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Role of cell surface GM3 ganglioside and sialic acid in the antitumor activity of a GM3-based vaccine in the murine B16 melanoma model

Abstract *Purpose*: To examine the role of GM3 monosialoganglioside and sialic acid in the antitumor activity of a vaccine based on GM3, hydrophobically conjugated with the outer-membrane-protein complex from *Neisseria meningitidis* (GM3/VSSP). *Methods*: In order to evaluate the relationship between antitumor activity and the presence of GM3 on the surface of tumor cells, we used two murine tumor cell lines with different ganglioside expression. Syngeneic mice were immunized with four i.m. doses of GM3/VSSP (120 μg) at 14-day intervals and challenged subcutaneously with tumor cells. *Results*: B16 melanoma cells showed GM3 on cell surface and GM3-dependent in vitro growth. As expected, preimmunization with the vaccine significantly inhibited tumor formation and prolonged survival in mice challenged with B16 cells. In contrast, no antitumor effect was observed in mice challenged with GM3-negative F3II mammary carcinoma cells. The reactivity of sera from immunized mice against B16 cells was confirmed by flow cytometry and immunoperoxidase staining. Depletion of sialic acid residues from the cell surface completely abolished antibody response against melanoma cells. *Conclusions*: These results indicate that the antitumor activity of GM3/VSSP is associated with GM3 expression on tumor cell surface and demonstrate a major role of sialic acid in the humoral response of vaccinated mice.

Keywords Immunotherapy · Sialic acid · GM3 ·
Melanoma · Breast cancer

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Introduction

Gangliosides are sialic acid-containing glycosphingolipids that have increased expression in the outer leaflet of cell membranes in various malignancies, including melanoma and breast cancer. Previous works have demonstrated that gangliosides play a role in cell signaling, adhesion, and migration of tumor cells (Iwabuchi et al. 1998a). Certain gangliosides, such as GD3, GM2, and GM3, are typified as tumor-associated antigens, which make them a good target for cancer immunotherapy (Chapman et al. 2000; Ragupathi et al. 2000). Anti-ganglioside-specific humoral immune response has been obtained in several preclinical or clinical protocols of active immunotherapy, and titers correlated with mice or patient survival (Zhang et al. 1998; Ritter et al. 1995; Helling et al. 1994; Livingston et al. 1994; Ravindranath et al. 1994a).

In 1999, Estevez et al. described a novel cancer vaccine based on the monosialoganglioside GM3 hydrophobically conjugated with the outer-membrane-protein complex (OMP) from *Neisseria meningitidis* to form very small-sized proteoliposomes (GM3/VSSP) (Estevez et al. 1999). It is known that GM3 is poorly immunogenic when administered as the unmodified nominal antigen (Livingston et al. 1989). Recently, we have demonstrated that ganglioside presentation in a proteolipid environment using GM3/VSSP vaccine can induce a strong antibody response and a potent antitumor effect against B16 mouse melanoma, a syngeneic tumor model expressing high amounts of GM3 (Carr et al. 2001; Alonso et al.. 1999). Correlation between antitumor activity of the vaccine and the presence of substantial amounts of GM3 on cancer cell surfaces, however, has not been yet explored using other animal models.

Antibodies are considered the primary mechanism for elimination of pathogens, as well as circulating tumor cells and micrometastases (Zhang et al. 1998). Furthermore, anti-ganglioside antibodies may be effective to

induce complement-mediated lysis of malignant cells and antibody-dependent cell-mediated cytotoxicity (Parajuli et al. 2001).

The great majority of cell surface antigens recognized by antibodies are carbohydrates. In this regard, carbohydrates have proven to be potent targets for immune recognition and attack, because of their immunogenicity and abundance at the cell surface (Livingston et al. 1997). Several monoclonal antibodies have been produced in mice immunized either with purified gangliosides or ganglioside-positive cells (Chapman et al. 1990; Cheung et al. 1985; Hirabashi et al. 1985). However, limited information is available concerning the specific reactive epitope of these monoclonal antibodies. In addition, there is incomplete evidence about the critical reactive residues recognized by antisera in the surface of tumor cells using ganglioside-based vaccines.

In the present work, we have examined the antigenic role of sialic acid in the antitumor activity of GM3/VSSP vaccine against GM3-positive B16 melanoma cells. Removal of sialic acid residues from tumor cell surface completely abolished the reactivity of serum from vaccinated mice against B16 cells. Interestingly, no significant antitumor effect was observed in mice preimmunized with GM3/VSSP and challenged with GM3-negative F3II mammary carcinoma cells.

Materials and methods

Tumor cells and culture conditions

The murine tumor cell lines B16 melanoma and F3II mammary carcinoma, syngeneic for C57BL/6 and BALB/c mice, respectively, were employed. We decided to use the parental B16 cell line (subline F0), that is characterized by the predominant presence of the monosialoganglioside GM3 on the cell surface (Nores et al. 1987; Hirabayashi et al. 1985). B16 melanoma cells were maintained in Dulbecco's modified Eagle medium (DMEM) from Gibco BRL (Grand Island, N.Y., USA), containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 10 µg/ml tetracyclin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The sarcomatoid mammary carcinoma cell line F3II is a highly invasive and metastatic variant positive for oligosialogangliosides (Vazquez et al. 2000; Alonso et al. 1996). F3II mammary carcinoma cells were maintained in minimal essential medium (MEM) from Gibco, supplemented with 5% FBS, 2 mM glutamine, and 80 µg/ml gentamycin.

Cell growth

The effect of exogenous GM3 on the growth of B16 and F3II cells was assessed in vitro. GM3 ganglioside was obtained from Sigma Chemical (St. Louis, Mo., USA). Tumor cells were plated onto 96-well plates ($5–10\times10^3$ cells/well) in medium supplemented with FBS. The next day, tumor cells were extensively washed with phosphate-buffered saline, pH 7.4 (PBS), and incubated for 48 h in the same medium with or without 5% FBS, and in the presence of indicated concentrations of GM3 ganglioside (0–100 µg/ml). Monolayers were then washed, fixed, and stained with toluidine blue. The amount of tumor cells was estimated from absorbance readings at 595 nm after solubilization with 1% SDS, as reported (Alonso et al. 1998).

Animals

C57BL/6 and BALB/c inbred mice were purchased from the Animal Care Division, Angel H. Roffo Institute of Oncology (Buenos Aires, Argentina), and kept 5–10 mice/cage with water and food ad libitum. Animals with an age of 8–14 weeks and an average weight of 25 g were used.

Vaccination

GM3/VSSP vaccine was produced and generously provided by the Center of Molecular Immunology (Habana, Cuba). Briefly, monosialoganglioside GM3 was purified from canine red blood cells and hydrophobically conjugated with the carrier OMP from *N. meningitidis* strain 385, as described in detail (Estevez et al. 1999). The method allowed gangliosides and proteins to incorporate into very small size proteoliposomes and conferred high solubility to the conjugate. Each GM3/VSSP vaccine contained 120 µg in 0.05 ml of PBS, mixed with an equal volume of the immunological adjuvant Montanide ISA 51 (SEPPIC, France) prior to injection. Vaccines were administered i.m. in the quadriceps four times at 14-day intervals. Control animals received only the adjuvant mixed with PBS.

Mouse sera and monoclonal antibody

Mice were immunized as described above, and 7–21 days after the fourth dose of the vaccine were bled through the optical artery and immediately sacrificed. Normal sera were also obtained from untreated mice. Sera were frozen at –20 °C in aliquots for further analysis. The GM3 ganglioside-specific mouse IgM monoclonal antibody (clone M2590) was purchased from International Bioscience (Tokyo, Japan).

Flow cytometry

Cultured B16 or F3II cells were harvested with a cell-scraper, resuspended in serum-free medium, and then incubated at 5×10^5 to 1×10^6 cells per sample with 1 µg of the monoclonal antibody M2590 or a control monoclonal antibody of the same isotype (IgM) for 30 min at 4 °C. Tumor cells were washed in medium containing 1% FBS and further incubated with 5 µl of FITC-conjugated goat antibodies anti-mouse IgM (Caltag Laboratories, Burlingame, Calif., USA). Alternatively, tumor cells were incubated with 15 µl of sera of either treated or control mice and then labeled with FITC-conjugated goat antibodies anti-mouse immunoglobulins (Caltag). The cells were then analyzed with a Cytoron flow cytometer (Ortho Diagnostic Systems, Raritan, N.J., USA).

Enzyme-linked immunosorbent assay (ELISA)

Flexible 96-well microtiter plates (Becton Dickinson, Oxnard, Calif., USA) were coated using 10 µg/ml of the GM3/VSSP conjugate in carbonate buffer, pH 9.6. Coated and control uncoated plates were blocked with 2% bovine serum albumin (BSA) in carbonate buffer and 5-fold serial dilutions of sera were applied. Bound IgM or IgG antibodies were detected with sequential incubations with either biotinylated anti-µ chain or anti-γ chain antibodies (Jackson Immunochemicals), followed by alkaline phosphatase-conjugated Extravidin (Sigma) and the chromogenic substrate 4-nitrophenyl-phosphate (Boehringer Mannheim). All steps were separated by six washes with 0.1% Tween-20 in PBS. Immunologicals were diluted in 0.2% BSA in PBS.

Specific antibodies against GM3 ganglioside were detected basically as described by Ravindranath et al. (Ravindranath et al. 1994b). Briefly, PolySorp 96-well microtiter plates (Nunc) were coated with 200 ng/well of unconjugated GM3 (4 µg/ml in ethanol). Ethanol was added to control wells. Once dry, plates were

blocked without previous washing with 2% BSA in PBS. Further steps were carried out as described above, except that 0.1% BSA/ 0.1% Tween-20 in PBS was used for washing.

Lymphoproliferation assay

Splenocytes were resuspended in DMEM supplemented with 10% FBS and 2 mM glutamine, and plated at 2×10^5 cells per well in U-bottom 96-well microtiter plates. GM3/VSSP was added at 5 µg ganglioside/ml. The plates were then incubated at 37 °C under 5% CO_2 for 3 days. Tritiated thymidine was added at 1 µCi/well and the plates were further incubated for another 18 h. Cells were then harvested, and tritium incorporation was monitored in a liquid scintillation counter. The stimulation index was defined as the ratio of mean cpm from wells supplemented with GM3/VSSP to mean cpm from wells with culture medium alone (triplicate determinations).

Indirect immunoperoxidase staining and neuraminidase treatment

For detection of B16- and F3II-reactive serum antibodies, the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA) was employed on formalin-fixed tumor cell monolayers and tumor sections following the instructions of the manufacturer. Monolayers were fixed with formalin and preserved at 4 °C. Tumor tissue specimens were fixed with formalin, included in paraffin, cut in 5-µm sections and rehydrated. Monolayers and tumor sections were incubated for 1 h with pooled sera at a dilution of 1:500 in PBS. Specimens were then incubated with biotinylated horse anti-mouse immunoglobulins for 30 min, followed by peroxidase-conjugated avidin-biotin complex for 30 min. Finally, bound antibodies were detected by incubation with the peroxidase substrate diaminobenzidine for 2–4 min and counterstained with hematoxylin. Pertinent specificity tests were performed, including blocking of endogenous peroxidase, omission of the first serum and utilization of diluted serum from control mice.

In order to evaluate the role of sialic acid in serum antibody response of GM3/VSSP vaccinated mice, fixed tumor cells were exposed to neuraminidase, a hydrolase that removes sialic acid residues from the cell surface. Tumor cell monolayers or tumor sections were incubated at 37 °C for 4 h or overnight, respectively, in the presence 0.1–1 UI/ml of type II neuraminidase (Sigma) previous to immunoperoxidase staining. As neuraminidase preparations may contain protease traces, the presence of proteolytic activity was discarded by a radial caseinolytic assay using casein-agarose substrates (Alonso et al. 1998).

Tumor cell challenge

For injection into mice, tumor cell suspensions were prepared by detaching the cells from culture flasks by trypsinization, followed by washing and resuspension in culture medium. Tumor cells were counted and viability was assayed by the trypan blue exclusion test. Twenty-one days after the fourth immunization with GM3/VSSP vaccine, mice were given inoculations in the subcutis of the right flank with 0.2 ml of tumor cell suspensions in serum-free medium. Preliminary experiments showed that a challenge of 2.5×10^3 B16 melanoma cells in C57BL/6 mice and 5×10^4 F3II mammary carcinoma cells in BALB/c mice induced a similar tumor incidence, of at least 50%. The respective syngeneic mice received fourfold the described tumor burdens, in order to achieve a tumor incidence of 100% (1×10^4 B16 cells and 2×10^5 F3II cells). In all cases, the time of appearance of local tumors was monitored by palpation and further confirmed by histopathology. Tumor size was measured with a caliper twice a week and tumor volume was calculated by the formula: $\pi/6 \times width^2 \times length$. When tumors exceeded 15,000 mm^3, animals were killed and necropsied.

Results

Expression of gangliosides in B16 melanoma and F3II mammary carcinoma cells and effect of exogenous GM3 on in vitro growth

To assess the presence or absence of GM3 ganglioside in the surface of B16 and F3II tumor cells, flow cytometric detection using the specific anti-GM3 monoclonal antibody M2590 was performed. As shown in Fig. 1a, the antibody reacted brightly against B16 melanoma cells and the reaction was notably inhibited by preincubation with soluble GM3. On the contrary (Fig. 1b), no interaction with the surface of F3II mammary carcinoma cells could be observed. These results confirm a significant expression of GM3 in B16 cells in contrast with the absence of this monosialoganglioside in the plasma membrane of F3II cells.

We then tested the effect of exogenous GM3 on the in vitro growth of B16 and F3II cells. The addition of GM3, at concentrations higher than 50 µg/ml, caused an increase in the proliferation of GM3-positive B16 cells in serum-free conditions. Conversely, exogenous GM3 induced a significant growth inhibition of GM3-negative F3II cells in the presence of 5% FBS (Fig. 2). Neither cytotoxic nor proapoptotic effects were observed using
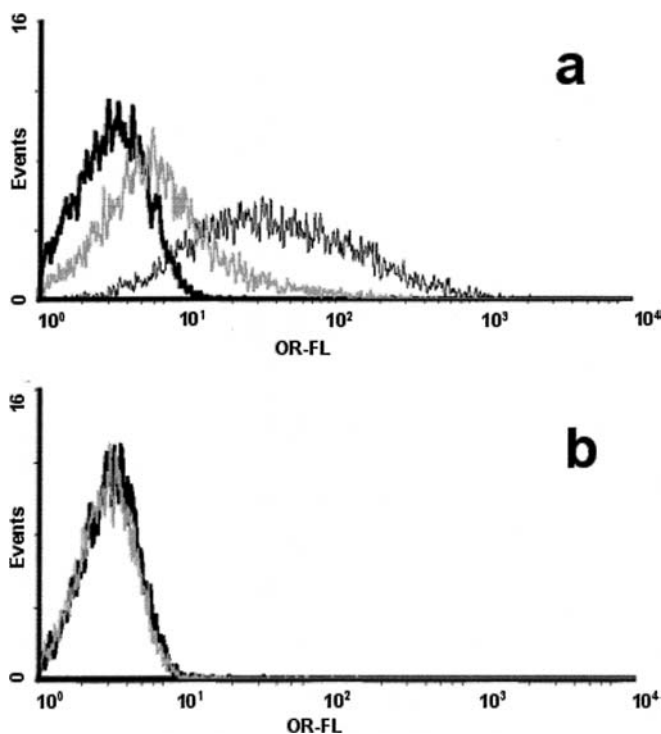

Fig. 1a,b Flow cytometry analysis of B16 melanoma and F3II mammary carcinoma cells labeled with the anti-GM3 monoclonal antibody M2590. **a** B16 cells were incubated with isotype control antibodies (*thick line*), M2590 (*thin line*) or M2590 preincubated with soluble GM3 (*gray line*); **b** F3II cells were incubated with isotype control antibodies (*thick line*) or M2590 (*thin line*). Results are representative of three independent experiments
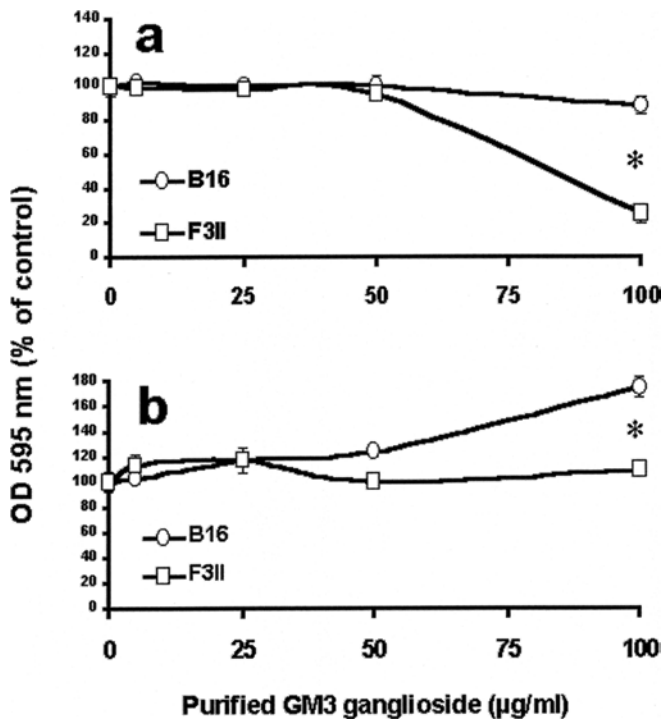
Fig. 2 Effect of exogenous GM3 ganglioside on the in vitro growth of F3II and B16 cells in the **a** presence or **b** absence of serum. Data represent means ± SEM ($n = 16$). *$P < 0.001$, ANOVA test

a non-radioactive cytotoxicity assay and a DNA fragmentation detection kit, respectively, suggesting a cytostatic action of GM3 on F3II monolayers (data not shown).

Serum antibody and lymphoproliferative response of GM3/VSSP immunized mice

In order to assess the antigenicity of the vaccine, we first studied the specificity of serum antibodies in immunized mice. The reactivity of sera was tested against both the GM3/VSSP conjugate or unconjugated GM3 ganglioside. Mice of either C57BL/6 or BALB/c strains were vaccinated with four doses of GM3/VSSP and serum samples were obtained 7–21 days after completion of the immunization schedule. All mice immunized with the GM3/VSSP vaccine developed IgG antibodies against the GM3/VSSP conjugate. High titers were obtained, reaching 32×10^6, and no significant differences were obtained between mouse strains. Interestingly, animals immunized with the unconjugated carrier VSSP plus Montanide developed similar titers against the GM3/VSSP conjugate, suggesting that a strong reactivity is directed against the carrier moiety of the conjugate.

The binding of serum antibodies to ELISA plates coated with purified GM3 was also assessed. Uncoated plates were used as a control for non-specific binding. Sera derived from all mice treated with the carrier pro-

tein plus Montanide or only with the immunological adjuvant, as well as from untreated mice, contained undetectable amounts of GM3-specific antibodies (IgM and IgG titers < 80). Analysis of serum samples derived from C57BL/6 mice immunized with GM3/VSSP vaccine yielded the detection of low-titer GM3-specific IgM antibodies in four of eight mice. Similarly, sera from BALB/c mice showed detectable anti-GM3 IgM antibodies in two of five mice. More interestingly, moderate-titer anti-GM3 IgG antibodies were detected in both strains, with titers reaching 1,280 (Table 1). In summary, we found the presence of at least one class of antibody against the purified GM3 ganglioside in five out of eight C57BL/6 (62.5%) and in four of five (80%) BALB/c immunized mice.

Next, we studied whether serum antibodies of immunized mice mediate direct recognition of tumor cells. In a previous report, we showed that sera from C57BL/6 immunized mice have a strong reactivity against B16 melanoma using tumor sections from tissues embedded either in Epon or paraffin (Alonso et al. 1999). Here, we analyzed the reactivity of sera from GM3/VSSP immunized mice against B16 and F3II tumor cells. Fig. 3 shows the serum reactivity of immunized mice against these cells as analyzed by flow cytometry. Clearly, immune sera recognized the surface of the GM3-positive B16 cells while GM3-negative F3II cells were not stained. The observed difference in serum antibody binding was confirmed by immunoperoxidase treatment of fixed cell monolayers. As shown in Fig. 4a, most melanoma cells display a distinct positive staining using sera from C57BL/6 immunized mice. Although the reactivity was associated with the cell membrane, a diffuse cytoplasmatic staining was also observed. In contrast, there was no reactivity of immunized sera against the surface of F3II mammary carcinoma cells. However, a heterogeneous pattern of reactivity was found in endomembranes (mainly endoplasmic reticulum and Golgi apparatus) in F3II cell cultures (Fig. 4b). In all cases, there was no reactivity with sera obtained from untreated mice or mice receiving only the immunological adjuvant.

Preliminary results obtained in lymphoproliferative assays showed an increase in the stimulation index of

Table 1 Specific anti-GM3 antibodies in sera of mice immunized with GM3/VSSP. Mice were immunized with four doses of GM3/VSSP vaccine and specific antibodies against purified GM3 ganglioside were detected by ELISA in 96-well microtiter plates coated with GM3 as described in Materials and methods. All control mice yielded undetectable levels of GM3-specific antibodies (IgM and IgG titers < 80)

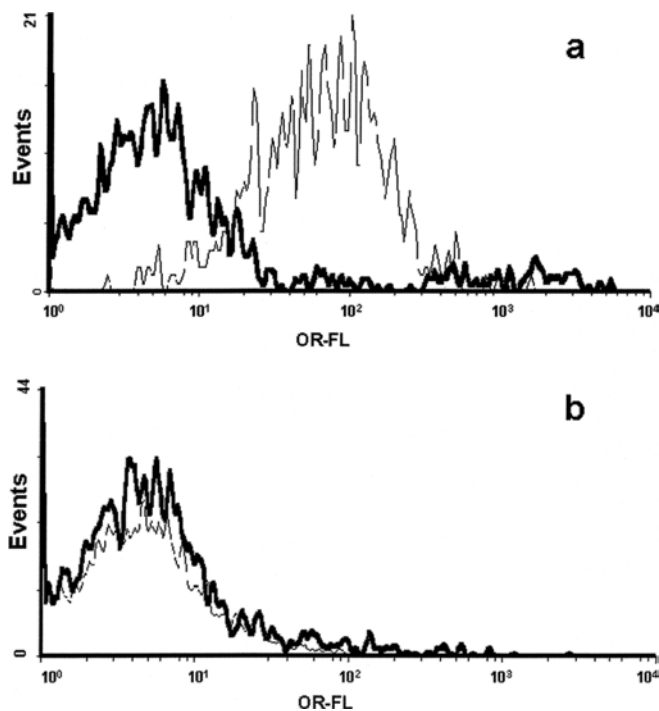| Strain	n	Titer (n)	
		IgM	IgG
C57BL/6	8	< 80 (4); 80 (3); 320 (1)	< 80 (4); 80 (1); 160 (2); 1280 (1)
BALB/c	5	< 80 (3); 80 (1); 640 (1)	< 80 (2); 80 (2); 1280 (1)

Fig. 3 Flow cytometry analysis of B16 melanoma and F3II mammary carcinoma cells labeled with sera of GM3/VSSP vaccinated mice. **a** B16 cells or **b** F3II cells were incubated with control serum (*thick line*), or from vaccinated mice (*thin line*)

splenocytes derived from vaccinated mice exposed to 5 μg/ml of GM3/VSSP. Stimulation index in C57BL/6 immunized mice doubled the index of the control group (9.7 ± 1.7 versus 5.3 ± 0.1). A similar response was observed in BALB/c mice, in which case vaccination increased the stimulation index by fourfold (20 ± 1.0 versus 4.5 ± 0.5).

Abolition of serum reactivity of GM3/VSSP vaccinated mice against B16 melanoma cells by removal of cell surface sialic acid

To establish whether sialic acid present in melanoma plasma membrane plays a key antigenic role in serum antibody response of mice immunized with GM3/VSSP, we treated formalin-fixed B16 monolayers with neuraminidase, a hydrolase that remove sialic acid residues from the cell surface. Neuraminidase pretreatment completely abolished the reactivity of serum from vaccinated mice against B16 melanoma cells (Fig. 4e). Desialylation of paraffin-embedded tissue sections of B16 subcutaneous tumors also inhibited humoral response against melanoma cells (data not shown). The possibility that protease traces contaminate neuraminidase preparations and may be partially responsible of the described effect was ruled out. We tested the proteolytic activity of neuraminidase preparations using a highly sensitive caseinolytic assay and no signs of degradation were observed.

Relationship of in vivo antitumor activity induced by GM3/VSSP vaccine with the presence of GM3 ganglioside on tumor cell surface

C57BL/6 or BALB/c inbred mice were vaccinated four times with GM3/VSSP vaccine on days –63, –49, –35, and –21, prior to B16 or F3II tumor cell challenge on day 0, respectively. In both cases, syngeneic animals received the minimal challenge to obtain a tumor incidence of 100% (1×10^4 B16 cells or 2×10^5 F3II cells), as indicated by preliminary experiments. As shown in Table 2, preimmunization with GM3/VSSP vaccine significantly inhibited tumor formation in C57BL/6 inoculated with B16 melanoma cells. Vaccinated mice survived longer than controls (Fig. 5a), and several animals were sacrificed on day 90 after challenge with no evidence of melanoma tumors. On the contrary, vaccination failed to block tumor formation in BALB/c mice challenged with GM3-negative F3II mammary carcinoma cells (see also Table 2 and Fig. 5b). Similar results (not shown) were obtained inoculating smaller tumor cell burdens, although tumor incidence was < 50% in control animals.

Discussion

GM3 is a monosialoganglioside, being the most abundant membrane glycolipid in melanoma cells (Hersey et al. 1998; Nakakuma et al. 1992). Previously, we have demonstrated that a GM3-based vaccine induced tumor protection in mice challenged with melanoma cells (Carr et al. 2001; Alonso et al. 1999). As with other vaccines, the understanding of the underlying mechanisms involved in immune responses is crucial to elaborate a model of potential clinical application (Bitton et al. 2002).

In this work, we confirmed the expression of GM3 ganglioside in the surface of B16 melanoma cells using a GM3-specific monoclonal antibody. Conversely, we observed that F3II mammary carcinoma cells do not express GM3 on their surfaces, although reactive epitopes could be detected in intracellular compartments. Other authors have described GM3-enriched microdomains in B16 cells, and postulated that this microclusters take part in vital cell functions, including adhesion, migration and, possibly, cell signaling, by interaction of GM3 with c-Scr and Rho transducer proteins (Iwabuchi et al. 1998b; Yamamura et al. 1997). GM3 has been cited also as a regulatory molecule for epidermal growth factor (Meuillet et al. 2000; Zhou et al. 1994), the basic fibroblast growth factor (Meuillet et al. 1996), and integrin receptors (Zheng et al. 1993). More recently, GM3 expression in melanoma has been shown as a determinant of malignant phenotype (Deng et al. 2002; Deng et al. 2000).

Exogenous GM3 stimulated the proliferation of B16 melanoma cells when cultured in serum-free conditions. On the other hand, growth arrest induced by GM3 ganglioside in GM3-negative F3II mammary carcinoma

Fig. 4a–e Indirect immunoper-oxidase staining of tumor cell monolayers by serum antibodies of GM3/VSSP vaccinated mice. B16 melanoma cells stained using **a** GM3/VSSP and **c** control serum; F3II mammary carcinoma cells stained with **b** GM3/VSSP and **d** control serum; **e** Neuraminidase-treated B16 cells stained with GM3/VSSP serum, 400×
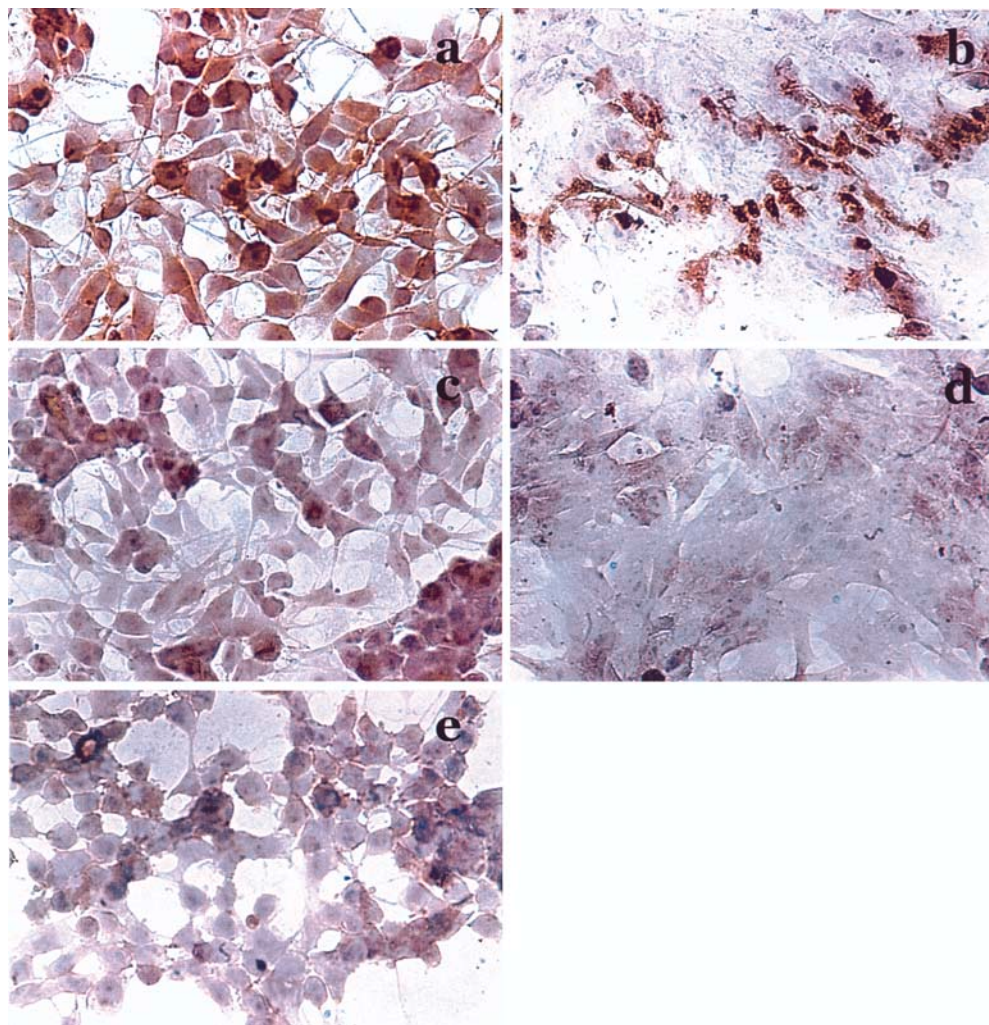
Table 2 Effect of GM3/VSSP vaccine on F3II mammary carcinoma and B16 melanoma tumor formation in syngeneic mice. Mice were immunized with four doses of GM3/VSSP vaccine and tumor cells were inoculated in the subcutis, as described in Materials and methods

Tumor cell line	Treatment	Tumor incidence[a]	
		Day 10	Day 30
B16 melanoma	Control	0/6 (0)	6/6 (100)
	GM3/VSSP	0/10 (0)	3/10 (30)[b]
F3II mammary carcinoma	Control	10/10 (100)	10/10 (100)
	GM3/VSSP	9/10 (90)	9/10 (90)

[a]Tumor incidence is expressed as positive/total animals at indicated days after tumor cell challenge; the number in parentheses indicates the percentage

[b]$P < 0.01$ versus control, Fisher's exact test

cells in complete medium suggests that growth control mechanisms involved in both cell lines are substantially different. Exogenous gangliosides have been shown to cause cell apoptosis in certain immortalized cell lines (Molotkovskaya et al. 2000). However, no signs of apoptosis or cytotoxicity were seen in GM3-treated F3II cell cultures, suggesting a cytostatic effect of the ganglioside.

Taking into account that signaling pathways involved in transformation vary in different tumor variants, GM3 may display variable effects on tumor cell growth, being stimulatory for some malignant cells such as B16. Therefore, high concentrations of GM3 ganglioside accumulated in the tumor milieu may be beneficial for melanoma but not for F3II mammary carcinoma cells.

Immunization of C57BL/6 and BALB/c mice with the GM3/VSSP vaccine elicited both IgM and IgG anti-GM3 antibodies. Immune sera also recognized B16 cell surface antigens but did not react with the plasma membrane of F3II cells, in accordance with the presence of GM3 ganglioside in B16 cells and its absence in F3II cells. Interestingly, immunocytochemistry studies using permeabilized F3II cells revealed an endomembrane staining by the immune sera. This may suggest the presence of GM3 in intracellular organelles that does not reach the plasma membrane, and could be used as precursor of complex gangliosides.

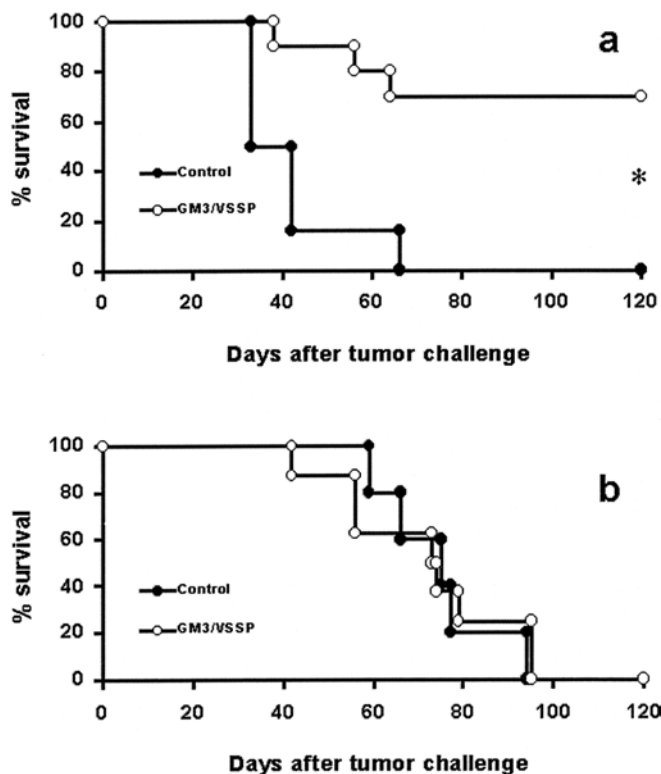Neuraminidase, a bacterial enzyme used to specifically remove sialic acid residues on cell surface antigens,

Fig. 5 Survival of mice after subcutaneous challenge with **a** B16 melanoma and **b** F3II mammary carcinoma cells. Animals were preimmunized with GM3/VSSP plus Montanide (*empty circles*) or received only the adjuvant (*filled circles*), as described in Material and methods. *$P < 0.001$, log-rank test

abolished the recognition of B16 cells by sera of GM3/VSSP immunized mice. Our results indicate that sialic acid residues present in the surface of GM3-positive B16 melanoma cells play a central role in the serum antibody response of GM3/VSSP-vaccinated mice. In addition, lymphoproliterative assay showed an increase in the stimulation index of splenocytes derived from immunized mice in the presence of GM3/VSSP.

Gangliosides are T-cell independent antigens considered not to be presented to T lymphocytes by the major histocompatibility complex as regular peptide antigens do (Ishioka et al. 1992; Harding et al. 1991). However, there are several examples of carbohydrate vaccines that do not support this proposed dogma. As an example, Taniguchi et al. (Taniguchi et al. 1988) obtained a specific GM3 cytotoxic T lymphocyte response in mice. Similarly, Zhao et al. (Zhao et al. 1995) demonstrated a specific cytotoxic T lymphocyte response against GD2 positive cells dependent on sialic acid residues. We have previously shown evidence of T cell involvement in GM3/VSSP immunized mice that elicited antibodies of the IgG1, IgG2a, and particularly IgG2b subclasses (Carr et al. 2001; Alonso et al. 1999). Nevertheless, antitumor activity generated against carbohydrate membrane antigens does not fit the classical type 1 or 2 model described for immune responses, and multiple cytolytic mechanisms seem to be required for antigen-specific

tumor rejection. In this regard, other in vivo mechanisms may also contribute to antitumor immunity, such as natural killer cell-mediated cytotoxicity (Van Lith et al. 2002).

Finally, we compared the antitumor activity of GM3/VSSP vaccine against B16 and F3II tumor cells in syngeneic animals. Using the minimal cell challenge that causes 100% of tumor incidence, GM3/VSSP vaccinated mice rejected tumor cells in the GM3-positive B16 melanoma but it did not in the GM3-negative F3II mammary carcinoma model. Although the present experiments were done with the parental B16 melanoma model (subline F0), we have also observed a significant antitumor activity of GM3/VSSP vaccine in previous experiments using the F1 and F10 variants with low- and high metastatic abilities, respectively (Carr et al. 2001).

With these results, an imposing question arises. Is this specific antitumor activity due to differences in the immunological response of the mouse strains tested or to a differential GM3 expression in the cell lines used? In both C57BL/6 and BALB/c mice, GM3/VSSP immunized sera were reactive against fixed and live B16 cells, suggesting that humoral response in both strains were very similar. Besides, Estevez et al. (Estevez et al. 1999) demonstrated a similar immunoglobulin profile in response to GM3/VSSP immunization in BALB/c mice. On the contrary, the presence of GM3 ganglioside on the surface of B16 cells and its absence in F3II cells allows us to hypothesize that this expression is the cause of the specificity of the antitumor activity of the vaccine.

The development of cancer vaccines is based on the premise that there are qualitative or quantitative fundamental differences between malignant and normal cells. However, progress in the development of cancer vaccines has so far been limited. Increased understanding of targets, immune recognition, and the underlying mechanisms leading to tumor rejection may well help to overcome these hurdles. In conclusion, using different tumor models with and without expression of GM3 monosialoganglioside, we were able to demonstrate that GM3/VSSP vaccine exerted an antitumor activity only in a GM3-positive tumor model, that serum from immunized mice recognize only the surface of GM3-positive tumor cells, and that sialic acid play a central role in such recognition.

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