Characterization of GD2 Peptide Mimotope DNA Vaccines Effective against Spontaneous Neuroblastoma Metastases

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Abstract
Disialoganglioside GD2 is an established target for immunotherapy in neuroblastoma. We tested the hypothesis that active immunization against the glycolipid GD2 using DNA vaccines encoding for cyclic GD2-mimicking decapeptides (i.e., GD2 mimotopes) is effective against neuroblastoma. For this purpose, two GD2 peptide mimotopes (MA and MD) were selected based on docking experiments to anti-GD2 antibody ch14.18 (binding free energy: −41.23 kJ/mol for MA and −48.06 kJ/mol for MD) and Biacore analysis (Kd = 12.3 × 10−5 mol/L for MA and 5.3 × 10−5 mol/L for MD), showing a higher affinity of MD over MA. These sequences were selected for DNA vaccine design based on pSecTag2-A (pSA) also including a T-cell helper epitope. GD2 mimicry was shown following transfection of CHO-1 cells with pSA-MA and pSA-MD DNA vaccines, with twice-higher signal intensity for cells expressing MD over MA. Finally, these DNA vaccines were tested for induction of tumor protective immunity in a syngeneic neuroblastoma model following oral DNA vaccine delivery with attenuated Salmonella typhimurium (SL 7207). Only mice receiving the DNA vaccines revealed a reduction of spontaneous liver metastases. The highest anti-GD2 humoral immune response and natural killer cell activation was observed in mice immunized with the pSA-MD, a finding consistent with superior calculated binding free energy, dissociation constant, and GD2 mimicry potential for GD2 mimotope MD over MA. In summary, we show that DNA immunization with pSA-MD may provide a useful strategy for active immunization against neuroblastoma. (Cancer Res 2006; 66(21): 10567-75)

Introduction
Development of an effective treatment against neuroblastoma is an important challenge in pediatric oncology. Neuroblastoma is the most common solid extracranial tumor in childhood. Over 50% of neuroblastoma patients initially present with disseminated stage IV disease characterized by dismal prognosis despite the introduction of novel therapeutic strategies, such as high-dose chemotherapy followed by autologous blood stem cell transplantation and differentiation therapy with retinoic acid (1).

Disialoganglioside GD2 is a well-established immunotherapeutic target to attack neuroblastoma because it is highly expressed on such neuroectodermal tumors as melanoma or neuroblastoma (2, 3). GD2 has been exploited for passive immunotherapy with two different monoclonal anti-GD2 antibodies (mAAb; i.e., the murine mAAb 3F8 and the human mouse chimeric mAAb ch14.18; refs. 4–7), which is entering a European phase III clinical trial (HR-NBL-1/ESIOP; ref. 8). To improve efficacy of passive immunotherapy directed against GD2, active vaccination was investigated using GD2-keyhole limpet hemocyanin (KLH) conjugate, anti-idiotypic antibody IAT7, and its encoding plasmid (9–12). Because the results of these studies revealed a moderate improvement of disease outcome, none of these active immunization approaches have entered extensive clinical application thus far. Here, we report a novel active immunization strategy applying advantageous aspects of DNA vaccination to a well-established antigen in neuroblastoma. DNA-based vaccines may provide a promising new approach to neuroblastoma therapy for several reasons. First, DNA activates the innate immune system because of unmethylated CpG motifs that function as vaccine adjuvants via stimulation of Toll-like receptor 9 (13, 14), which results in the release of such proinflammatory (TH1) cytokines as interleukin-12 (IL-12) and IFN-γ followed by increased MHC class I expression on neuroblastoma cells. Second, the versatility of DNA allows for coexpression of appropriate cytokines or chemokines as well as costimulatory molecules, which enhance the efficacy of antitumor immune responses (15). Third, DNA vaccines induce long-lived T cell–mediated tumor-protection memory immune responses; they are cost-effective and easy to handle (16–18).

One general obstacle to overcome in the generation of a vaccine inducing an anti-GD2 immune response is the antigen itself because GD2 is a glycolipid, which evokes poor immune responses and functions as a T cell–independent antigen (19). One possibility to involve T cells and provide for effective B-cell help followed by the generation of proinflammatory CD3+CD4+ T helper (TH1) cells is to change the nature of the antigen from glycolipid to peptide with a peptide mimotope approach (20). In previous work, we described for the first time the identification of 13 independent circular GD2 decapeptide mimotopes (21). This was accomplished by biopanning a phage-display library, presenting 1.04 × 109 different circular peptide sequences (22) with anti-GD2 mAAb ch14.18 followed by calculated docking experiments of peptide mimotopes to a GD2 Fab fragment ME36.1 (23). The efficacies of two mimotopes showing the best GD2 mimicry were shown to induce an anti-GD2 antibody IgG response in BALB/c mice (24). Similar work has been reported (25) on the identification of GD2 peptide mimotopes using a phage-display library presenting linear
peptides. The use of DNA vaccines encoding for these linear peptides was without determination of in vivo efficacy in an immunocompetent animal model. Here, we extend these findings and report on docking experiments to anti-GD2 antibody ch14.18 with new circular GD2 peptide mimotopes compared with the nominal antigen GD2. We show immunologic effects of these circular GD2 peptide mimotopes in vitro and in vivo in a syngeneic neuroblastoma mouse model.

We selected a nonreplicating strain of *Salmonella typhimurium* (SL 7207) as a vehicle for an oral DNA vaccine delivery (26–28). These attenuated bacteria provide a “danger signal” (29, 30), enhance antigen presentation, and promote a TH1 cellular immune response. We successfully used this vehicle to immunize against other neuroblastoma associated antigens (i.e., tyrosine hydroxylase; refs. 31, 32). Based on these considerations, we selected SL 7207 for oral vaccine delivery.

In the present study, we report the generation and characterization of DNA vaccines encoding the circular decapeptides mimicking the nominal structure of GD2. Selection of the peptide mimotopes for DNA vaccine design was based on novel results from calculated docking experiments to mAb ch14.18. After cloning of the mimotope sequences into psSecTag A, the constructs were analyzed for peptide expression and their potential of mimicking GD2. Then, the plasmids were evaluated to induce both a humoral and innate cellular immune response after a lethal challenge of neuroblastoma cells in a syngeneic mouse neuroblastoma model.

### Materials and Methods

#### Computer modeling

All template structures were identified for the generation of a model for the variable domain of ch14.18 mAb using the PSI-BLAST program (33). The amino acid sequence was that described previously (25). The heavy and light chains (VH and VL) were considered separately. Protein Data Bank (34) structure 1f3d was found to be the best template structure for the VH and 1jguf for the VL, resulting in 71% and 96% sequence identity, respectively. In contrast, the 1jg5 template previously used (25) shows an identity of only 67%. Homology modeling was done with the aid of the Swiss-PdbViewer (35). When necessary, loop structures were extracted from the LIP database (36). In a second step, the binding of GD2 to mAb ch14.18 was analyzed. The planar structure of GD2 was obtained to mAb ch14.18 was analyzed. The planar structure of GD2 was obtained (37). Three-dimensional conformers were generated using the software Catalyst (Accelrys Software, Inc., San Diego, CA). These were docked onto the antibody model with aid of GRAMM (38). Models for the linear and circular peptides MA (C-GRLKMVPDLE-C) and MD (C-DGGWLSKGSWC-C; ref. 21) were built using the Swiss-Pdb Viewer. We calculated the ranking according to GOLD score and the binding free energy of the interaction between the peptides (MA and MD) and the nominal GD2 antigen with mAb ch14.18 (39).

#### Peptide synthesis and mimine construction of GD2 mimicking DNA vaccines

Minigene encoding for GD2 mimicking peptides (Table 1), flanked by two (Gly3Ser)4 linkers and the T1 epitope, were created by overlapping PCR as previously described (32). PCR fragments were isolated and cloned into pGEM T easy vector following the manufacturers’ guidelines (Promega, Mannheim, Germany) and subjected to molecular sequencing. Correct inserts were subcloned into pSA using restriction enzymes HindIII/ XhoI. The decapetides MA and MD were synthesized, cyclized, and conjugated to KLH by JPT GmbH (Berlin, Germany).

#### Protein expression analysis

The expression of mimotope peptides was tested after transient transfection of the DNA vaccines (pSA-MA and pSA-MD) into CHO-1 cells using the Fugene 6 transfection reagent (Roche, Mannheim, Germany). After 72 hours, cells were lysed [0.1% SDS in PBS (pH 7.4), 4°C, 30 minutes] and centrifuged (10,000 × g, 4°C, 45 minutes). Lysates were separated by SDS-PAGE using the Novex system (Invitrogen, Karlsruhe, Germany), and blots were incubated with anti-myc and anti-His antibodies (Invitrogen) according to the manufacturer’s protocol. POD-labeled anti-mouse IgG antibody (Sigma, Steinheim, Germany) was added [1:10,000, 0.1% Tween 20, 5% milk, PBS (pH 7.4)]. The membrane was washed (3×, 15 minutes, room temperature), and signals were analyzed with the ECL Detection Reagents (Amersham, Munich, Germany).

#### Analysis of GD2 mimics

Mimicry analysis was accomplished following lysis of 1 × 106 transfected cells in 100 μL lysis buffer [SDS 0.1% in PBS (pH 7.4)]. After centrifugation (10,000 × g, 4°C, 45 minutes), 100 μL of the supernatant were dotted onto a nitrocellulose membrane by vacuum blotting using the Delrin dot blotting apparatus (Schleicher and Schuell, Kassel, Germany). The membrane was blocked [1% bovine serum albumine (BSA), TBS (pH 7.4)] and incubated (1 hour, room temperature) with anti-GD2 antibody (14G2a, BD Pharmingen, Heidelberg, Germany) at 1 μg/mL TBS (pH 7.4, 4°C). After washing, the membrane was incubated with POD-labeled anti-mouse IgG antibody (1:1,000; 8 μL, 1 hour, room temperature; Sigma). Signals were analyzed with the AEC developing kit (Vector Laboratories, Burlingame, CA). Signal intensity was measured with the GS-800 densitometer (Bio-Rad Laboratories GmbH, Munich, Germany). The adjusted absorbance of one dot was calculated as follows: mean absorbance inside the volume area multiplied with the dot area (mm2) minus adjusted absorbance of the membrane background (GS-800 software from Bio-Rad Laboratories). Each dot represents the adjusted absorbance of lysate prepared from 1 × 106 CHO-1 cells.

#### Surface plasmon resonance measurement

The affinities of the peptides to mAb ch14.18 were determined by surface plasmon resonance measurement in a BiaCore X-system (Uppsala, Sweden) in HBS buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% Surfactant P20]. Each cyclized peptide CDGGWLSKGSWC (peptide MD) and CGLRKMVPDLE (peptide MA) was immobilized on a CM5 sensor chip (measurement cell) via the amine coupling method as described by the producer. Irrelevant peptide (SATPWDLKTL) was attached with the same method and used in the reference cell. Affinity of chimeric anti-GD2 mAb ch14.18 to mimotope peptides was determined using a dilution series of mAb ch14.18 from 90 to 1.4 μmol/L (MD) and 46.6 to 1.5 μmol/L (MA). All binding experiments were executed at 25°C with a flow rate of 5 μL/min. Data were analyzed using the equilibrium method of the program BIAevaluation 3.0.

#### Cell culture and mice

The NXS2 murine neuroblastoma model was previously described (40). Syngeneic female A/J mice (8-10 weeks old) were obtained (Harlan Winkelmann, Borchcn, Germany) and housed according to the German guide for the care and use of laboratory animals (i.e., “Tierschutzgesetz”). Once liver metastases were detectable in control groups, mice were sacrificed, and spleenocytes were isolated and cultured in RPMI (10% FCS, 100 μg/mL penicillin-streptomycin, 50 μmol/L β-mercaptopetanol, 100 IU/mL IL-2) in the presence or absence of irradiated (50 Gy, 10 minutes) NXS2 neuroblastoma cells (1:100) for 7 days.

#### Immunization procedure and analysis of the tumor growth and metastasis

First, DNA vaccines were delivered to A/J mice (n = 6-8) every 2 weeks (3×–4×) by oral gavage of 1 × 106 *S. typhimurium* SL7207 carrying the mimigene vector constructs or empty vector controls. Peptide vaccines were given to mice by i.p. or s.c. injection with KLH-conjugated mimotope A and D (MA and MD; 10 μg/100 μL PBS) adsorbed to 100 μL Al(OH)3 (Injekt Alum, Pierce, Bonn, Germany) at identical time points (3×–4×; ref. 24). Second, peptide vaccination was applied as described above with or without the coadministration of DNA adjuvants (pSA empty vector delivered by attenuated *S. typhimurium*). Blood samples were taken at indicated time points: T0, 7 days before 1st immunization; T1 to T5 weekly after the immunization period. The induction of spontaneous liver metastases using 2 × 105 NXS2 cells s.c. or 1 × 105 NXS2 cells i.v. was done as previously described (40). Spontaneous liver metastases were analyzed by determination of liver weight.

#### Determination of the anti-GD2 serum response

Disialoganglioside GD2 (Sigma) was coated onto standard ELISA plates (96 wells, flat-bottomed; Greiner, Frickenhausen, Germany) at 50 ng/50 μL methanol per well. Plates were blocked with 1% bovine serum albumine (BSA)/PBS (pH 7.4, 2 hours, 37°C). Serum incubation was accomplished after four serial 1/2 dilutions (2 hours, 37°C). After washing [3×, PBS, 0.1% BSA (pH 7.4)], the secondary anti-mouse IgG peroxidase conjugate (Sigma) antibody was added (1:10,000, 2 hours, 37°C). Substrate development was induced after...
washing [3 ×, PBS, 0.1% BSA (pH 7.4)] using substrate reagent pack DY999 (R&D Systems, Minneapolis, MN) according to the manufacturer's guidelines. The reaction was stopped with 4 N H₂SO₄. Absorbance was determined in a standard ELISA reader at 450 nm. The adjusted absorbance was calculated as follows: adjusted absorbance = absorbance of T1-T5 / absorbance of T1.

**Cytotoxicity assays.** Lysis of NXS2- and YAC-1-target cells was determined in a standard 51Cr-release assay (8). Percent lysis was calculated as follows: percent lysis = (sample release / spontaneous release) / (maximum release / spontaneous release) × 100.

**IFN-γ secretion assay.** IFN-γ secretion was determined using the IFN-γ ELISA kit (BD OptEIA SET, BD PharMingen). Supernatants (300 μL) of splenocytes cocultured in the presence and absence of irradiated NXS2 cells were collected at several time points and stored at −80°C. On the day of measurement, 50 μL samples of the supernatant were analyzed in duplicates.

**Flow cytometry.** The percentage of tumor necrosis factor-α (TNF-α) and IL-10 producing CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells was determined by fluorescence-activated cell sorting analysis. Freshly isolated splenocytes were resuspended in DMEM (10% FCS) and treated for 3 hours with a cocktail of monensin (2 μmol/L), ionomycin (1 μg/mL), and phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich, Taufkirchen, Germany). Cells were fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich; 30 minutes, 4°C, dark). Cells were washed [1% BSA, 0.1% sodium azide, PBS (pH 7.4)] and incubated (4°C, 30 minutes) with antibodies directed against lymphocyte surface molecules (1:100): anti-CD3-PerCP, anti-CD4-FITC or anti-CD8a-FITC, anti-CD25-PE (BD Biosciences, Heidelberg, Germany). Cells were washed and permeabilized [PBS (pH 7.4), 0.1% saponin; Sigma-Aldrich]. Intracellular cytokines were determined with anti-TNF-α PE and anti-IL-10 PE antibodies (1:200, 20 minutes, 4°C, dark; BD Biosciences). Samples were analyzed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) equipped with "Cell Quest."

**Statistics.** Nonparametric data were analyzed by the Kruskal-Wallis test and the Mann-Whitney U test. Parametric data were analyzed with the Student’s t test. The data from pooled splenocytes, investigated by cytotoxicity assays and flow cytometry, were analyzed by the m² test. Differences were considered significant at P < 0.05.

## Results

**Selection of GD2 peptide mimotopes based on calculated binding interactions with mAb ch14.18.** The binding interaction of circular GD2 peptide mimotopes (MA and MD) and nominal

### Table 1. Potential contacts of MA, MD, and GD2 with the modeled anti-GD2 antibody ch14.18

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* Amino acids of decapeptides MA and MD were numbered 1 to 10.
† Carbohydrate residues of GD2 are GalNac 1, galactose 2, glucose 3, 2,3 NeuAc 4, 2,8 NeuAc 5 (8).
‡ Potential contacts between GD2 peptide mimotopes MA and MD and ganglioside GD2 with mAb ch14.18 variable light (VL) and heavy (VH) chain is assumed when the distance between any pair of heavy atoms is <4.5 Å.
§ Amino acids of VL and VH with potential contact to both mimotopes and GD2 are highlighted (gray).
GD2 with mAb ch14.18 was calculated based on a 4.5 Å criterion. For GD2, only docking results were considered, which allowed for localization of the fatty acid chains of glycolipid GD2 in the neuroblastoma cell membrane, and the best docking result is presented (Fig. 1A; Table 1). There are 15 amino acids responsible for the interaction of mAb ch14.18 with GD2. (Fig. 1A; Table 1), by which the majority interacts with VL. This is in contrast to the mimotope peptides, which interact with a similar number of amino acids of both chains (VL and VH). Eight residues of mAb ch14.18 were identified to interact with both peptides MA and MD as well as with GD2: Tyr37, His51, Ser96, Val99, Pro100, Leu102 (VL), Gly98, and Glu100 (VH; Table 1).

Docking experiments with circular peptides MA and MD (Fig. 1B; Table 1) resulted in lower calculated binding free energies of MA (−41.23 kJ/mol) and MD (−48.06 kJ/mol) compared with that of GD2 (−0.17 kJ/mol). Docking of the linear variants of MA and MD revealed higher free energy binding values (linear MA: 6.76 kJ/mol; linear MD: −6.82 kJ/mol), indicating that the circular peptides fit better into the binding pocket of mAb ch14.18. Circular MD showed a 15% lower calculated binding free energy (ΔΔE = 6.83 kJ/mol), suggesting a 15% higher affinity to ch14.18 than circular MA.

Finally, we determined the dissociation constant (Kd) among anti-GD2 mAb, ch14.18, and the synthetic cyclic GD2 peptide mimotopes MA and MD. Both peptides bound to mAb ch14.18; however, the capacity of MD to bind to mAb ch14.18 was three times higher than that of MA (Fig. 1C). Based on these findings, MA and MD were used for further in vitro and in vivo studies.

**Generation and characterization of DNA vaccines encoding GD2 peptide mimotopes.** To confirm results from computer modeling, a total of eight GD2 peptide mimotope sequences were selected (21) to generate DNA vaccines encoding for GD2 mimotope peptides by overlapping PCR (41). The DNA vaccines are based on pSecTag2 (pSA; Fig. 2A) and include a kappa leader sequence, a T-cell helper epitope from HIV-1 gp120 (42), and a mimotope DNA sequence. This is flanked by a linker sequence (GlySer)₄ and followed by myc and 6×his tags, respectively. The correct size of the insert (301 bp) was verified following restriction enzyme digests with HindIII and XhoI, and the integrity of the designed DNA was determined by molecular sequencing.

GD2 peptide mimotope expression induced by GD2 peptide mimotope DNA vaccines was analyzed by Western blotting in CHO-1 cells, which revealed characteristic bands of the mimotope peptides at the calculated size of 12 kDa with leader sequence and 10 kDa without it. This was in contrast to controls such as depicted exemplarily for GD2 peptide mimotopes MA and MD (Fig. 2B).

**Determination of GD2 mimicry.** To address the question whether the recombinant peptide derived from the generated DNA vaccines could mimic the structure of the nominal antigen GD2, the lysates of 1 × 10⁶ transfected CHO-1 cells were dotted onto a nitrocellulose membrane and probed with murine anti-GD2 mAb 14G2a. It is important to note that VL- and VH- protein sequences involved in GD2 binding of mAb 14G2a are identical to mAb ch14.18. The highest adjusted absorbance was found with the pSA-MD lysate, which was twice higher than the adjusted absorbance of pSA-MA lysate (Fig. 2C). Therefore, the pSA-MD construct was considered to provide for the best GD2 mimicry within. The lysate of GD2 positive M21 melanoma cells (1.25 × 10⁶) was used as a positive control.

In summary, we could clearly show mimicry of the nominal antigen GD2 using synthetic cyclic peptide MA and MD as well as...
lysates of CHO-1 cells transfected with GD2 peptide mimotope DNA vaccines pSA-MA and pSA-MD in vitro with a higher mimicry potential of MD and pSA-MD in contrast to that of MA and pSA-MA. Based on these findings, the efficacies of these GD2 peptide mimotopes were tested in a syngeneic model of murine neuroblastoma.

**Effect of GD2 peptide mimotope vaccination in vivo.** The effect of GD2 peptide mimotope vaccination was determined in a peptide and a DNA vaccination approach. Thus, we tested the KLH peptide mimotope conjugates versus the mimotope encoding plasmids in a spontaneous GD2-positive liver metastasis model using NXS2 neuroblastoma cells. Immunization of mice with the DNA vaccines encoding for MA or MD (pSA-MA and pSA-MD) was accomplished by their oral delivery using attenuated S. typhimurium (SL7207) as a carrier. Mice receiving these DNA vaccines were protected from spontaneous liver metastases in contrast to the control group (Fig. 3A) and revealed no macroscopic liver metastases in four of six animals in both DNA vaccine groups.

We could also show a 1.4- to 1.6-fold increase in the anti-GD2 serum response of pSA-MA or pSA-MD vaccinated mice already 1 week after the last immunization in a mouse model of murine neuroblastoma (Fig. 3B). The highest anti-GD2 response was observed in the pSA-MD group, consistent with a higher free binding energy and GD2 mimicry potential of MD over MA (Figs. 1 and 2). Interestingly, i.p. immunizations with mimotope peptides (MA and MD) conjugated to KLH adsorbed to Al(OH)3 did neither elicit a tumor protective immune response (data not shown).

To further investigate the absence of a vaccination effect in mice receiving the peptides, we addressed the question whether this is associated with the adjuvants or the route of peptide delivery. Therefore, we carried out a second set of peptide mimotope vaccination experiments using s.c. injections of MD conjugated to KLH adsorbed to Al(OH)3 (Fig. 4). Similar to results obtained with i.p. injection (Fig. 3), mice receiving MD peptide mimotope s.c.
showed an increased level in liver metastases (Fig. 4A), indicating that changing the route of application had no effect on peptide vaccine efficacy in this model.

However, coadministration of oral DNA (CpG-rich pSA empty vector delivered by attenuated SL7207) with s.c. injections of MD peptide reestablished vaccine efficacy as indicated by normal liver weights and the complete absence of macroscopic liver metastases (Fig. 4A). This finding was in contrast to untreated controls (Fig. 4A) and controls receiving pSA empty vector (data not shown), which all presented with liver metastases. The antitumor effect was accompanied by a strong increase in the anti-GD2 serum response over time in MD peptide immunized mice receiving the oral DNA adjuvants in contrast to controls (Fig. 4B), arriving at a maximum of a 4.5-fold increase (T<sub>5</sub>). These data are in line with the previously described induction of a humoral immune response by mimotope peptide immunizations in BALB/c mice (24).

In summary, oral GD2 mimotope DNA vaccination is more efficient than the GD2 mimotope peptide vaccination to protect the A/J mice against spontaneous neuroblastoma liver metastases (Fig. 3). This contention was further strengthened by the beneficial adjuvant effect of oral DNA also in combination with a peptide vaccination approach (Fig. 4).

**Mechanisms of GD2 peptide mimotope DNA vaccine induced anti-neuroblastoma immune responses.** To evaluate the mechanisms of antitumor immune responses observed in the DNA vaccination approach, we looked for an anti-GD2 humoral

![Figure 3. Effects of DNA versus peptide vaccination with plasmids encoding for GD2 mimotopes and synthetic GD2 peptide mimotopes in a syngeneic neuroblastoma model. Mice were immunized either by oral gavage of pSA-MA– and pSA-MD–transfected attenuated S. typhimurium or by i.p. injection of KLH-conjugated synthetic GD2 peptide mimotopes (MA and MD) adsorbed to Al(OH)₃. All mice (control, n = 6; experimental groups, n = 8) were challenged by s.c. injection of NXS2 neuroblastoma. A, spontaneous liver metastasis was determined by measurements of liver weights of fresh specimens. Columns, mean; bars, SE. *, P < 0.05. The horizontal line indicates the liver weight of mice without metastases (1.2 g). Photographs of one representative liver specimen from each group of mice. B, blood samples taken one week before the 1st immunization and 1 week after the last immunization were analyzed by GD2 ELISA. Results of the anti-GD2 serum response following DNA vaccination. Columns, mean adjusted absorbance values; bars, SE. *, P < 0.05 (Student’s t test).
production of circulating CD4+ and CD8+ T cells suggest a TH1 bias in favor of DNA vaccinated mice (Fig. 5C). Specifically, in groups of mice immunized with the GD2 peptide mimotope DNA vaccines (pSA-MA and pSA-MD), we could show an increase in CD3+CD4+ and CD3+CD8+ T-cell populations in contrast to control mice. This increase was accompanied by an increase in the production of TNF-α and a decrease in the expression of IL-10 by these T-cell populations (Fig. 5C). These findings suggest the induction of both a humoral and an innate cellular immune response following GD2 peptide mimotope DNA vaccination.

Discussion

The induction of tumor-protective immunity against neuroblastoma by using disialoganglioside GD2 as a target is an important goal in pediatric oncology. To achieve this, we were first to change the nature of the GD2 antigen from glycolipid to peptide to strengthen its antigenicity. This was accomplished by identification of circular GD2 peptide mimotopes (21), which showed efficacy to induce a humoral anti-GD2 IgG response in BALB/c mice (24). In an alternative approach, linear peptides that also mimicked the structure of GD2 were identified (25). Efficacies of DNA vaccines encoding for these linear peptides were shown by reduction of primary tumor growth in a melanoma xenograft model. Here, we extend these findings reporting the efficacy and mechanisms of GD2 peptide mimotope DNA vaccines against neuroblastoma metastases in a syngeneic immunocompetent mouse model.

For this purpose, we generated DNA vaccines encoding circular decapeptides mimicking the nominal structure of GD2. We first designed a model to measure the free energy of binding of the nominal glycolipid antigen GD2 to anti-GD2 mAb ch14.18 to establish a baseline for further mimotope selection (Fig. 1). In such docking experiments, we confirmed the majority of amino acids of mAb ch14.18 that are essential for binding of GD2 mimotope peptides (25) with some distinct differences (Table 1). Our model identified Asn35, Tyr41, Pro100, and Leu102 of VL as well as Gly31, Asn33, Trp47, Ala50, Thr58, and Ser97 of VH to be involved in peptide binding, but not Arg32, Pro101 (VL), and Pro53 (VH). All other 16 amino acids were identical. Interestingly, these docking experiments yielded a lower calculated binding free energy for MD than that of MA, suggesting a higher affinity of MD to mAb ch14.18 than that of MA. This result was confirmed by Biacore experiments. Finally, DNA vaccines encoding GD2 peptide mimotope MD achieved higher anti-GD2 antibody levels in vivo than those encoding mimotope MA. This further strengthens the contention of peptide mimotope docking to mAb ch14.18. Importantly, the circular structure of the decapeptides induced by the flanking cysteine residues is crucial for GD2 mimicry. This notion is supported by an increase in the free energy of binding attained by the linear versions of MA and MD, as well as by results from Biacore experiments. In fact, neither linear MA nor linear MD peptides were able to bind to mAb ch14.18 (data not shown).

Based on these results, the sequences of MA and MD were selected for the design of a DNA vaccine similar to the design of a DNA vaccine encoding for carbohydrate peptide mimotopes (42, 43). In these reports, the genetic linkage of the LeY mimotope to the T1 epitope of HIV gp120 is important for induction of an anti-LeY immune response. Based on these findings, we also fused the DNA sequence of the T1 epitope gene with the GD2 mimotope (21), which showed efficacy to induce a humoral anti-GD2 IgG response in BALB/c mice (24). In an alternative approach, linear peptides that also mimicked the structure of GD2 were identified (25). Efficacies of DNA vaccines encoding for these linear peptides were shown by reduction of primary tumor growth in a melanoma xenograft model. Here, we extend these findings reporting the efficacy and mechanisms of GD2 peptide mimotope DNA vaccines against neuroblastoma metastases in a syngeneic immunocompetent mouse model.

Figure 4. Effect of coadministration of oral DNA adjuvants in GD2 peptide mimotope–vaccinated A/J mice. Mice were immunized by s.c. injection of KLH-conjugated synthetic GD2 peptide mimotopes (MD) adsorbed to Al(OH)3 with and without coadministration of oral DNA (pSA empty vector delivered by attenuated S. typhimurium; pSA). All mice (control, n = 6; experimental groups, n = 8) were challenged by i.v. injection of NXS2 neuroblastoma. A, spontaneous liver metastasis was determined by measurements of liver weights of fresh specimens. Columns, mean; bars, SE. *, P < 0.05. The horizontal line indicates normal liver weights of mice without metastases (1.2 g). Representative liver photographs from each group of mice. B, the course of anti-GD2 serum responses in peptide-vaccinated mice with and without oral application of oral DNA (pSA). Samples were taken on time points indicated by an arrow. Results (mean) are illustrated as x-fold increase in contrast to the preimmunization anti-GD2 serum level (T1). *, P < 0.05 (Student's t test). T = time point.

response (Fig. 3B and Fig. 4B) and determined natural killer (NK) cell activation and IFN-γ secretion and analyzed the cytokine expression profile in T-cells of immunized mice (Fig. 5). NK cell activity increased in mice vaccinated with pSA-MD as indicated by elevated lysis of YAC-1 target cells (Fig. 5A). In contrast, effector cells were ineffective in NXS2 target cell lysis, suggesting the absence of a CD8+ T-cell immune response. Interestingly, YAC-1 target cell lysis was only observed with effector cells, which were cocultured with irradiated NXS2 cells. Similarly, the secretion of IFN-γ was increased in the pSA-MD–vaccinated group of mice in contrast to the control group (Fig. 5B). Finally, the cytokine
The proof of concept of DNA vaccines encoding GD2 peptide mimotopes was investigated in a syngeneic model of neuroblastoma, which compared DNA vaccination with a peptide vaccination approach. In this model, the level of spontaneous liver metastases was decreased in the DNA-vaccinated mouse groups (Fig. 3). The mechanisms involved in induction of an anti-neuroblastoma immune response following GD2 peptide mimotope DNA vaccination revealed an anti-GD2 serum response combined with NK cell activation assuming a role for antibody-dependent cellular cytotoxicity. As expected, we could not show a cellular immune response mediated by CD8+ T cells. This is indicated by the absence of NK cell activation in the absence of irradiated NXS2 cells (data not shown). It is unclear at this stage whether NK-T cells are also involved in the anti-neuroblastoma immune response following treatment with the GD2 peptide mimotope DNA vaccine. Only coculturing effector cells from such vaccinated mice with irradiated NXS2 cells (Ag) resulted in the activation of NK cell function (Fig. 5). A role for antigen-specific NK-T cell activation possibly mediated by a CD1-restricted antigen presentation (44) and subsequent secretion of large amounts of IFN-γ upon activation (Fig. 5) needs further investigation (45), also including the possibility of involvement of different T-cell subpopulations. Interestingly, control groups immunized with KLH-coupled mimetic peptides did not show protection against metastasis, consistent with the absence of a GD2-specific IgG antibody response. This is an interesting difference to our previous results of successful GD2-specific IgG induction by i.p. peptide immunization in BALB/c mice (24), possibly due to immunologic differences of the inbred mouse strains. The observation of increased liver metastases (Fig. 3) in mice immunized with KLH-coupled mimetic peptides suggests the induction of tolerance. The mechanism of tolerance induction in this model is unclear and may be related to poor antigenicity of the distinct peptides used. Importantly, the efficacy of the peptide vaccination approach was established by simultaneous oral delivery of DNA (pSA empty vector) using attenuated Salmonella SL7207 (Fig. 4). Here, we could show that the combination of oral DNA delivery with peptide vaccination can switch tolerance to immunity. Therefore, the effective anti-neuroblastoma immune response induced by GD2 peptide mimotope DNA vaccines is most likely related to the use of the carrier system (32). Thus, the bacterial membrane contains LPS, which together with unmethylated CpG motifs in the bacterial DNA stimulates two different Toll-like receptors (TLR-4 and TLR-9), leading to a TH1 shift and effective costimulation of adaptive immunity (49). We could indeed show such a TH1 shift for CD4+ and the CD8+ T-cell populations (Fig. 5C), emphasizing the advantage of attenuated S. typhimurium as a DNA vaccine carrier.

In summary, we describe the efficacy of novel DNA vaccines encoding GD2 peptide mimotopes and their immunologic effects in a syngeneic model of neuroblastoma. The vaccines induce an

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**Figure 5.** Immune response following vaccination with DNA vaccines encoding GD2 peptide mimotopes. Mice were immunized with the appropriate DNA vaccines and then subjected to the analysis of NK cell activation, release of IFN-γ, and intracellular cytokine expression of T-cell subpopulations. All mice (control, n = 6; experimental groups, n = 8) were challenged with NXS2 cells. A, NK cell activation was determined with pooled splenocytes of mice immunized with pSA-MD (■ and □) and pSA (○ and △) control at varying effector to target cell ratios (1 = 100:1, 2 = 50:1, 3 = 25:1, 4 = 12.5:1) 7 days after coculture in the presence (■ and ○) and absence (□ and △) of irradiated NXS2 cells. *, P ≤ 0.05 (Mann-Whitney). B, IFN-γ-secretion from splenocytes of mice immunized with GD2 peptide mimotope DNA vaccine pSA-MD was determined over time in the presence of irradiated NXS2 cells. *, P ≤ 0.05 (χ² test). C, intracellular cytokine expression was determined in T-cell subpopulations. TNF-α and IL-10 expression was analyzed in CD4+ and CD8+ T cells of vaccinated mice. The percentages of CD4+ or CD8+ T cells as well as the percentages of TNF-α and IL-10 producing CD3+CD4+ or CD3+CD8+ T cells were calculated relative to the total number of CD3+ T cells. *, P ≤ 0.05 (χ² test). ND, nondetectable.
anti-GD2 humoral immune response in combination with activation of NK cells. These findings provide an important baseline for the further development of this immunotherapeutic approach for the treatment of neuroblastoma.

Acknowledgments

References

Characterization of GD2 Peptide Mimotope DNA Vaccines Effective against Spontaneous Neuroblastoma Metastases

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