

Complete Antitumor Protection by Perioperative Immunization with GM3/VSSP Vaccine in a Preclinical Mouse Melanoma Model

Mariano R. Gabri,¹ Zaima Mazorra,² Giselle V. Ripoll,¹ Circe Mesa,² Luis E. Fernandez,² Daniel E. Gomez,¹ and Daniel F. Alonso¹

Abstract Purpose: The GM3/VSSP vaccine is composed of very small sized proteoliposomes resulting from the hydrophobic conjugation of GM3 ganglioside with membrane proteins from *Neisseria meningitidis*. Previously, we showed that preventive vaccination with GM3/VSSP induces a specific antitumor response and elicits the rejection of syngeneic GM3-positive melanoma cells in immunized mice. Our aim was to explore the antitumor properties of perioperative GM3/VSSP vaccination in a preclinical mouse model.

Experimental Design: The highly metastatic B16F10 mouse melanoma was used to investigate perioperative vaccination with GM3/VSSP. The vaccine was administered i.m. in doses of 120 µg emulsified with the adjuvant Montanide ISA 51 at weekly or biweekly intervals, and s.c. tumors were excised 25 to 31 days after tumor cell implantation. The persistence of antitumor protection and dose dependency was also examined in preimmunized animals. To evaluate the immune performance of tumor-bearing and tumor-operated mice, ovalbumin-specific delayed-type hypersensitivity, cytokine secretion, and cell proliferation responses were studied.

Results: Surgical excision of B16F10 tumors improved survival, and perioperative immunization with four biweekly GM3/VSSP doses yielded survival for all animals ($P = 0.04$; log-rank test). Mice showed neither local recurrence nor lung metastasis at the end of the experiment. An impairment of CD4⁺ T-cell responses was observed in tumor-bearing animals measured as neoantigen-specific delayed-type hypersensitivity, with a significant recovery after surgery. A strong interleukin-4 secretion was induced in B16F10-operated mice, whereas IFN- γ remained unaffected.

Conclusion: Preclinical evidence suggests that GM3/VSSP vaccine might have therapeutic potential to induce antitumor immunity in patients with minimal residual disease after surgery, thereby preventing or prolonging the time to recurrence.

Active immunotherapy in cancer patients has not been as successful as previously imagined in the clinical setting. A possible explanation for this is the timing of immunotherapeutic intervention with respect to disease progression. Preventive immunotherapy is an attractive strategy for patients at a high risk of having cancer, including melanoma (1), as well as for viral-associated malignancies such as hepatocellular

carcinoma (2) and cervical cancer (3), with some studies still in progress. However, a number of antitumor vaccination trials have included patients in late stages of the disease when all other treatment modalities have failed and the development of tumor-induced immunosuppression significantly interferes with immunization (4). The reduction of tumor burden by surgery or other conventional therapies, before or during vaccine administration, could substantially improve the therapeutic benefit. Malignant melanoma is a tumor with a steeply increasing incidence and scarce therapeutic options once metastatic. Currently, no vaccine is widely commercially available for melanoma treatment or prevention (5).

Ganglioside vaccines have been clinically tested in different types of advanced cancers, mainly melanomas (5, 6), based on the observation that altered expression of these glycolipids in melanoma cells correlated with their metastatic potential. Additionally, GM3 and GD3, the major gangliosides in melanomas, are shed into the tumor microenvironment and can promote severe immune dysfunctions (7–11). It has been claimed that the induction of anti-GM3 antibodies circumvents this specific immunosuppression (8, 11). For these reasons, the therapeutic success of a GM3-based vaccine in patients with early-stage melanoma remains an interesting and open question, but more preclinical evidence in a relevant animal model is needed.

Authors' Affiliations: ¹Laboratory of Molecular Oncology, Department of Science and Technology, Quilmes National University, Buenos Aires, Argentina and ²Department of Vaccines, Center of Molecular Immunology, Havana, Cuba
Received 5/4/06; revised 8/31/06; accepted 9/12/06.

Grant support: Quilmes National University, National Health Ministry (Argentina), Elea Laboratories, and Recombio.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: M.R. Gabri and Z. Mazorra contributed equally to this work. D.F. Alonso and D.E. Gomez are members of the National Council for Scientific and Technical Research (CONICET, Argentina).

Requests for reprints: Mariano R. Gabri, Laboratory of Molecular Oncology, Department of Science and Technology, Quilmes National University, R. Saenz Peña 180, Bernal B1876BXD, Buenos Aires, Argentina. Fax: 54-11-4365-7132; E-mail: mrgabri@unq.edu.ar.

©2006 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-06-1075

To answer this question, the immunosuppressive, GM3-positive B16 mouse melanoma and a GM3-based vaccine were used. The vaccine is composed of very small sized proteoliposomes (VSSP) resulting from the hydrophobic conjugation of GM3 gangliosides with *Neisseria meningitidis* membrane proteins (9). We have previously shown experimental data indicating that preventive immunization of mice with the GM3/VSSP vaccine elicited the rejection of B16 melanoma cells (10, 11). The vaccine consistently induced an antiganglioside response in mice (9). In addition, serum of vaccinated animals recognized B16 cells by flow cytometry and immunohistochemistry, and caused complement-mediated cytotoxicity. The specific response could be ascribed to antibodies of the IgG2b subclass (10, 11).

Here, we found that perioperative biweekly (but not weekly) immunizations with the GM3/VSSP vaccine in mice bearing early-stage B16F10 melanoma tumors induced a complete antitumor protection in all mice. Interestingly, an adequate vaccination protocol seems to overcome tumor-induced immunosuppression in operated animals and, consequently, allows the immune system to prevent tumor recurrence and metastasis.

Materials and Methods

Vaccine. The GM3/VSSP vaccine was produced by the Center of Molecular Immunology (Havana, Cuba). Briefly, GM3 monosialoganglioside containing the *N*-acetylneuraminic acid was purified from canine RBC and hydrophobically conjugated with outer membrane proteins from *N. meningitidis*, as previously reported (9). The method allowed gangliosides to hydrophobically incorporate into VSSP and conferred high solubility to the conjugate. An appropriate amount of GM3/VSSP was resuspended in 0.05 mL of PBS, mixed with an equal volume of the immunologic adjuvant Montanide ISA 51 (Seppic, Paris, France) before injection. Vaccines were administered i.m. in the quadriceps, and control animals received only the adjuvant mixed with PBS.

Animals. Specific pathogen-free C57BL/6 mice were obtained from Universidad Nacional de La Plata (La Plata, Argentina) and 5 to 10 mice per cage were kept with water and food *ad libitum* in the animal house facility at Quilmes National University according to an institutionally approved animal protocol. Female mice ages 8 to 14 weeks and with an average weight of 25 g were used.

Tumor cells and culture conditions. B16 mouse melanoma cells, sublines F0 (poorly metastatic) and F10 (highly metastatic), were maintained in DMEM (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, and 10 μ g/mL tetracycline. Cell viability was assessed using the trypan blue exclusion technique.

Tumor cell challenge. B16 cells were trypsinized, washed with PBS, resuspended in serum-free medium, and injected in the subcutis of the flank. The time of appearance of local tumors was monitored by palpation and further confirmed by histopathology. Mice were monitored for 90 to 120 days after tumor challenge. The largest perpendicular diameters of the resulting tumors were periodically measured with a caliper, and tumor volumes were calculated using the formula $\pi/6 \times \text{length} \times \text{width}^2$. When tumors exceeded 15,000 mm³, or when the general condition was affected, animals were sacrificed by cervical dislocation and necropsied. To investigate the presence of spontaneous metastasis in mice bearing highly metastatic B16F10 tumors, lungs were fixed in Bouin's solution and the presence of surface nodules was determined under a dissecting microscope.

Preimmunization with GM3/VSSP. To evaluate dose-dependent effects on tumor protection, groups of 5 to 10 mice received four

doses of 120, 240, or 360 μ g of GM3/VSSP vaccine every 14 days and, 21 days after the last dose, were challenged with 10⁴ viable B16F0 cells. To examine the duration of antitumor protection, mice were preimmunized using the same protocol with four doses of 120 μ g of the vaccine, and then challenged with 2.5×10^3 B16F0 cells at days 21, 35, or 49 after the last vaccine dose. Negative animals of each group were rechallenged in the left flank 91, 105, or 119 days after the last vaccine dose, respectively. In addition, the antitumor effects of one, two, three, or four biweekly or weekly doses of 120 μ g GM3/VSSP administered before challenge with highly metastatic B16F10 cells were tested.

Perioperative immunization with GM3/VSSP. At day 0, mice were implanted in the subcutis with 5×10^4 highly metastatic B16F10 melanoma cells. A set of experiments was initially conducted to make the surgical technique reproducible and to define the best time frame for surgery in the present melanoma model. S.c. tumors were excised at days 25 to 31 under anesthesia by i.p. injection of ketamine/xylazine (100:10 mg/kg of body weight), when tumor volumes reached ~ 250 mm³. Groups of 5 to 10 mice were immunized with four weekly doses of 120 μ g GM3/VSSP beginning on the day of the surgery, or at 14-day intervals beginning when tumors became palpable at days 10 to 16, as depicted in Fig. 1.

Immunization with ovalbumin in Freund's adjuvant. Mice were implanted in the subcutis with 5×10^3 B16F10 melanoma cells and s.c. tumors were excised 1 week after palpation. As controls, nonoperated tumor-bearing mice and operated healthy mice were used. For delayed-type hypersensitivity (DTH) assays, mice were immunized with ovalbumin in complete Freund's adjuvant (Sigma, St. Louis, MO) on days 2 and 16 after surgery, and 7 days later, were intradermally challenged with 50 μ g ovalbumin in the right hind foot pad. After 48 hours, the volume of inflammation (swelling of the mice foot) was measured using a plethysmometer (Ugo Basile, Comerio, Italy). To measure cell proliferation response and cytokine secretion, a single dose of 100 μ g ovalbumin mixed with complete Freund's adjuvant was administered s.c. near the base of the tail. Immunization protocols began 2 days after surgery. In the kinetic experiments, immunizations began 2, 15, or 30 days after surgery. One week later, mice were killed and inguinal lymph nodes were removed.

Cell proliferation and cytokine secretion assays. Inguinal lymph nodes from immunized mice, as described above, were smashed and made into a single-cell suspension in RPMI 1640 (Life Technologies) with Glutamax I and 25 mmol/L HEPES supplemented with 5% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 50 μ mol/L 2-mercaptoethanol. Cells were then cultured in the presence of ovalbumin at a concentration of 100 μ g/mL. Proliferation was monitored by measuring [methyl-³H]TdR (1 μ Ci per well; Amersham,

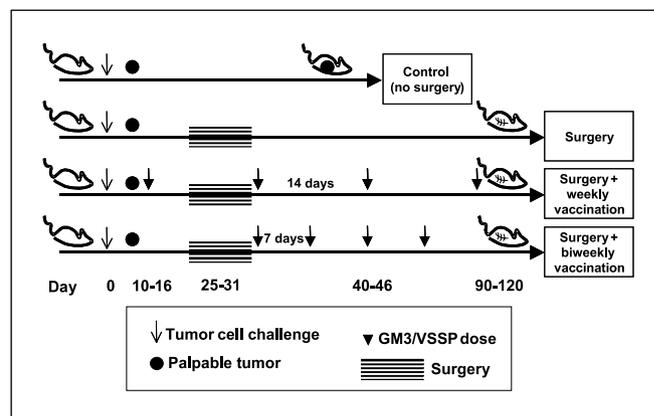


Fig. 1. Perioperative immunization protocol with GM3/VSSP vaccine. At day 0, mice were implanted in the subcutis with melanoma cells. Animals were vaccinated with weekly or biweekly doses of GM3/VSSP and s.c. tumors were excised under anesthesia. For experimental details, see Materials and Methods.

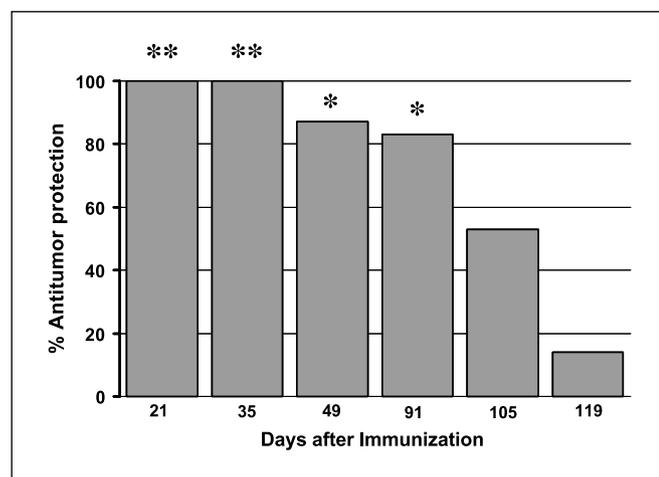


Fig. 2. Antitumor protection in mice preimmunized with GM3/VSSP vaccine at different times before melanoma cell challenge. Mice were preimmunized with four doses of 120 μ g of the vaccine at 14-day intervals, and then challenged with 2.5×10^3 B16F0 cells at days 21, 35, or 49 after the fourth vaccine dose. Negative animals were rechallenged in the left flank 91, 105, or 119 days after the fourth vaccine dose. More than 80% of control animals developed s.c. tumors and died (**, $P < 0.01$; *, $P < 0.05$ versus controls; χ^2 test).

Cambridge, United Kingdom) incorporation on day 4 of culture. The stimulation index was calculated dividing the mean counts per minute of stimulated cells with that of cells cultured with complete medium alone. For the cytokine assays, at day 3 of culture, 500 ng/mL ionomycin and 50 ng/mL phorbol myristate acetate (Sigma) were added to the cells. Supernatants were removed 16 hours later and cytokines were evaluated using interleukin-4 (IL-4) and IFN- γ mouse ELISA kits from BD Biosciences (Bedford, MA), following the protocol recommended by the manufacturer.

Statistical analysis. The equality of variances was analyzed with Bartlett's test, and Kolmogorov-Smirnov test was used to analyze normal distribution of data. Comparison of tumor incidence or antitumor protection was done using the χ^2 test. Differences in the survival of mice were analyzed using the Kaplan-Meier method, and groups were compared using the log-rank test. Differences in the DTH and cell proliferation were evaluated by ANOVA and Tukey test for multiple comparisons. Student's t test was used to assess differences between groups in kinetics cytokine assays. Statistical analysis was done using the GraphPad InStat software program.

Results

Persistence of antitumor protection after preimmunization with GM3/VSSP and dose dependency. We first explored antitumor protection against local melanoma disease using poorly metastatic B16F0 melanoma cells. Previous experiments indicated that pretreatment of C57BL/6 mice with several doses of GM3/VSSP could inhibit s.c. tumor formation after challenge with a low burden of syngeneic melanoma cells (10, 11). We investigated the duration of antitumor protection after preimmunization with four biweekly i.m. doses of 120 μ g GM3/VSSP by injecting a low tumor burden of 2.5×10^3 B16F0 cells in the subcutis. In different sets of experiments, at least 80% of control animals developed s.c. tumors and died 46.5 ± 5.7 days (mean \pm SE) after challenge. As shown in Fig. 2, complete antitumor protection was obtained 21 to 35 days after the administration of the last dose of the vaccine. A significant antitumor protection was still maintained 91 days after

preimmunization, but the antitumor effect disappeared at days 105 to 119.

We asked whether antitumor protection by GM3/VSSP vaccine is dose dependent in the present mouse melanoma model using higher tumor burdens of B16F0 cells. In control mice injected with 10^4 tumor cells, tumor incidence was 92% and animals died 39.8 ± 3.4 days after challenge. On the other hand, 70%, 83%, and 89% of mice preimmunized with the 120, 240, and 360 μ g/dose of GM3/VSSP, respectively, showed no evidence of tumor and survived until the end of the experiment at day 90 (Fig. 3A). Antitumor protection was significant with the three dose levels ($P = 0.002$, $P = 0.001$, and $P < 0.001$ for the 120, 240, and 360 μ g/dose, respectively; χ^2 test) and effects on mice survival were dose dependent ($P < 0.001$; log-rank test for trend). Similar results were obtained with a higher tumor burden of 5×10^4 B16F0 cells, but antitumor protection was lower ($\sim 40\%$ of mice showed no tumors with doses of 360 μ g GM3/VSSP).

Survival benefit with repeated biweekly preimmunization with GM3/VSSP. We examined the number of vaccine doses required to induce antitumor protection against the aggressive B16F10 cells. Different groups of mice were preimmunized with one, two, three, or four doses of 120 μ g GM3/VSSP. Vaccines were administered at 14-day intervals, and then mice were challenged with 5×10^3 B16F10 cells. The survival of animals receiving at least three biweekly GM3/VSSP doses was significantly higher than in the control group. No survival benefit was obtained with one or two biweekly vaccine doses (Fig. 3B). Weekly preimmunization with several doses of GM3/VSSP was also ineffective in inducing tumor protection and prolonging survival (Fig. 3C).

Complete antitumor protection and survival benefit with perioperative biweekly immunization using GM3/VSSP. We have evaluated the antitumor properties of GM3/VSSP vaccine in a preclinical mouse model of melanoma surgery using the highly metastatic B16F10 cells. We first examined the best time frame for surgery in the present melanoma model. The best surgical results were achieved when s.c. tumors reached a volume of ~ 250 mm 3 (25-31 days after B16 cell challenge), whereas resection of tumors $>1,000$ mm 3 was ineffective and often presented a rapid progression after surgery.

To investigate the effects of perioperative GM3/VSSP immunization, we induced melanoma tumors by inoculating 5×10^4 B16F10 cells in the subcutis. Perioperative GM3/VSSP (120 μ g per dose) was administered using two different protocols, with four weekly doses beginning from the day of the surgery, or with biweekly doses beginning before surgery when tumors became palpable. The third GM3/VSSP vaccination, the minimum required to obtain an effective antitumor effect against B16F10, was administered ~ 15 days after surgery, at the time when mice were at high risk of local recurrence or metastasis (see also Fig. 1). As expected, all nonoperated control animals rapidly developed aggressive s.c. tumors, and died 34.5 ± 3.3 days after challenge as a consequence of the high local tumor burden, without showing signs of macroscopic lung metastasis. Surgical excision of melanoma tumors significantly improved survival, but perioperative immunization with weekly doses of the vaccine were not effective to induce a survival benefit with respect to surgery alone. As shown in Fig. 4, perioperative immunization with four biweekly GM3/VSSP doses beginning 15 days before surgery

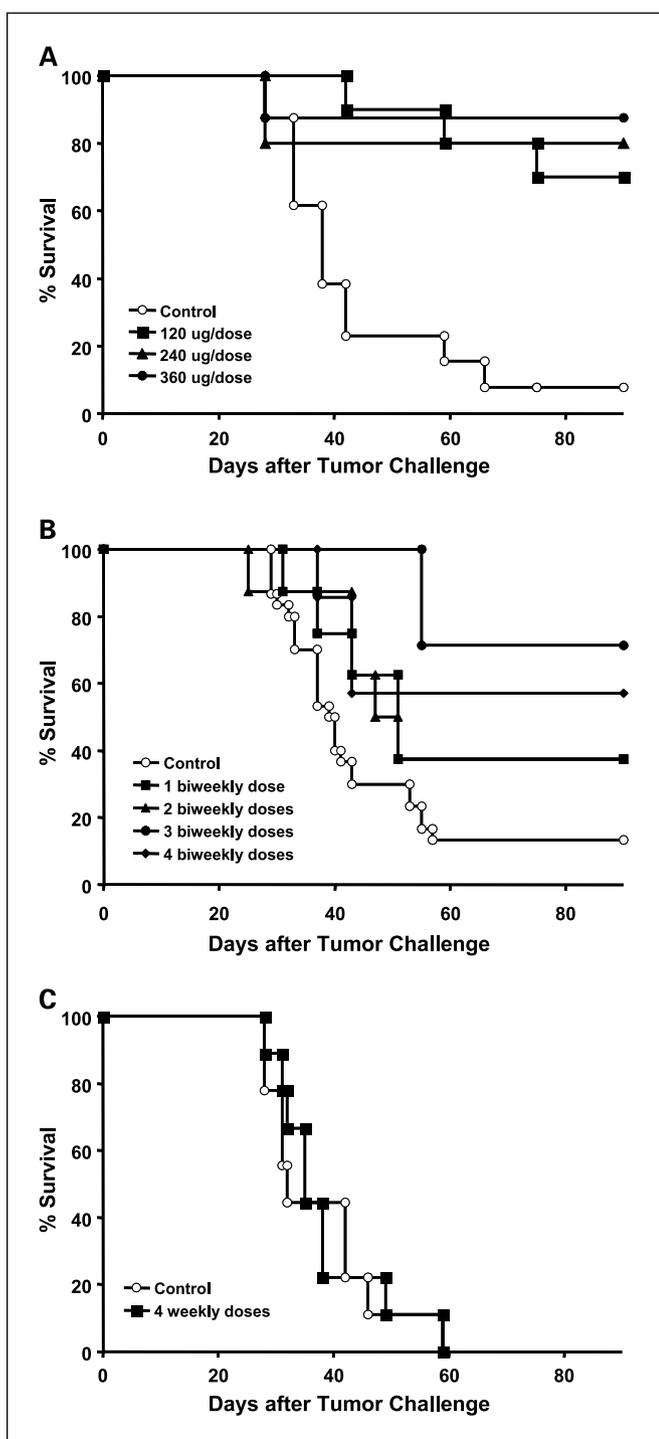


Fig. 3. Survival of mice after different preimmunization protocols. *A*, survival of mice preimmunized with different doses of GM3/VSSP vaccine before melanoma cell challenge. Mice were preimmunized with four doses of the vaccine at 14-day intervals, and challenged with 10^4 B16F0 cells 21 days after the fourth dose. Overall survival was significantly dose dependent ($P < 0.001$; log-rank test for trend). *B*, survival of mice preimmunized with a different number of biweekly GM3/VSSP doses. Mice were preimmunized with one, two, three, or four biweekly doses of 120 μ g vaccine, and then challenged with 5×10^3 B16F10 cells 63 days after the first dose. Overall survival of animals preimmunized with three and four biweekly doses was significantly higher than in controls ($P = 0.002$ and $P = 0.02$, respectively; log-rank test), whereas one or two doses have no significant effects. *C*, survival of mice preimmunized with weekly GM3/VSSP doses. Mice were preimmunized with four weekly doses of 120 μ g vaccine, and then challenged with 5×10^3 B16F10 cells 21 days after the fourth dose. No antitumor effects were obtained with repeated weekly immunization.

yielded survival of all animals ($P = 0.04$ versus surgery alone and surgery plus weekly vaccination; log-rank test). Table 1 also presents the incidence of local recurrence and lung metastasis in the different treatment groups.

Impairment of neoantigen-specific CD4⁺ T-cell response in B16F10-bearing mice after tumor removal. To evaluate the immune system performance of B16F10-bearing mice before and after surgery, we measured antiovalbumin CD4⁺ T-cell functions. For this purpose, ovalbumin-specific DTH or proliferation responses were assayed by immunizing two doses or a single dose of ovalbumin in complete Freund's adjuvant, respectively. Compared with tumor-bearing mice, CD4⁺ T-cell response in B16F10-operated mice showed a significant recovery after surgery, measured by ovalbumin antigen-specific DTH ($P < 0.05$, Tukey test). However, this response is largely distant from the DTH developed in healthy, sham-operated mice ($P < 0.01$, Tukey test; Fig. 5A). Proliferative response, evaluated 1 week after a single immunization, showed a 2-fold reduction on H³T incorporation by lymph nodes cells from B16F10-bearing mice versus control mice ($P < 0.01$, Tukey test). In this case, no differences between operated and nonoperated B16F10-bearing mice were found (Fig. 5B).

To analyze whether surgical excision of primary tumor recovers CD4⁺ T-cell function, B16F10-operated mice were immunized 2, 15, and 30 days after surgery with ovalbumin emulsified in complete Freund's adjuvant. Seven days later, inguinal lymph nodes were removed, cells were cultured for 4 days with ovalbumin, and the supernatant was collected for cytokine quantification by ELISA. Strikingly, immunization induced a strong IL-4 secretion in B16F10-operated mice as compared with healthy animals ($P < 0.05$, Student's *t* test), but values returned to normal 30 days after tumor excision. On the contrary, IFN- γ secretion remained unaffected (Fig. 5C and D).

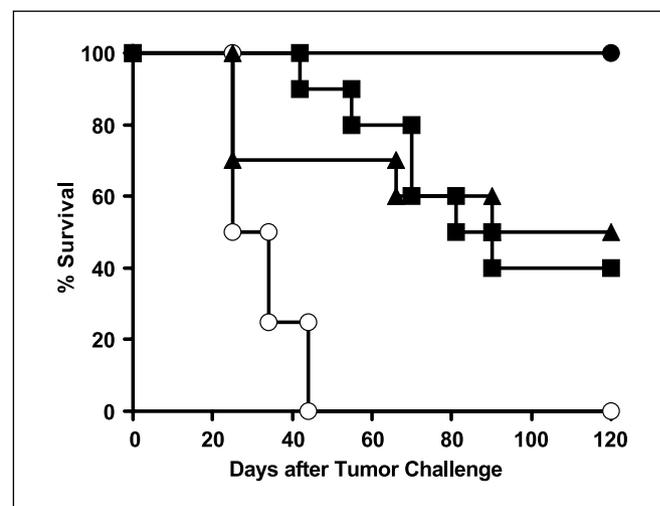


Fig. 4. Survival of mice implanted with B16F10 melanoma tumors and treated with surgery and perioperative GM3/VSSP vaccination. Results show control mice without treatment (○), and mice treated with surgery (▲), surgery plus weekly immunization (■), or surgery plus biweekly immunization (●) with GM3/VSSP vaccine. For details, see Materials and Methods. Overall survival of animals treated with surgery plus biweekly vaccination was significantly higher than in controls ($P = 0.002$; log-rank test) or animals treated with surgery and surgery plus weekly vaccination ($P = 0.04$; log-rank test).

Table 1. Survival, local recurrence, and lung metastasis in mice implanted with B16F10 melanoma tumors and treated with surgery and perioperative GM3/VSSP vaccination

Treatment group*	Overall survival at the end of the experiment [†] (>120 d)	Incidence of local recurrence and/or rapid death after surgery [†]	Incidence of lung metastasis in autopsy study [†]
Control (no surgery)	0% (0/4)	—	0% (0/4) [‡]
Surgery	50% (5/10)	40% (4/10)	20% (2/10)
Surgery + weekly vaccination	40% (4/10)	30% (3/10)	40% (4/10)
Surgery + biweekly vaccination	100% (5/5) [§]	0% (0/5)	0% (0/5)

*S.c. tumors were excised 25 to 31 days after challenge when tumor volumes reached ~ 250 mm³, and mice were immunized with four weekly GM3/VSSP doses beginning at the day of the surgery, or with four biweekly doses beginning when tumors became palpable at days 10 to 16.
[†]Survival and incidences are expressed as a percentage (positive/total animals).
[‡]Nonoperated control mice rapidly died as a consequence of the high s.c. tumor burden, without macroscopic distant metastasis.
[§]For survival curves and statistical significance, see Fig. 4.

Discussion

Immunotherapy trials represent >50% of all clinical trials of biotherapies, frequently including patients with advanced cancer (12). We do not yet have a cancer vaccine that can reliably and consistently induce tumor remission or improve patient survival (13, 14). Focusing on melanoma, treatment did not prove to be efficacious in large, randomized phase III trials. A large randomized study was conducted by the intergroup mechanism (Intergroup Trial E1694) using the GMK vaccine. In that study, 880 patients with stage III melanoma were randomized. The trial was closed after interim analysis indicating the inferiority of GMK compared with high-dose IFN- α (15). Similarly, CancerVax announced the discontinua-

tion of its phase III clinical trial of Canvaxin in patients with stage III melanoma. The decision followed the recommendation of the independent Data and Safety Monitoring Board, based on the data reviewed at the third interim analysis, in which Canvaxin did not show efficacy as a postsurgical adjuvant treatment for patients with advanced-stage melanoma.

However, this lack of results seems to be due, in part, to the incorrect patient characteristics evaluated, and not due to the lack of therapeutic potential of the vaccine. It is known that mice with experimental tumors, as well as patients with cancer, show a decreased immunologic potency (16). This tumor-induced immunosuppression is reinforced in patients with high tumor burdens (17, 18). Consequently, in the last few years, our view of the range of applications of tumor vaccines has

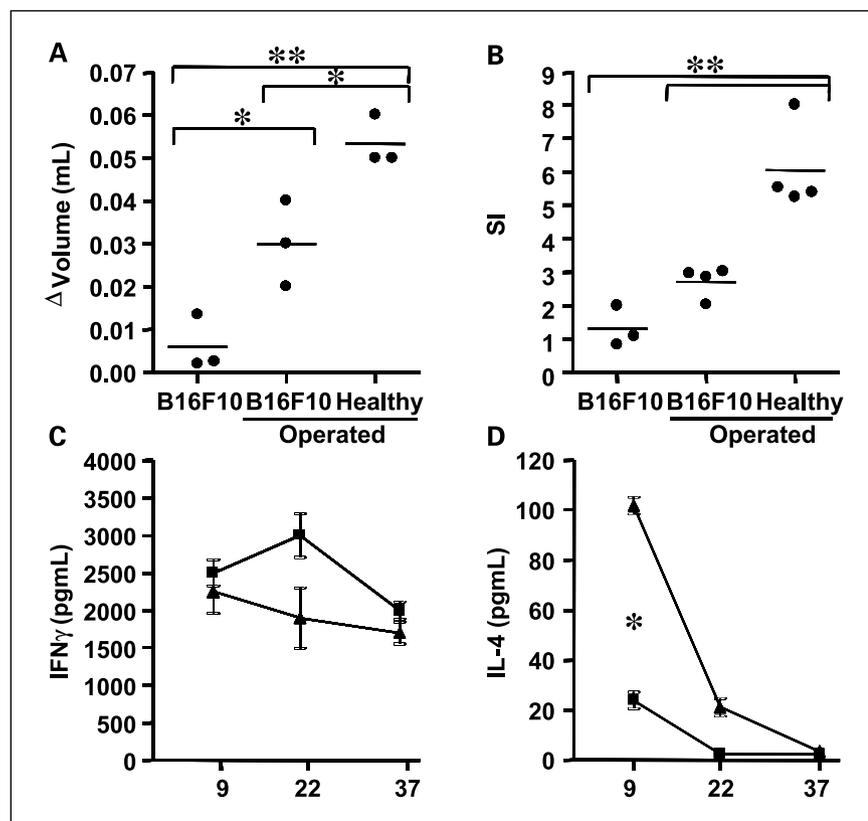


Fig. 5. Immunosuppression induced by B16F10 melanoma tumor measured after tumor removal. Ovalbumin-specific DTH (A) or proliferation (B) responses were assayed by immunizing with two or single doses of ovalbumin in complete Freund's adjuvant, respectively. Both experiments showed that induced CD4⁺ cellular response was significantly diminished (**, $P < 0.01$, Tukey test) in operated and tumor-bearing mice compared with healthy mice (sham surgery). Points, individual mice. In the kinetic experiments, immunizations began 2, 15, or 30 days after surgery. One week after each immunization, ovalbumin-specific IFN- γ (C) and IL-4 (D) secretion by cells from draining lymph nodes was measured. Cytokine secretion showed that 9 days after tumor removal, immunization induces strong IL-4 secretion (*, $P < 0.05$, Student's t test) compared with healthy mice. Although IFN- γ secretion remained invariable, IL-4 levels returned to normal 30 days after surgery. Points, means per group from a single experiment; bars, SD. Representative of three independent experiments.

changed because treatment of patients with minimal residual disease or with low staging showed better responses to vaccination. Furthermore, an open question remains regarding whether vaccines have sufficient benefit for exploration in adjuvant or even in preventive conditions. The answers to these questions can be found, in part, by exploring the responses observed with the correct use of animal models.

We have previously described the antitumor activity of a vaccine based on GM3 monosialoganglioside inserted in VSSP derived from *N. meningitidis* plus Montanide in the B16 melanoma mouse model. The GM3/VSSP vaccine was capable of inducing antibodies against the defined melanoma antigen GM3 ganglioside. We showed that preimmunization protects mice against low burdens of syngeneic B16 tumor cells, correlating with subsequent prolonged survival (10, 11). This promising therapeutic potential in a mouse melanoma model has led to a phase I clinical trial in patients with melanoma, which has shown attractive results (19). Based on these patients' experiences, some questions have arisen, and animal models could provide the answers.

In the present animal model, we have explored immunization with GM3/VSSP vaccine in combination with surgical excision of the primary tumor mass. The results showed that this model could clear the response to vaccination in combination with resection of the highly aggressive B16F10 melanoma. In this scenario, we observed that mice subjected to surgery plus a perioperative vaccination protocol consisting of four GM3/VSSP doses every 14 days beginning before surgery have an increased survival period compared with any other group. Furthermore, all mice showed neither local recurrence nor visible lung metastasis at the end of the experiment. Interestingly, mice under a similar perioperative immunization with four doses of the vaccine, but administered every 7 days, showed reduced survival, similar to animals treated with surgery alone. This fact is in accordance with the preventive vaccination model in which biweekly preimmunization with at least three GM3/VSSP doses before tumor challenge was enough to induce antitumor resistance against local melanoma disease, whereas weekly preimmunization was clearly ineffective.

With respect to the persistence of the GM3/VSSP effect and dose dependency, antitumor protection was maintained for at least 90 days, and mice survival was significantly dose dependent in the preventive model using B16F0 cells. However, the combination of surgery with perioperative vaccination using the lower dose of 120 μ g was sufficient to induce a complete antitumor protection in the aggressive B16F10 model. Nevertheless, it would be interesting to prove different dose levels and immunization protocols in clinical trials.

All these preclinical data strongly suggest a combined therapeutic effect of tumor excision and vaccination with GM3/VSSP every 14 days. This is an important issue to translate to the clinical setting because patients with stage II melanoma were reported to have an ~50% chance of survival 5 years after surgery. Some patients with stage II melanoma are at high risk for recurrent disease, and occult micrometastases cause recurrence following treatment with surgery alone (20, 21). According to the results obtained with the present preclinical model, vaccination with GM3/VSSP could significantly increase survival after the surgical management of primary cutaneous melanoma.

Because several clinical protocols involve active immunotherapy in cancer patients with large primary tumors or postsurgery patients, the functional effect of the tumor mass in the immune response would be an important feature. A better understanding of the immune competence of cancer patients could be modeled in mice. Previous studies with experimental animals have led to the conclusion that vaccine efficacy is inversely proportional to tumor burden (10). The relationship between tumor burden and immune function raises the important question of whether tumor-induced immunosuppression is reversible by surgical removal of the primary tumor. Recently, Danna et al. (22) investigated the existence of tumor-induced immunosuppression in tumor-bearing mice in response to vaccination with hen egg white lysozyme as model antigen. They showed that surgical removal of primary tumor restores immune competence even when disseminated metastatic disease is present (22). In this sense, we studied the functional immunologic variables following immunization with a "foreign" antigen, such as ovoalbumin, in tumor-bearing and healthy mice. The purpose of the experiment was to examine the response to vaccination with ovoalbumin before and after tumor surgery and the possible kinetics of immune recovery after excision of the tumor mass. In immunocompetent mice, this antigen should induce a strong and specific response.

Our data revealed dysfunctional cell-mediated immunity for the CD4⁺ compartment induced by the presence of the B16F10 tumor. The cytokine profile was different in postsurgery groups, with an increase in IL-4 secretion by total lymph node cells in B16F10 postsurgery mice compared with the control group. These results are in accordance with other results that reveal antigen-specific CD4⁺ T-cell unresponsiveness as an early event in tumor progression, which has clear implications for cancer immunotherapy (23).

The results of the kinetic experiment, in which we removed the primary tumor and immunized mice at days 2, 15, or 30 postsurgery, revealed that complete polarization of the CD4⁺ T cells requires a period of time after surgery. This experimental evidence suggests that choosing the right moment for immunotherapeutic intervention relative to the perioperative period might be an important point to take into consideration in the design of new clinical protocols. Few studies indicate that immune system functions in cancer patients recover after surgery (24, 25). However, there are no precedent studies of this phenomenon for melanoma. Our results showed that the B16F10 tumor induces a CD4⁺ T-cell dysfunction that cannot be rapidly overcome, lasting for at least 30 days after surgical treatment. In this scenario, four biweekly immunizations with GM3/VSSP during the immunosuppression window induced a strong antitumor response. This fact suggests that our vaccine, given in a correct immunization protocol, is able to increase the immune responsiveness and allows the immune system to prevent tumor recurrence or metastasis. This could be ideally applied to stage II melanoma patients with minimal residual disease after surgery. Additionally, the long-term persistence of the antitumor response shown in the preventive scenario suggests that this kind of patient can be vaccinated periodically, with at least three doses every 3 months beginning before or during surgical excision of the primary lesion. This vaccination will probably prevent tumor recurrence and also exert an antimetastatic effect.

Altogether, these preclinical experiments propose the GM3/VSSP vaccine as a therapy designed to elicit and/or boost antitumor immunity in patients with minimal residual disease after surgery, thereby preventing or prolonging the time to

recurrence. Future clinical trials will be needed for a definitive confirmation about the beneficial role of the GM3/VSSP vaccine in the perioperative handling of patients with stage II melanoma.

References

- Cassarino DS, Miller WJ, Auerbach A, Yang A, Sherry R, Duray PH. The effects of gp100 and tyrosinase peptide vaccinations on nevi in melanoma patients. *J Cutan Pathol* 2006;33:335–42.
- Lavanchy D. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. *J Clin Virol* 2005;34:S1–3.
- Mahdavi A, Monk BJ. Vaccines against human papillomavirus and cervical cancer: promises and challenges. *Oncologist* 2005;10:528–38.
- Malmberg KJ. Effective immunotherapy against cancer. *Cancer Immunol Immunother* 2004;53:879–92.
- Kananath D, Hill AD, Dijkstra B, Kenelly R, McDermott EM, O'Higgins NJ. Adjuvant therapies in the treatment of stage II and III malignant melanoma. *Surgeon* 2005;3:245–56.
- Ravindranath MH, Hsueh EC, Verma M, Ye W, Morton DL. Serum total ganglioside level correlates with clinical course in melanoma patients after immunotherapy with therapeutic cancer vaccine. *J Immunother* 2003;26:277–85.
- Kudo D, Rayman P, Horton C, et al. Gangliosides expressed by the renal cell carcinoma cell line KK-RC-45 are involved in tumor-induced apoptosis of T cells. *Cancer Res* 2003;63:1676–83.
- Dohi T, Nores G, Hakomori S. An IgG3 monoclonal antibody established after immunization with GM3 lactone: immunochemical specificity and inhibition of melanoma cell growth *in vitro* and *in vivo*. *Cancer Res* 1988;48:5680–5.
- Estevez F, Carr A, Solorzano L, et al. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). *Vaccine* 2000;18:190–7.
- Alonso DF, Gabri MR, Guthmann MD, Fainboim L, Gomez DE. A novel hydrophobized GM3 ganglioside/*Neisseria meningitidis* outer membrane-protein complex vaccine induces tumor protection in B16 murine melanoma. *Int J Oncol* 1999;15:59–66.
- Carr A, Mazon Z, Alonso DF, et al. A purified GM3 ganglioside conjugated vaccine induces specific, adjuvant-dependent and non-transient antitumor activity against B16 mouse melanoma *in vitro* and *in vivo*. *Melanoma Res* 2001;11:219–27.
- Ottaiano A, Mollo E, Di Lorenzo G, et al. Prospective clinical trials of biotherapies in solid tumors: a 5-year survey. *Cancer Immunol Immunother* 2005;54:44–50.
- Restifo NP, Lewis JJ. Cancer: principles and practice of oncology. In: De Vita VT, Rosenberg SA, Hellman S, editors. Baltimore: Lippincott; 2004. p. 45–57.
- Lewis JJ. Therapeutic cancer vaccines: using unique antigens. *Proc Natl Acad Sci U S A* 2004;101:14653–6.
- Kirkwood JM, Ibrahim JG, Sosman JA, et al. High-dose interferon α -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.
- Kiessling R, Wasserman K, Horiguchi S. Tumor induced immune dysfunction. *Cancer Immunol Immunother* 1999;48:353–62.
- Pawelec G, Heinzel S, Kiessling R, et al. Escape mechanism in tumor immunity: a year 2000 update. *Crit Rev Oncog* 2000;11:97–133.
- Egorov IK. Mouse models of efficient and inefficient anti-tumor immunity, with emphasis on minimal residual disease and tumor escape. *Cancer Immunol Immunother* 2006;55:1–22.
- Guthmann MD, Bitton RJ, Carnero AJ, et al. Active specific immunotherapy of melanoma with a GM3 ganglioside-based vaccine: a report on safety and immunogenicity. *J Immunother* 2004;27:442–51.
- Ross MI. Early-stage melanoma: staging criteria and prognostic modeling. *Clin Cancer Res* 2006;12:312–9.
- Lamb LA, Halpern AC, Hwu WJ. Diagnosis and management of stage I/II melanoma. *Semin Oncol Nurs* 2003;19:22–31.
- Danna EA, Sinha P, Gilbert M, Clements VK, Pulaski BA, Ostrand-Rosenberg S. Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. *Cancer Res* 2004;5:2205–11.
- Staveley-O'Carroll K, Sotomayor E, Montgomery J, et al. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc Natl Acad Sci U S A* 1998;95:1178–83.
- Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 2001;166:678–89.
- Barbieri C, Fujisawa MM, Yasuda CL, et al. Effect of surgical treatment on the cellular immune response of gastric cancer patients. *Braz J Med Biol Res* 2003;36:339–45.