available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Systematic amino acid substitutions improved efficiency of GD_2-peptide mimotope vaccination against neuroblastoma

Matthias Bleeke[a,e], *Stefan Fest*[a,b,c,e], *Nicole Huebener*[a], *Christiane Landgraf*[d], *Burkhart Schraven*[c], *Gerhard Gaedicke*[d], *Rudolf Volkmer*[d,*], *Holger N. Lode*[a,*]

[a]*Charité – Universitätsmedizin Berlin, Department of Pediatrics, Experimental Oncology, Berlin, Germany*
[b]*Otto-von-Guericke University of Magdeburg, Faculty of Medicine Magdeburg, Department of Pediatrics, Magdeburg, Germany*
[c]*Otto-von-Guericke University of Magdeburg, Faculty of Medicine Magdeburg, Institute of Molecular and Clinical Immunology, Magdeburg, Germany*
[d]*Charité – Universitätsmedizin Berlin, Department of Medical Immunology, Berlin, Germany*

ARTICLE INFO

Article history:
Received 18 March 2009
Received in revised form 14 July 2009
Accepted 22 July 2009
Available online 18 August 2009

Keywords:
Immunotherapy
Neuroblastoma
Ganglioside
Mimotope
SPOT

ABSTRACT

The likelihood of identifying peptides of sufficient quality for the development of effective cancer vaccines by screening of phage display libraries is low. Here, we introduce the sequential application of systematic amino acid substitution by SPOT synthesis. After the substitution of two amino acids within the sequence of a phage display-derived mimotope of disialoganglioside GD_2 (mimotope MA), the novel mimotope C3 showed improved GD_2 mimicry *in vitro*. Peptide vaccination with the C3 mimotope induced an 18-fold increased anti-GD_2 serum response associated with reduction of primary tumour growth and spontaneous metastasis in contrast to MA mimotope controls in a syngeneic neuroblastoma model. In summary, SPOT provides an ideal optimisation tool for the development of phage display-derived cancer vaccines.

1. Introduction

The translation of carbohydrates or glycolipids into peptide surrogates by application of phage display libraries is an established tool to overcome the weak antigenicity of the nominal antigens. The generated peptides are mimotopes of the nominal antigen and their protective efficacy as vaccines against infective agents, including bacteria and viruses, as well as against cancer was demonstrated.[1–3] However, the application of phage display libraries is restricted by the technically limited number of actually expressed and screened peptide antigens and may therefore fail to identify the most efficient peptide mimotope.[4–6] We addressed the question if optimisation of a phage display-derived mimotope can be carried out by systematic alteration of the amino acid sequence resulting in improved antibody binding and vaccine efficacy. Here, we establish proof of concept that such an optimisation procedure is effective for a peptide mimotope of disialoganglioside GD_2 (mimotope MA) used as a vaccine against neuroblastoma.

Ganglioside GD_2 is an established target for immunotherapy against neuroblastoma, the most common extracranial solid tumour during early childhood. Infusions of monoclonal anti-GD_2 antibodies (mAbs), namely chimeric mAb ch14.18

* *Corresponding authors:* Address: Forum 4, R 2.0407, Augustenburger Platz 1, 13353 Berlin, Germany. Tel.: +49 (0) 30 450 666233; fax: +49 (0) 30 450 559917 (H.N. Lode). Address: Institut für Medizinische Immunologie, Abt. Molekulare Bibliotheken, Hessische Str. 3-4, 10115 Berlin, Germany. Tel.: +49 (0) 30 450 524267; fax: +49 (0) 30 450 524942 (R. Volkmer).
E-mail addresses: rve@charite.de (R. Volkmer), holger.lode@charite.de (H.N. Lode).
[e] Equally contributed to this paper.
0959-8049/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2009.07.026

and murine mAb 3F8, could improve the outcome of stage 4 patients.[7–10] Active vaccination with GD_2 encounters various obstacles including poor antigenicity and T-cell independency of glycolipid antigens.[11,12] In order to overcome this problem, GD_2-peptide mimotopes were identified by application of phage display libraries.[13–15] All these mimotopes were able to induce immune responses against GD_2-positive tumours, such as neuroblastoma and melanoma.[16–18]

In order to improve GD_2 mimicry of mimotopes phage display may be supported by sequential application of the SPOT technology,[19] which is based on synthesis of a defined peptide library onto a solid phase (reviewed in[20,21]). Therefore, our objective was to apply SPOT synthesis on a recently reported GD_2-peptide mimotope[16] by systematic replacement of each amino acid within the peptide mimotope sequence by all other genome-encoded amino acids.

Here, we present for the first time that amino acid substitution analysis carried out by SPOT synthesis for a phage display-derived mimotope of disialoganglioside GD_2 enhances the mimicry potential *in vitro* and improves anti-neuroblastoma efficacy of the optimised mimotope in a syngeneic murine model of neuroblastoma. Our results indicate that SPOT is a useful optimisation tool for vaccines generated from phage display-derived peptide mimotopes.

2. Materials and methods

2.1. Amino acid substitution analyses

Systematic alteration of the amino acid sequence and subsequent probing for GD_2 antibody binding were performed by SPOT synthesis and anti-GD_2 mAb ch14.18 binding assay on the SPOT membranes as described below.

2.2. SPOT synthesis

Peptide syntheses on a cellulose membrane were performed according to a standard SPOT synthesis protocol.[21] Briefly, peptides were synthesised on a β-alanine prepared membrane using the MultiPep SPOT-robot (INTAVIS Bioanalytical Instruments AG, Köln, Germany). Array design was performed with the aid of the in-house software LISA 1.82. Synthesis started with the definition of spots (ß-alanine, two cycles, double coupling, 15-min reaction each) followed by standard SPOT synthesis of the desired peptides. Solutions of Fmoc-amino acid-Opfp esters (Fmoc = fluoromethoxycarbonyl, Opfp = pentafluorophenyl) in N-methylpyrrolidone were used. After completion of peptide synthesis, cleavage of side chain-protecting groups was achieved through treatment with a mixture of trifluoroacetic acid (90% TFA w/v), tri-isobutylsilane (3%, TIBS w/v) and H_2O (2% v/v) in dichloromethane (DCM) for 1 h. Subsequent washing was done with DCM (3 × 3 min), dimethylacetamide (DMA 3 × 3 min), EtOH (3 × 3 min) and diethyl ether (2 × 3 min), followed by TFA (60% w/v), TIBS (3% w/v) and H_2O (2% v/v) in DCM for additional 2.5 h. Finally the membrane was washed with DCM (3 × 3 min), DMA (3 × 3 min), EtOH (2 × 3 min), phosphate buffer (pH 7.4, 0.1 m, 2 × 3 min), H_2O (2 × 3 min), EtOH (2 × 3 min) and diethyl ether (2 × 3 min) and dried.

2.3. Anti-GD2 mAb ch14.18 binding assay on the cellulose membrane

The membrane-bound GD_2 mimotope MA wild type and substitution variants were washed with ethanol and thrice (10 min, RT) with Tris-buffered saline (TBS, pH 8) containing 0.05% v/v Tween (T-TBS). Then, the membrane was blocked for 3 h with blocking buffer (10% blocking reagent v/v (CRB, Norwich, Great Britain) and 1% sucrose in 1: 10 TBS) and subsequently incubated with the chimeric anti-GD_2-mAb ch14.18 (1 μg/ml) in the same blocking buffer at 4 °C for 14 h. After washing (3×, T-TBS), the membrane was incubated with a peroxidase-conjugated anti-human IgG mAb (Sigma–Aldrich, Steinheim, Germany, 1 μg/ml). The membrane was washed again. Antibody binding was visualised using a chemiluminescence substrate (Pierce, Rockford, IL, USA) and the Lumi-Imager™ (Roche Diagnostics, Mannheim, Germany). Analysis and quantification of spot signal intensities were executed with the software Genespotter (MicroDiscovery GmbH, Berlin, Germany). Genespotter has a fully automatic grid finding routine resulting in reproducible signal intensities. The spot signal is calculated from a circular region around the spot centre detected on the image. The background signal for each spot is determined with a safety margin to this circular region.

2.4. Peptide synthesis

For further analyses selected peptides were synthesised by standard solid phase peptide synthesis on TentaGel S Ram resin (Rapp Polymere, Tübingen, Germany) using a multiple peptide synthesiser (Syro II, MultiSynTech, Witten, Germany). Synthesis was performed according to standard Fmoc-chemistry and PyBOP activation for all amino acids (2-fold coupling). Peptides were purified to >95% by preparative HPLC. Analytical HPLC analysis (Waters, Milford, MA, USA) was carried out using a linear gradient of solvents: A, 0.05% TFA (v/v) in water and B, 0.05% TFA (v/v) in acetonitrile; gradient 5–60% over 30 min. HPLC conditions: UV detector 214 nm, RP-18 column. Peptide identity was determined by ESI mass-spectrometry (Q-TOFmicro™, Waters, Milford, MA, USA). For *in vivo* experiments, peptides MA and C3 were synthesised and coupled to keyhole limpet haemocyanin (KLH) by Jerini Peptide Technologies (Berlin, Germany).

2.5. Surface plasmon resonance measurement

The affinities of the mutated peptide mimotopes to the ch14.18 mAb were determined by surface plasmon resonance measurement in a BiacoreX-system (Biacore, Uppsala, Sweden) in HBS buffer (10 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% (v/v) Surfactant P20). Analyses were carried out in two rounds. In the first round of measurements the ch14.18 mAb was immobilised on a CM5 sensor chip (measurement cell) via the amine coupling method as described by the producer. An irrelevant antibody (anti-GST-IgG) was used in the reference cell. Affinity of the peptide mimotopes was determined using a dilution series of cyclic and linear decapeptides from 2 mM to 4 μM. In the second round the peptide mimotope with the highest affinity to the ch14.18 mAb of round one (i.e. 'C3') and both of the original