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Peptides mimicking GD2 ganglioside elicit cellular, humoral and tumor-protective immune responses in mice

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Abstract

Introduction—Because of its restricted distribution in normal tissues and its high expression on tumors of neuroectodermal origin, GD2 ganglioside is an excellent target for active specific immunotherapy. However, GD2 usually elicits low-titered IgM and no IgG or cellular immune responses, limiting its usefulness as a vaccine for cancer patients. We have previously shown that anti-idiotypic monoclonal antibody mimics of GD2 can induce antigen-specific humoral and cellular immunity in mice, but inhibition of tumor growth by the mimics could not be detected.

Methods and results—Here, we isolated two peptides from phage display peptide libraries by panning with GD2-specific mAb ME361. The peptides inhibited binding of the mAb to GD2. When coupled to keyhole limpet hemocyanin (KLH) or presented as multiantigenic peptides in QS21 adjuvant, the peptides induced in mice antibodies binding specifically to GD2 and delayed-type hypersensitive lymphocytes reactive specifically with GD2-positive D142.34 mouse melanoma cells. Induction of delayed-type hypersensitivity (DTH) reaction was dependent on

CD4-positive lymphocytes. The immunity elicited by the peptides significantly inhibited growth of GD2-positive melanoma cells in mice.

Conclusion—Our study suggests that immunization with peptides mimicking GD2 ganglioside inhibits tumor growth through antibody and/or CD4-positive T cell-mediated mechanisms. Cytolytic T lymphocytes most likely do not play a role. Our results provide the basis for structural analysis of carbohydrate mimicry by peptides.

Keywords

GD2; Peptide mimics; Antibodies; DTH; Tumor growth inhibition

Introduction

GD2 ganglioside is a major constituent of neuroectodermal tumors [29]. GD2-specific mAb have inhibited melanoma metastasis formation in mice [22, 50] and have shown some in vivo anti-tumor effects in melanoma and neuroblastoma patients [1, 8, 9, 15, 17, 23, 44]. Furthermore, GD2 is a potential target for treatment of gliomas, medulloblastomas, small cell lung carcinomas, retinoblastomas, neuroblastomas and osteo- and soft tissue sarcomas which all express this antigen [7, 19, 43, 45]. Induction of GD2-specific sustained immunity by active immunization may be advantageous over passive immunotherapy with mAb. However, GD2 is difficult to purify in amounts sufficient for immunization of cancer patients. When presented by tumor cells or as a vaccine in adjuvant, GD2 usually elicited a short-lived, low titered IgM response of poor memory [21, 29, 40, 42, 47]. In light of the high expression of GD2 by tumors of various tissue origins, induction of GD2-specific long-lived IgG responses and cellular immunity to GD2 is desirable; such immune responses may inhibit tumor growth. GD2 presented by syngeneic mouse EL-4 lymphoma cells elicited MHC class I restricted CTL in mice [51]. These CTL responses were blocked by GD2-specific mAb. However, to our knowledge, whole tumor cell vaccines have not elicited GD2-specific cellular immunity in cancer patients.

Peptides or anti-idiotypic antibodies (Ab₂)³ may functionally mimic carbohydrates and induce IgG and cellular immune responses which the carbohydrate itself is usually unable to induce [2, 5, 25]. Carbohydrate mimetic peptides derived from panning peptide libraries with antibodies or lectins [35] and by pharmacodesign principles have been described [10, 30]. Ab₂ have been generated by immunization with GD2-specific antibodies, while peptides have been isolated by panning phage display peptide libraries with GD2-specific antibodies. Thus, a DNA vaccine encoding a peptide isolated with the GD2-specific mAb 14G2a primed mice for challenge with GD2, resulting in the induction of antibodies that exhibited complement-dependent cytotoxicity against GD2⁺ melanoma cells and inhibited growth of human melanoma cell xenografts after transfer to tumor-bearing SCID mice [6]. However, the DNA vaccine alone was unable to induce protective antibodies and induction of cellular immunity by the vaccine has not been demonstrated. Recently, a DNA vaccine encoding a peptide isolated with the GD2-specific mAb ch14.18 inhibited spontaneous neuroblastoma liver metastasis formation in mice by inducing antibody formation and activating natural killer cells [13]. We have shown that Ab₂ mimicking GD2 induced IgG binding to GD2⁺ melanoma cells, and GD2-specific delayed-type hypersensitivity (DTH) reaction in mice [2]. However, inhibition of tumor growth following immunization with the Ab₂ could not be demonstrated. Similarly, a plasmid encoding single chain Fv of Ab₂ mimicking GD2 induced GD2-specific antibodies in mice [48].

In the present study, we have isolated peptides by panning phage display peptide libraries with the GD2-specific mAb ME361. Two peptides were selected for immunization of mice based on their capacity to inhibit binding of mAb ME361 to GD2. The peptides mimic GD2

as they induced GD2-specific antibodies and DTH reactions in mice. Importantly, peptide immunization of mice protected them against challenge with GD2⁺ melanoma cells. The peptides are candidate vaccines for patients with GD2⁺ tumors.

Materials and methods

Animals and adjuvant

C57BL/6 or CD4^{-/-} C57BL/6 female mice aged 6–8 weeks were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and maintained at Wistar Animal Facility in accordance with the IACUC guidelines.

Cell lines, mAbs and adjuvant

The GD2-specific mAb ME361, a mouse IgG2a, was developed and characterized as described [20]. C57BL/6 D142.34 murine melanoma cells (kindly provided by Drs. Koichi Furukawa, Nagasaki University, Nagasaki, Japan) have been doubly transfected with genes encoding β -1,4-N-acetylgalactosaminyltransferase, using the genitacin selection marker, and α -2,8-sialyltransferase, using the hygromycin selection marker, resulting in the constitutive expression by the transfectants of both GD2 and GD3 [3,18]. B78.H1 cells are parental GD2-negative cells. Transfected D142.34 cells were maintained in RPMI 1640 medium with GlutaMax (Invitrogen Corporation, Grand Island, NY) supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA), 400 μ g/ml G418 and 50 μ g/ml hygromycin (Mediatech, Inc. Cellgro, Herndon, VA). B78.H1 cells were grown in RPMI 1640 medium supplemented with 10% FBS. Neither cell line expresses MHC class I and II antigens, even after IFN- γ treatment [2]. Thus, they resemble human melanoma cells which often are HLA class I and II negative [39]. The YAC-1 mouse lymphoma cell line was maintained in RPMI 1640 medium supplemented with 10% FBS. QS-21 adjuvant was obtained from Antigenics Inc. (Lexington, MA).

Panning of a phage display peptide library with ME361 mAb

Peptides P9 and P10 were isolated as we have described previously [26, 41]. A random 15mer peptide phage display library was panned using the GD2-specific mAb ME361 as a target. The initial choice of using the 15mer library was predicated on the notion that this length is similar to complementarity determining regions in antibodies with antigen mimicry (anti-idiotypic antibodies). The 15mer library has been constructed with the phage fUSE5 vector. The library was made by ligating a synthetic 33 bp BglI fragment into fUSE5 and transfecting *E. coli* K91/Kan⁺ cells with ligation product by electroporation. The 15mer phage library was reacted with biotinylated mAb ME361 immobilized on streptavidin-coated polystyrene f100 mm Petri dishes. In the first round, approximately 1,012 transducing units were incubated overnight with 10 μ g/ml biotinylated mAb in 100 μ l reaction mixtures. Phage eluted in the first round were amplified in liquid phase and subjected to a second round of affinity purification with mAb ME361 at a concentration of 5 μ g/ml. Phage from the second round of amplification were amplified and subjected to further rounds with decreasing mAb concentration. The decrease in mAb concentration in the second and third rounds, and the use of excess phage introduced binding competition, and were intended to select for high-affinity binding epitopes. Phage in the eluates from the second and third rounds of affinity purification were cloned and propagated for sequencing [41].

Synthetic peptides

Peptides were synthesized by SynPep Corporation (Dublin, CA). A cysteine residue was added to the original sequence at the NH₂-terminal of the synthetic peptides to facilitate

conjugation with keyhole limpet hemocyanin (KLH). Multiple-antigen peptides (MAP) were prepared by Bio-Synthesis, Inc. (Lewisville, TX). The HIV derived peptide S266 was included as a specificity control. The sequences of the peptides are shown in Table 1. All peptides were dissolved in 50% DMSO at a concentration of 20 mg/ml and further diluted in buffer or medium for use in the various assays.

Inhibition of mAb ME361 binding to GD2 by peptides

ELISA plates were coated with 0.3 µg GD2 ganglioside (Advanced Immunochemical Inc., Long Beach, CA) per well dissolved in ethanol and dried by evaporation at room temperature overnight. Biotin-mAb ME361 (at the final concentration of 0.15 µg/ml) was pre-incubated with an equal volume of peptides P9, P10 or S266 at various concentrations (see Fig. 1) in 1% BSA/PBST (PBS with 0.05% Tween-20) overnight at 4°C. Wells were blocked with 200 µl of 2% BSA in PBS-T for 2 h at room temperature. The mixture of peptide and biotin-mAb ME361 (100 µl/well) was added and incubated overnight at 4°C. Biotin-mAb ME361 (0.15 µg/ml) was used as non-inhibited control and the same concentration of biotin-H24B5 anti-influenza virus antibody as an isotype-matched control mAb. HRP-Streptavidin (1:18,000 in 1% BSA/PBST) was added and the incubation was continued for 1 h at room temperature. Then TMB substrate buffer was added and the mixture was incubated for 15–30 min in the dark. The absorbance was read at 450 nm.

Inhibition of mAb ME361 binding to GD2⁺ tumor cells by immune sera elicited with peptides

Since control sera exhibited high non-specific binding to D142.34 cells (GD2⁺) in assays which measure direct binding of the sera to the cells, an immunofluorescence inhibition test was used to measure serum antibody binding to D142.34 cells. This assay measures inhibition of binding of mAb ME361 to the cells by the sera. D142.34 cells (GD2⁺) (5×10^5 cells per well of microtiter plate) were mixed with dilutions of pre-immune and post-immune mouse sera and incubated for 50 min on ice. Cells were then washed three times in PBS and biotinylated mAb ME361 at a concentration that gives a 50% maximal binding (0.7 µg/ml) or the isotype-matched biotinylated control mAb H24B5 (0.7 µg/ml), and mouse γ-globulin (50 µg/ml) were added to the cells and incubated for 50 min on ice. After three washes, Extravidin-FITC (1:200) was added to the cells and incubated for 30 min on ice in the dark. Cells were washed three times and resuspended in 0.5 ml PBS. The stained cells were analyzed in the cytofluorograph. Percentage inhibition of mAb ME361 binding was calculated using the formula $(1\% \text{ binding of mAb ME361 in the presence of sera} / \% \text{ binding of mAb ME361 in the presence of buffer}) \times 100$.

Conjugation of peptides to KLH

Peptides were conjugated to maleimide-activated KLH using the Imject Maleimide Activated mcKLH Kit from Pierce Biotechnology Inc. (Rockford, IL). The peptide-carrier conjugate was purified following the manufacturer's instructions.

Immunizations

For the study of humoral and cellular immune responses, C57BL/6 mice were immunized subcutaneously (s.c.), in the vicinity of axillary and inguinal lymph nodes with peptides conjugated to KLH or MAP. QS-21 was used as an adjuvant (Antigenics Inc., Lexington, MA). For induction of humoral immune response 10 mice per group were immunized s.c. with 15 µg of peptide in 10 µg of adjuvant in a total volume of 100 µl twice at a one-week interval. Sera were obtained from blood samples of mice before immunization and 2 weeks after the last immunization. For the induction of lymphoproliferative responses, groups of 10 mice each were immunized s.c. twice at 1-week interval with 15 µg of peptide in 10 µg of

adjuvant in a total volume of 100 μ l. Mice were euthanatized 1 week after the second immunization. For induction of DTH response, C57BL/6 or CD4^{-/-}C57BL/6 mice (eight per group) were immunized s.c. twice at a one-week interval with 15 μ g peptide-KLH plus 10 μ g QS-21 in 0.2 ml volume. For tumor protection study, C57BL/6 mice (seven mice/group) were immunized s.c., in the vicinity of axillary and inguinal lymph nodes with 15 μ g (prophylactic study) or 50 μ g (therapeutic study) of peptides conjugated to KLH or MAP four times at 2-week intervals. QS-21 (10 μ g/mouse) was used as an adjuvant.

Detection of GD2-specific antibodies in sera

Sera from immunized mice were assessed for reactivity with GD2 ganglioside as previously described [26]. Briefly, MaxiSorp ELISA plates were coated with GD2 (Advanced Immunochemicals, Long Beach, CA) or GD1a (Sigma, St. Louis, MO) at 3 μ g/ml in ethanol and dried by evaporation. Diluted sera were added after blocking the plates with 2% BSA (Sigma) in PBS containing 0.05% Tween 20 (Sigma) and plates were incubated for 1 h at room temperature. To detect antibody binding to GD2, goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Southern Biotech Associates, Birmingham, AL) was added to the plates, followed by the addition of substrate. Post-immunization values were scored as positive if they exceeded the pre-immunization values by factor >3.

Lymphocyte proliferation assay

The proliferative responses of splenocytes from immunized mice were assessed using modifications of the methods described by Kruisbeek et al. and Zhang et al. [27, 49]. Splenocytes were harvested 7 days (MAP-immunized mice) or 14 days (KLH-peptide-immunized mice) after the second immunization by squashing spleens in RPMI 1640 medium containing 5% FBS and 50 μ g/ml gentamycin, and filtering the cells through a 70 μ m cell strainer (BD Biosciences, Bedford, MA) fitted onto 50 ml tubes. Cells were washed twice in HBSS containing 50 μ g/ml gentamicin and resuspended in complete medium (RPMI 1640 medium supplemented with 5% FBS, 5×10^{-5} 2-mercaptoethanol and 50 μ g/ml gentamicin; Sigma). One day before the proliferation assay, irradiated (2 Gy, Cesium source) splenocytes from non-immunized syngeneic mice were plated at 8×10^5 cells/well in 96-well flat bottom microtiter plates (BD Labware, Franklin Lakes, NJ) and incubated overnight at 37°C. The non-adherent cells were carefully removed and adherent cells were washed once with warm RPMI 1640 medium. Adherent cells were incubated for 8 h at 37°C in triplicates with peptide (10 or 2 μ g/ml), 1×10^4 irradiated (600 Gy, Cesium source) syngeneic D142.34 cells (GD2⁺) or B78.H1 cells (GD2⁻) or Con A (Sigma) (5 μ g/ml). Excess unbound antigens or cells were removed by aspiration, followed by the addition of 2.5×10^5 responder splenocytes from immunized mice to each well. Cells were incubated at 37°C for 3–5 days. For cytokine production analysis, supernatants were removed after 72 h of culture and stored at -70°C. Cells were pulsed with 1 μ Ci ³H-thymidine per well for 12–18 h, and radioactivity incorporation was measured in a beta counter (Packard Perkin Elmer, Shelton, CT).

DTH assay

Mice were injected two weeks after the last immunization with 5×10^5 irradiated (600 Gy, Cesium source) D142.34 (GD2⁺) or B78.H1 (GD2⁻) cells in 10 μ l PBS intradermally into the base of the left or right ear. The increase in ear thickness was measured using a digimatic caliper (Mitutoyo, Kawasaki, Japan) at 24, 48 and 72 h post-challenge. The increase in ear thickness was calculated by comparing the values after challenge with the values before challenge. The specificity of the response was determined by comparing the thickness increase in the left and right ears.

Tumor protection studies

In the prophylactic experiments, mice (seven per group) were challenged s.c. in the right flank with 2×10^5 D142.34 cells two weeks after the fourth immunization with 15 μ g of peptide (KLH-coupled or MAP). Tumor growth was monitored on a weekly basis by measuring the tumor volume (width $\times$ length $\times$ depth) using a digimatic caliper. Mice were euthanatized on day 55 and tumors were removed and weighed.

In the therapeutic experiments, C57BL/6 mice (7/group) were inoculated s.c. with 1.5×10^5 D142.34 (GD2⁺) tumor cells. Ten days later, mice were immunized s.c. four times at 2-week intervals with 50 μ g of GD2-mimicking peptide P10 or with control peptide S266, coupled to KLH and mixed with QS-21 adjuvant.

Statistical analysis

For the proliferation assays, data were expressed as mean values with standard deviations. Mean values were compared for validation of statistically significant differences using student's *t*-test, two-sided and tailed. The analysis of variance was used to compare experimental groups on percent specific lysis (CTL assay), tumor volume on day 55, and increase in ear thickness (DTH). Mouse-specific linear regressions were fit using the natural logarithm of tumor volume and day, and the slopes used to estimate tumor growth rates. The analysis of variance was used to compare slopes among the experimental groups. Kaplan-Meier survival curves were computed and used to estimate the median times to tumor detection. The log-rank test was used to compare the percentage of tumor-free animals. All in vitro determinations were performed in triplicates. All *P* values presented are for two-sided statistical tests. *P* values < 0.05 were considered significant. The analyses were done using SAS/STAT software, Version 9.1 of the SAS System for Windows.

Results

In vitro characterization of GD2-mimicking peptides

The 15mer phage library was panned on biotinylated mAb ME361 immobilized on streptavidin-coated polystyrene f100 mm Petri dishes. Phage in the eluates from the second and third rounds of affinity purification were cloned and propagated for sequencing. The peptides P9 and P10 were selected for additional studies since they inhibited the binding of mAb ME361 to GD2 (Fig. 1). The inhibition is specific since the S266 peptide derived from HIV (Table 1) showed a significantly ($P < 0.05$) lower inhibitory effect on the binding of mAb ME361 to GD2. As shown in Fig. 1, the peptide P10 showed a higher inhibitory activity than the peptide P9 and therefore may be a better mimotope.

GD2-specific antibody responses after immunization of mice with KLH-coupled peptides or MAP

Sera collected from peptide-immunized mice were assessed for their reactivity to GD2. Figure 2 shows the binding reactivity of pre- and post-immune sera from groups of ten mice each. Values from post-immune sera at least three times the values of pre-immune sera were scored as positive. Sera from the mice immunized with P9-KLH (Fig. 2a), P10-KLH (Fig. 2b), P9-MAP (Fig. 2d) or P10-MAP (Fig. 2e) showed substantial increase ($>3x$) in binding to GD2 as compared to preimmunized sera at most of the dilutions tested. The immune sera did not bind to GD1a (not shown). Furthermore, sera from mice immunized with the S266 control peptide did not bind significantly to GD2 as compared to buffer controls (Fig. 2c, f).

To determine whether sera from the mice immunized with the peptides P9-KLH and P10-KLH bind to GD2⁺ cells, the capacity of the immune sera to inhibit the binding of mAb ME361 to GD2⁺ D142.34 cells was determined. Sera from one of the ten mice immunized

with peptide P9-KLH and from five of the ten mice immunized with the peptide P10-KLH inhibited the binding of mAb ME361 to D142.34 cells by >15% at serum dilution of 1:3 (Fig. 3). The lack of inhibitory activity of the sera from the majority of the mice immunized with the P9-KLH peptide was surprising since the sera significantly bound to isolated GD2 (see Fig. 2a). The inhibition by the sera from P10-KLH immunized mice was specific, since sera from the mice immunized with the control peptide S266 had no detectable effect on the binding of mAb ME361 to D142.34 cells (Fig. 3e, f).

Absence of GD2-specific proliferative responses of splenocytes from mice immunized with P9 and P10 peptides

Proliferative responses of splenocytes from mice immunized with P9-KLH, P10-KLH, P9 MAP, or P10 MAP were measured after in vitro stimulation with GD2-mimicking peptides P9 and P10, control peptide S266, D142.34 cells (GD2⁺) or B78.H1 cells (GD2⁻ control cells). There were no GD2-specific responses when the splenocyte responses of a group immunized with GD2-mimicking peptide to various in vitro stimulants were compared or when comparing the responses of experimental and control-immunized groups.

DTH responses in mice immunized with P9 and P10 peptides

DTH responses to intradermal challenge with GD2⁺ D142.34 or GD2⁻ B78.H1 melanoma cells were evaluated in C57BL/6 (Fig. 4a–c) and C57BL/6 CD4 KO (Fig. 4d–f) mice following immunization of the mice with KLH-coupled GD2-mimicking or control peptides. Significant ($P < 0.001$) DTH responses were observed in P9-KLH and P10-KLH-immunized mice (Fig. 4a, b), but not in control peptide-immunized mice (Fig. 4c). In CD4 KO mice, P9-KLH peptide, but not P10-KLH peptide, elicited significant ($P < 0.01$) DTH response (Fig. 4d, e) suggesting that DTH responses elicited by P10-KLH peptide may be dependent on CD4⁺ T cells while those elicited by P9-KLH peptide may not.

Inhibition of tumor growth in mice immunized with P9 and P10 peptides

KLH-coupled and MAP peptides were evaluated for their capacity to inhibit D142.34 (GD2⁺) melanoma cell growth in mice (Fig. 5). Tumor volumes were measured on various days after tumor inoculation (Fig. 5a, b). In addition, mice were sacrificed on day 55, and tumors were excised and weighed. Tumor volumes were significantly ($P < 0.05$ and < 0.01) reduced between days 35 and 49 of measurements in mice immunized with P10-KLH peptide as compared to mice immunized with control peptide (Fig. 5a). This finding was reflected in significantly reduced tumor weights on day 55 in mice immunized with P10-KLH peptide as compared to both control mice and mice immunized with P10-KLH peptide (Fig. 5c). Tumor growth rates differed significantly ($P < 0.05$; Fig. 5a) between mice immunized with P10-KLH peptide as compared to S266-KLH peptide. Tumor growth inhibition by P9-KLH peptide was significant ($P < 0.05$) only on day 35; furthermore, no effect of immunization with this peptide was detected on tumor weight (Fig. 5c).

P10-MAP (Fig. 5b), similar to P10-KLH peptide (Fig. 5a), significantly ($P < 0.05$ and 0.01) inhibited tumor growth as determined on days 28, 35, 44 and 49. Tumor growth rates differed significantly (< 0.01 ; Fig. 5a) between mice immunized with P10-MAP as compared to S266-MAP. Although mean tumor weight on day 55 was reduced in mice immunized with P10-MAP as compared to control mice, this effect was not significant ($P > 0.05$), due to high S.D. of the mean in control mice (Fig. 5c). Following immunization with P9-MAP, tumor growth inhibition was apparent on day 49 only (Fig. 5b), but tumor weights on day 55 were not significantly ($P > 0.05$) different from those in control mice (Fig. 5c). Thus, by measuring tumor weights, which is considered more accurate than measuring volumes, P10-KLH peptide, of all four peptides tested, was the most effective in inhibiting tumor growth in the prophylactic setting. This peptide was therefore evaluated for its capacity to inhibit

growth of established D142.34 tumors. There was a marginally significant ($P = 0.077$) difference between the percentage of tumor-free mice in the group of mice immunized with P10-KLH peptide versus that in the group of mice immunized with the S266-KLH peptide (Fig. 6). When absolute tumor volumes were compared between the two groups, P values were between 0.07 and 0.85 on various days of measurements (not shown). The median times to tumor detection were 84 and 28 days in the groups of mice immunized with P10-KLH and S266-KLH peptides, respectively.

Discussion

We have shown here that peptides, isolated from a phage display peptide library by panning with the GD2-specific mAb ME361, induce humoral, cellular and tumor-protective immunity to GD2 and/or GD2-positive melanoma cells in mice. The peptides inhibited tumor growth in the prophylactic and, albeit marginally significant, also in the therapeutic setting. To the best of our knowledge, this is the first report demonstrating that immunization with GD2 mimicking peptides induces cellular and tumor protective immune responses in mice.

We have shown previously that immunization of mice with peptides mimicking tumor-associated carbohydrates other than GD2 can induce humoral, cellular and/or tumor-protective immune responses in mice. Thus, peptide mimics of difucosylated ganglioside elicited IgM and IgG antibodies that bound to synthetic and natively expressed Lewis^x related and Le^y structures and mediated complement-dependent cytotoxicity of human tumor cells [26, 33]. Furthermore, splenocytes from mice immunized with peptide mimics of sialyl-Lewis^x and sialyl-Lewis^a produced IFN- γ following in vitro stimulation with the peptide or sialylated Lewis^x [34]. A peptide mimicking O- β -linked *N*-acetylglucosamine and administered with IL-12 inhibited established tumor growth mediated by CD8⁺ T cells [36]. Recently, we have shown that immunization of mice with a mimic of Lewis^y and Lewis^b, selected based on its reactivity with *Griffonia simplicifolia* lectin I and wheat germ agglutinin induced serum IgM antibodies that mediated the apoptosis of mouse and human tumor cells in vitro. Vaccine-induced anti-carbohydrate antibodies reduced the outgrowth of micrometastases in the 4T1 spontaneous tumor model, significantly increasing survival time of tumor-bearing animals [37]. Other investigators have isolated peptide mimics of GD1a which inhibited GD1a-mediated adhesion of mouse tumor cells to endothelial cells and prevented metastasis formation [24].

We immunized mice with two GD2-mimicking peptides (P9 and P10). Each of the peptides was administered either as KLH conjugate or as MAP with QS-21 as adjuvant. Immunizations with MAP and QS-21 have been shown by us previously to induce carbohydrate-specific humoral, cellular and/or tumor-protective immunity in mice [11, 26, 34]. We were expecting the immune responses to GD2-mimicking MAP to be more specific for GD2 as compared to the responses to KLH-coupled peptides as KLH is expressing multiple potentially immunogenic and crossreactive epitopes [31]. However, antibody responses in both KLH-peptide and MAP-immunized mice were significant and specific for GD2 as they were absent in control mice (Fig. 2). MAP were more effective than KLH-coupled peptides in eliciting GD2-specific antibodies (Fig. 2), presumably because of the high copy number of peptides within the MAP complex, as compared to monomeric peptides coupled to KLH [14]. The DTH reaction elicited by peptides-KLH was specific for GD2⁺ cells and may be dependent on CD4⁺ cells in P10-KLH, but not in P9-KLH immunized mice (Fig. 4). The demonstration of a GD2-specific DTH response in the mice immunized with the GD2 mimicking peptides-KLH is in contrast to the absence of GD2-specific lymphoproliferative responses in these mice. This discrepancy may be explained by the higher sensitivity of the DTH assay as compared to the lymphoproliferation assay [12,

28]. Furthermore, the DTH reaction reflects the lymphocyte activity in vivo, whereas the proliferation assay measures the lymphocyte activity in vitro and therefore is more prone to artifacts.

The immune response elicited by P10 peptide more effectively inhibited tumor growth than that elicited by P9 peptide, both as MAP or KLH-coupled peptide (Fig. 5). This was evident both for tumor volume and tumor weight measurements. P10 peptide also was more potent in eliciting antibodies binding to GD2 on tumor cells (Fig. 3).

The safety and immunogenicity of KLH-coupled anti-idiotypic antibodies in cancer patients has been well established [4, 32], whereas there is no clinical experience with MAP. The results obtained with MAP in mice and humans predict that they should display a similar immunogenicity and safety in patients with malignant diseases. Preclinical immunopathology studies with P10-MAP do not show any evidence of autoimmunity or demyelization (Kieber-Emmons unpublished observations).

We can only speculate on the mechanism of tumor growth inhibition in vivo observed in mice immunized with the GD2-mimicking peptides. Antibodies may play an important role (Figs. 2, 3). MHC class I-restricted CTL most likely are not involved since tumor cell lysis by immune lymphocytes in vitro was not significant and the tumor cells are MHC class I-negative. It is possible that indirect mechanisms may have contributed to growth inhibition of the MHC class I and II-negative tumor cells in vivo. Thus, other investigators have described inhibition of growth of MHC class II-negative tumor cells in mice immunized with T helper (Th) peptides. In that study, Th cells most likely were activated by MHC-class II-positive APC at the tumor site, and the activated Th cells in turn stimulated a variety of non-specific effectors of the innate immune system, such as dendritic cells or neutrophils, leading to tumor cell lysis [38]. A similar mechanism may be operational in our study as we have shown that the peptides induced delayed-type hypersensitive T lymphocyte responses in vivo. DTH responses seen in P10-KLH-immunized mice were dependent on the presence of CD4⁺ T cells (Fig. 4), in agreement with other studies [16, 46]. These T cells may have stimulated non-specific effectors of the innate immune system [38] leading to tumor growth inhibition.

In conclusion, our study suggests that peptides mimicking GD2 ganglioside inhibit tumor growth through antibody and/or CD4⁺ T cell-mediated mechanisms. Our results provide

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Abbreviations

Ab2	Anti-idiotypic antibody
DTH	Delayed-type hypersensitivity
KLH	Keyhole limpet hemocyanin
MAP	Multiple-antigen peptides

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Fig. 1.

Inhibition of mAb ME361 binding to GD2 by peptides P9 and P10. ELISA plates were coated with 0.3 μg per well of GD2 dissolved in ethanol. Biotinylated mAb ME361 (0.15 μg/ml) was pre-incubated with various concentrations of peptides in PBS containing 1% BSA and 0.05% Tween-20 (PBS-T) overnight at 4°C. After blocking the wells with 2% BSA in PBS-T, the mixture of peptide and biotin-mAb ME361 (100 μl/well) were added and plates were incubated overnight at 4°C. HRP-Streptavidin was added and incubation was continued for 1 h at room temperature. The TMB substrate buffer was then added, and the reaction was developed for 15–30 min in the dark. The absorbance was read at 450 nm. Inhibition of biotin-mAb ME361 binding to GD2 by peptides was determined relative to the binding in the presence of buffer

Fig. 2.

Induction of GD2-specific antibodies in mice by immunization with peptide mimics of GD2. C57BL/six mice (ten per group) were immunized twice at 1-week interval with 15 μ g of peptides (P9, P10 or the control peptide S266) coupled to KLH (a–c) or as MAP (d–f) and formulated in 10 μ g of QS-21 adjuvant. Pre-immunization and post-immunization sera obtained 2 weeks after the last immunization were collected and stored at -70°C . MaxiSorp ELISA plates were coated with GD2 at 0.3 μ g/well in ethanol. Plates were blocked with 2% BSA in PBS containing 0.05% Tween 20, diluted sera were added and plates were incubated for 2 h at room temperature. To detect antibody binding to GD2, goat anti-mouse IgG antibody conjugated with alkaline phosphatase was added followed by the addition of substrate. Each value represents one mouse. *Asterisk* denotes mean post-immunization value that exceeds the pre-immunization value by factor >3. Horizontal lines represent means of ten mice per group. Sera did not significantly bind to GD1a (not shown)

Fig. 3.

Inhibition of mAb ME361 binding to GD2⁺ tumor cells by sera from mice immunized with peptides P9-KLH and P10-KLH. C57BL/six mice (ten per group) were immunized twice at 1-week interval with 15 μ g of peptides P9 or P10 coupled to KLH and formulated in 10 μ g of QS-21 adjuvant. Pre- and post-immune sera were collected before and 2 weeks after the second immunization and stored at -70°C . Tumor cells (5×10^5 /well) were incubated with diluted sera, washed, and incubated with biotinylated mAb ME361. Cells were washed and incubated with Extravidin-FITC. Staining was analyzed in the cytofluorograph and % inhibition of mAb binding by the sera was calculated. Each value represents one mouse. Horizontal lines represent means of ten mice per group

Fig. 4.

DTH responses in mice immunized with peptides P9-KLH and P10-KLH. C57BL/6 mice or CD4-knockout (KO) C57BL/6 mice (eight per group) were immunized twice at 1-week interval with 15 μ g GD2-mimicking peptides P9 or P10, or the control peptide S266 coupled to KLH and in QS-21 adjuvant. Two weeks after the last booster, irradiated (600 Gy, Cesium source) D142.34 (GD2⁺) or B78.H1 (GD2⁻) cells (5×10^5) in 10 μ l PBS were injected intradermally into the base of the left or right ear. The increase in ear thickness was measured with a digimatic caliper at 48 and 72 h post challenge. Increase in ear thickness was calculated by comparing the values after challenge with those before challenge. Values represent mean + SD

Fig. 5.

Effect of immunization with peptides P9 and P10 on D142.34 tumor cell growth in the prophylactic setting. C57BL/six mice (seven per group) were immunized s.c. four times at 2-week intervals with 15 μ g of GD2-mimicking peptides P9 or P10 or with the control peptide S266, coupled to KLH (a, c) or as MAP peptides (b, c) using QS-21 adjuvant. Two weeks after the last immunization, mice were inoculated s.c. with 2×10^5 D142.34 (GD2⁺) cells on day 0. Tumor cell growth was measured weekly (a and b). Tumor take ratios (number of animals with tumor to total number) for each group are indicated at each time point. Results are expressed as mean tumor volume + SD (** $P < 0.01$, * $P < 0.05$; a and b). Tumor growth rates differed significantly between mice immunized with P10-MAP as compared to S266-MAP ($P < 0.01$; b) and P10-KLH peptide versus S266-KLH peptide ($P < 0.05$; a). c Mice were euthanized on day 55 and tumors were removed and weighed

Fig. 6. Effect of immunization with peptide P10-KLH on D142.34 tumor cell growth in the therapeutic setting. C57BL/six mice (seven per group) were inoculated s.c. with 1.5×10^5 D142.34 (GD2⁺) tumor cells. Ten days after challenge, mice were immunized s.c. four times at 2-week intervals with 50 μ g of GD2-mimicking peptides P10 or with control peptide S266, coupled to KLH and mixed with 10 μ g of QS-21 adjuvant. Tumor growth was measured weekly. The percentage of tumor-free mice on various days of measurement is shown. There was a marginally significant ($P = 0.077$) difference between the percentage of tumor-free mice in the P10-KLH-immunized versus S266-KLH-immunized group

Table 1

Peptides used for immunization

Peptide	Phage ID	Sequence
9	ME361-posSEQ13	^a (C)LDVVLA WR ^b DGLSGAS
10	ME361-posSEQ30	(C)GVVWRYTAP V HLGDG
S266 (Control)		(C)IRIQRGPGRA F VTIG KI

^a A cysteine residue was added to the original sequence to facilitate conjugation with protein carriers

^b Aminoacids in bold denote potential anchor residues for MHC class I (H2D^b and H2K^b) of C57BL/6 mice (<http://Syfpeithi.bmi-heidelberg.com>)