the immunization protocol. Each group consisted of 10 mice. Overall survival in animals vaccinated with GM3/VSSP was significantly higher than in control mice ($P < 0.01$, log-rank test). Vaccination with GM3/VLDL was ineffective.

aggressive B16-F10 melanoma cells. Animals were inoculated with 10^3 viable cells on week 3 after initiation of the immunization protocol. Although immunization failed to block tumour formation, vaccinated mice demonstrated a significant reduction in subcutaneous tumour volume (Figure 2).

Molecular specificity of GM3/VSSP vaccine and preliminary evidence of the non-transient character of tumour protection

We evaluated whether the tumour protection induced by GM3/VSSP vaccine is specific, adjuvant dependent and non-transient. C57BL/6j mice were immunized with GM3/VSSP plus Freund's adjuvant or Montanide ISA 51. Control animals were given unconjugated OMP or NGcGM3/VSSP plus adjuvant, or GM3/VSSP without adjuvant. Mice were inoculated with 10^3 B16-F1 cells on week 9 after initiation of the immunization protocol (first challenge). The overall survival in the groups of mice vaccinated with GM3/VSSP plus adjuvant were significantly higher than in the control groups ($P < 0.05$, log-rank test). In these experiments, GM3/VSSP vaccine administered either with Freund's adjuvant or with Montanide ISA 51 prevented tumour growth in 100% of mice after the first challenge with melanoma cells. Although some animals remained free of tumour 60 days after tumour inoculation, no significant increase in survival was observed with the GM3/VSSP vaccine without adjuvant or with the NGcGM3/VSSP vaccine plus adjuvant, a ganglioside/proteoliposome complex in which OMP was conjugated to *N*-glycosylated GM3 (data not shown).

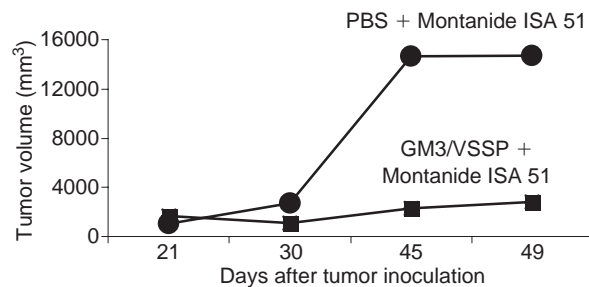


Figure 2. Effect of GM3/VSSP vaccine on B16-F10 melanoma tumour growth. Mice were given PBS (control) or GM3/VSSP vaccine plus Montanide ISA 51 and inoculated with 10^3 tumour cells on week 3 after initiation of the immunization protocol. Each group consisted of 20 mice. Data represent mean tumour volumes; standard errors were less than 10%. Tumour volumes were significantly higher in control mice from day 45 onwards ($P < 0.01$, Student's *t*-test).

Tumour-free animals who survived the first challenge were re-inoculated with 5×10^3 B16-F1 cells and monitored for another 90–180 days (second challenge). Figure 3 shows the Kaplan-Meier survival profile of mice in each group. Under the re-challenge conditions, all animals originally given OMP alone or GM3/VSSP without adjuvant developed tumours and died 80–150 days after the second challenge. Interestingly, GM3/VSSP with Freund's adjuvant or Montanide ISA 51 inhibited tumour formation in 60% and 33% of mice receiving the second tumour challenge, respectively. However, under these experimental conditions an increase in overall survival was only significantly correlated with the administration of GM3/VSSP plus Freund's adjuvant (see Figure 3). On the other hand, about 90% of animals given

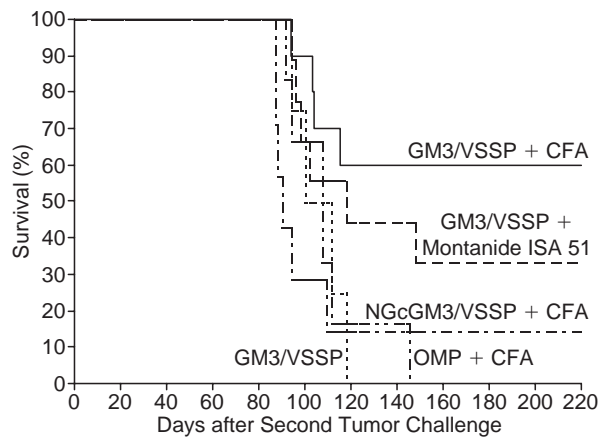


Figure 3. Survival of mice given GM3/VSSP plus Freund's adjuvant or Montanide ISA 51, GM3/VSSP alone, or NGcGM3/VSSP or OMP plus Freund's adjuvant that remained free of tumour more than 60 days after the first melanoma inoculation and were re-challenged with 5×10^3 B16-F1 cells. Each group consisted of 10 mice. Overall survival in animals vaccinated with GM3/VSSP plus Freund's adjuvant was significantly higher ($P < 0.05$, log-rank test).

NGcGM3/VSSP plus Freund's adjuvant developed tumours and died after re-inoculation of melanoma cells.

Immunization with GM3/VSSP vaccine induced high titres of anti-ganglioside IgM and IgG antibodies

Mice immunized with GM3/VSSP in the presence of either Freund's adjuvant or Montanide ISA 51 showed anti-GM3 IgM and IgG antibody responses at week 8 (Table 1). In contrast, GM3/VSSP vaccine without immunological adjuvant was not immunogenic. In sera from mice inoculated with unconjugated OMP or NGcGM3/VSSP plus adjuvant, antibodies against GM3 were absent. However, IgM and IgG antibodies against NGcGM3 (median titre 640) were induced in mice immunized with NGcGM3/VSSP (data not shown). Freund's adjuvant and Montanide ISA 51 were equally efficient in the induction of high titres of IgG and IgM, although the number of mice with IgM antibodies was higher in the group vaccinated with GM3/VSSP plus Freund's adjuvant. No evidence of toxicity or autoimmunity was observed in mice with high titres of anti-GM3 antibodies.

Sera from immunized mice contained highly specific anti-GM3 IgG antibodies

The specificity of IgG anti-GM3 antibodies detected in the high titre sera of GM3/VSSP vaccinated mice was studied by HPTLC immunostaining with ganglioside standards GM3, NGcGM3, NGcGM2, GM1, GD1a and GT1b. The results of a representative experiment are shown in Figure 4. Reactivity was restricted to GM3 ganglioside in all cases, demonstrating the elevated specificity of the antibodies

Table 1. Anti-GM3 IgM and IgG responses of C57BL/6j mice receiving four immunizations with various vaccine preparations

Vaccine ^a	n	Anti-GM3 IgM ^b ELISA titre	Anti-GM3 IgG ^b ELISA titre
OMP plus CFA + IFA	10	0	0
GM3/VSSP (without adjuvant)	10	0	0
GM3/VSSP plus CFA + IFA	10	80 (n = 3); 160; 320 (n = 3); 640 (n = 2); 1280	160; 640 (n = 4); 2560 (n = 2); 5120 (n = 3)
GM3/VSSP plus Montanide ISA 51	9	320; 1280 (n = 2)	160 (n = 2); 320; 640; 1280 (n = 3); 2560
NGcGM3/VSSP plus CFA + IFA	10	0	0

^aVaccines were administered at a dose of 120 µg, as described in Materials and methods.

^bSerological results correspond to week 8 after initiation of the immunization protocol.

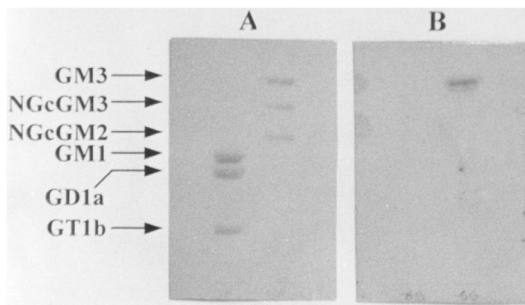


Figure 4. Specificity of serum from a mouse immunized with GM3/VSSP plus Freund's adjuvant determined by TLC immunostaining. Standard ganglioside mixtures were applied to HPTLC plates and developed as described in Materials and methods. **A** Gangliosides were visualized with resorcinol-HCl reagent. **B** After incubation with immunized serum, the plate was stained with alkaline phosphatase-labelled goat anti-mouse IgG antibody.

induced by vaccination. Preimmune sera showed no reactivity against gangliosides (data not shown).

IgG2b or IgG3 preponderance in sera from immunized mice was associated with the type of adjuvant

Sera from mice vaccinated with GM3/VSSP plus either Freund's adjuvant or Montanide ISA 51 were tested by ELISA using a panel of IgG subclass-specific secondary antibodies. In both groups of mice, anti-GM3 specific IgG antibodies belonging to the four subclasses (a Th1-like subclass pattern) were detected (Figure 5). Nevertheless, a clear predominance of IgG2b and IgG3 was observed. Serum IgG in the group with Freund's adjuvant was mainly of

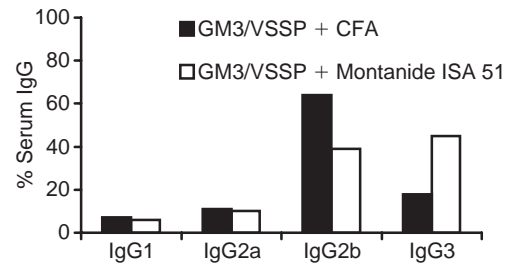


Figure 5. Serum IgG subclass distribution as a function of the type of adjuvant. Anti-GM3 IgG positive sera from mice immunized with GM3/VSSP vaccine either in Freund's adjuvant or Montanide ISA 51 were individually analysed for IgG subclass distribution by ELISA. The average of the contribution of each subclass was calculated from individual values in the corresponding group, and expressed as a percentage of the total IgG.

the IgG2b subclass, whereas in the case of Montanide ISA 51 an equilibrium between the IgG2b and IgG3 subclasses was observed.

GM3/VSSP immunization stimulated IgG antibodies with a strong antitumour complement-mediated cytotoxic effect

The effector function of IgG anti-GM3 antibodies in the serum of five Balb/c mice vaccinated with GM3/VSSP plus Montanide ISA 51 (diluted 1:5) was tested by CDC. Immune sera from all the mice lysed B16-F10 cells in the presence of rabbit complement (Table 2). Preimmune sera showed no toxicity with complement, and immune sera were not cytotoxic when complement was not added.

Table 2. Complement lysis of B16-F10 melanoma cells mediated by anti-GM3 antibodies in sera from Balb/c mice immunized with GM3/VSSP plus Montanide ISA 51

Mouse no.	IgG optical density ^a	Percentage lysis ^b		
		Preimmune serum with complement	Hyperimmune serum with complement	Hyperimmune serum without complement
2	0.678	0	89	0
3	1.000	0	63	0
6	0.934	0	62	0
7	0.826	0	70	0
10	0.400	0	87	0

^aIgG ELISA values of 1:80 diluted sera corresponding to week 8 after initiation of the immunization protocol. Anti-GM3 IgM antibodies were absent in these sera.

^bTarget cells were labelled with ⁵¹Cr and treated with 1:5 diluted antisera, as described in Materials and methods.

Discussion

Gangliosides are sialic acid-containing glycosphingolipids that have increased surface membrane expression on cancers of neuroectodermal origin. Interesting data are coming from experiments with ganglioside-based cancer vaccines in syngeneic murine models. Particularly, the B16 mouse melanoma model has attracted the attention of researchers due to its simple ganglioside profile (it expresses only GM3), its ample experimental use, and the existence of variants with distinct metastatic potential.

Previous efforts to render GM3 immunogenic using unmodified nominal antigen have failed⁵ or have been only partially successful.⁸ GM3-rich cellular vaccines based in B16 cells⁸ or human melanoma cells (pMCV),⁹ as well as a GM3-based anti-idiotypic monoclonal antibody (MAb D704)¹⁰ have been generated. They were tested for immunogenicity and antitumour activity in the B16 model. Both B16 and pMCV cellular vaccines administered with adjuvant in C57BL/6 mice induced IgM anti-GM3 antibodies. Ravindranath *et al.*⁸ showed an association between vaccine-induced anti-GM3 IgM antibodies and decreased tumour growth and prolongation of host survival. A protective humoral response, characterized by a low ratio of IgG/IgM to GM3 was suggested, although these cellular vaccines were not able to increase anti-ganglioside IgG preimmune levels. On the other hand, MAb D704 (Ab2) coupled to KLH and administered to C57BL/6 mice in the presence of Freund's adjuvant induced Ab1 and Ab3 IgG antibodies that lasted for more than 3 months. Ab2 generated anti-B16 cell responses, mainly delayed-type hypersensitivity. Also, significant suppression of tumour growth and prolongation of survival were observed.

In our work, we used a novel hydrophobic conjugated vaccine consisting of purified GM3 coupled to OMP from *Neisseria meningitidis* to produce VSSP.¹³ Administration of the vaccine significantly increased the overall survival of animals after challenge with B16 melanoma. In treated mice, GM3/VSSP vaccine dramatically increased the capacity to reject subcutaneous implantation of B16-F1 cells. Vaccinated mice challenged with highly aggressive B16-F10 cells also demonstrated a significant reduction in subcutaneous tumour volumes. In contrast, hydrophobic incorporation of the ganglioside into VLDL was ineffective, indicating that the immunological properties of OMP²² and the method of proteoliposome formation are crucial for optimal formulation.²³

Our vaccine consistently induced high titres of

IgM- and IgG-specific anti-GM3 antibodies. As in the case of almost all ganglioside vaccines, the efficacy of GM3/VSSP in the production of antibodies was adjuvant dependent.

With preparations of limited or unknown antigenicity, it is unclear whether patients respond immunologically to the selected antigen.⁴ With these criteria, cellular and anti-idiotypic vaccines might be considered as 'indirect' antigen defined vaccines. Our experiments clearly showed that immunization of mice with NGcGM3/VSSP neither produced anti-GM3 antibodies nor induced antitumour protection. Nuclear magnetic resonance and molecular modelling studies, performed on both GM3 and NGcGM3, have shown similar conformational behaviour for these two molecules.²⁴ However, our results strongly supported the molecular dependence of tumour protection obtained with GM3/VSSP vaccine in the B16 model.

In previous preclinical trials with tumour-associated ganglioside vaccines,²⁵ efforts were devoted to measuring the persistence of the antibodies induced by immunization and its association with the extent of tumour protection. Using the GM3/VSSP preparation in re-challenge experiments, we explored whether the protection conferred by vaccination is non-transient. A significant number of mice treated with GM3/VSSP vaccine who survived a first tumour cell challenge were able to resist a second challenge with five times more B16 cells, inoculated more than 80 days after the first immunization. Hence, in the present model, tumour protection could be maintained without prolonged re-immunizations.

The precise mechanisms by which GM3/VSSP vaccine confers tumour protection are unknown. With respect to ganglioside antigens, antibody-mediated protection has been claimed, mainly focused on the activation of the complement system.⁴ Particularly with GM3, other mechanisms of antibody action against B16 melanoma have been suggested, including direct inhibition of tumour cell growth and reversion of GM3-induced immunosuppression.^{6,8} Interestingly, in our experiments only immunization with GM3/VSSP plus adjuvant induced anti-GM3 antibodies and conferred protection in the C57BL/6j animal model. Additionally, the vaccine generated IgG antibodies in another mouse strain (Balb/c) with potent CDC against highly aggressive B16-F10 cells.

Freund's adjuvant contributed significantly more to the increase in overall survival than Montanide ISA 51 in mice immunized with GM3/VSSP. This is perhaps associated with the observed adjuvant-

dependent differences in the rate of IgM seroconversion, IgG subclass distribution profile, or both. Nevertheless, no serological differences in IgM and IgG titres in the sera from the respective immunized mice were detected.

The efficacy of using VSSP immunogens might also involve a direct cell-mediated immunity. Recent observations in models of tumour rejection mediated by interleukin-12 have shown the essential role of V α 14 natural killer T-cells in the cytotoxic effect. These cells were activated by glycosylceramides in both T-cell receptor-mediated and CD1d-restricted fashions.^{26,27} These facts have opened the door to the evaluation of this cell-mediated mechanism of tumour protection.

In summary, our GM3/VSSP vaccine produced a significant increase in overall survival and a reduction in tumour growth in mice challenged with B16 melanoma cells, without generating any toxicity or morbidity. These data suggest a potential clinical application of this novel GM3-based vaccine in the treatment of patients with malignant melanoma.

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