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GD3-replica peptides selected from a phage peptide library induce a GD3 ganglioside antibody response

Iuliana Popa[a,c,1], Dai Ishikawa[b,*,1], Michinori Tanaka[b], Koichi Ogino[b], Jacques Portoukalian[a], Takao Taki[b]

[a] *INSERM U.346, Edouard Herriot Hospital, Lyon, France*
[b] *Molecular Medical Science Institute, Otsuka Pharmaceutical Co. Ltd., 463-10 Kagasuno, Kawauchi, Tokushima-shi, Tokushima 771-0192, Japan*
[c] *Institute of Molecular Chemistry "Petru Poni" Iasi, Romania*

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Abstract GD3-replica peptides were obtained from a phage peptide library and an anti-GD3 monoclonal antibody (Mab) (4F6), and anti-GD3 Mabs were generated by immunizing a peptide GD3P4. A Mab, 3D2 was found to recognize GD3 by immunohistochemical approaches. Amino acid analysis of heavy and light chain variable regions of 4F6 and 3D2 showed that the respective chains had the same length, and only a few different amino acid substitutions were found. The present data indicate that the immunogenic GD3P4 is processed in a certain size and exposed on the antigen-presenting cells with a molecular shape quite similar to that of the GD3 epitope in 4F6.

Keywords: GD3-replica peptide; Antibody response; Antigen presentation

1. Introduction

Carbohydrates on tumor cell surfaces have been described as tumor-associated antigens [1], and in particular, the presence of disialogangliosides GD3 and GD2 has been well documented in melanoma, small cell lung carcinoma, neuroblastoma, and glioma [2–5], raising the possibility of immunotherapy using the tumor-associated gangliosides as target antigens [6–8]. Some monoclonal antibodies (Mabs) specific for these gangliosides have been reported as anti-tumor drugs that led to suppression of cell growth and induction of apoptosis of human tumor cells [9–11].

When anti-idiotypic (Id) antibodies against anti-ganglioside Mab are injected in animals, some anti-Id antibodies recognize the original antigen gangliosides and such anti-Id antibodies are good candidates for vaccine therapy [12–14]. However, the whole anti-Id antibodies contain extra motifs, except in the idiotope (antigen-mimicking domain) that may account for the unexpected allergic reactions that are often observed when anti-Id antibodies are administrated. This problem could be overcome if the idiotopes are replaced by oligopeptides.

We have tried to prepare peptides that mimic glycosphingolipids using Mabs against glycosphingolipids using a phage peptide library [15,16]. And the obtained peptides showed function similar to the antigen glycosphingolipids, such as inhibitory effects on glycosidase [15], adhesion activity of tumor cells to endothelial cells and tumor growth in in vivo study [16]. In this study, we proposed to call glyco-replica peptides to these peptides with similar functions with original glycosphingolipids [16]. The possibility to produce peptides that can mimic the carbohydrate structure in terms of molecular shape is one of great interests. If this working hypothesis is correct, this phenomenon would explain a possible mechanism of autoimmune disease such as Guillian Barre Syndrome and other neuropathies. Another point is a production mechanism of antibody against glycoconjugates via glyco-replica peptide like process.

In these respects, in the present study, we have prepared GD3-replica peptides using a phage peptide library and the anti-GD3 Mab 4F6 [17]. The peptides thus obtained were shown to induce antibodies recognizing GD3 following immunization of mice with multi-antigen peptides (MAPs).

2. Materials and methods

2.1. Materials

The pentadecamer random peptide library displayed on filamentous phage (fd phage) surface protein (pIII) and the host bacterial strain *Escherihia coli* K91Kan were provided by Dr. Y. Saya, Kumamoto University, Kumamoto, Japan [18]. The phage library contains approximately 1.5×10^8 individual peptides.

MAPs were synthesized from MAP resin (Watanabe Chemical Co., Japan) using a peptide synthesizer ACT357 (Advanced Chem.-Tech). Horseradish peroxidase (HRP)-conjugated antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse Monoclonal Antibody Isotyping Test Kit was purchased from DAINIPPON Pharmaceutical Co. Ltd. (Osaka). KOD plus was purchased from TOYOBO (Tokyo) and oligonucleotides mixture for cloning of mouse immunoglobulin heavy chain was from Amersham Biosciences (Piscataway, NJ). The other oligonucleotides were purchased from NIHON BIO SERVICE (Saitama, Japan).

*Corresponding author. Fax: +81 88 665 5662.
E-mail address: d_ishikawa@research.otsuka.co.jp (D. Ishikawa).

[1] Authors equally contributed to this work.

Abbreviations: Mab, monoclonal antibody; MAP, multi-antigen peptide; VSSP, very small size protoliposomes; ITLC, immunostaining on thin-layer chromatography plate; TMB, tetramethyl benzidine; CDR, complementarity-determining regions; V_H, heavy chain variable region; V_L, light chain variable region

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2.2. Selection of GD3-replica peptides (biopanning)

Biopanning used here was performed as described elsewhere [15]. Briefly, a phage library (6.2×10^{10} titers) was subtracted by incubation with mouse immunoglobulin-conjugated protein A-Sepharose beads and the non-bound phage clones were incubated with 4F6-beads. After which, the bead-bound phage clones were amplified by infection to host bacterial cells. Experiments following the described above repeated three times. After the third round of biopanning, 30 phage clones were randomly picked up and amplified. The amino acid alignments inserted in pIII protein of the phage clones were determined by DNA sequence analysis. The classified phage clones were subjected to phage ELISA.

2.3. DNA sequence

Each single strand DNA from the amplified phage clones was analyzed by dideoxynucleotide chain termination. An oligonucleotide 5′-TAACACTGAGTTTCGTCACCAGTA was used as an anti-sense primer.

Both heavy chains and light chains of Mab were amplified by RT-PCR from total RNA obtained from hybridoma by a QIAGEN RNA-Easy Kit. The amplified fragments were inserted into pT7 Blue3 blunt vector (Novagen) and subjected to DNA sequence. T7 primer, U19 primer, and oligonucleotides shown in Figs. 3 and 4 were used for the sequencing reaction.

2.4. Generation of hybridoma producing antibody against GD3

MAPs in a buffered solution of very small size protoliposomes (VSSP) particles (kindly provided by Dr. Luis Fernandez [19], Center of Molecular Immunology, Havana, Cuba) and Montanide ISO 51 were mixed to form an emulsion. Three CD-1 mice were immunized by injection i.p. of emulsion containing 30 μg of MAPs. The schedule of immunization was one injection every two weeks for two or six months. The sera were assayed by ELISA for their capacity to bind the peptides and GD3. The spleen cells of the mice whose sera reacted the strongest with GD3 were fused with mouse myeloma SP2 cells using polyethylene glycol. Hybridoma fusions were screened against GD3. The positive clones were grown and expanded for mass production. All cells used in the present experiments were cultured in RPMI 1640 medium containing 10% FBS.

2.5. ELISA

The phage clones, MAPs, and gangliosides were used as antigens in ELISA. The individual phage clones (10^{10} titers/well), MAPs (100 ng/well), and gangliosides (1 μg/well) were fixed on 96 well micro titer plates (Maxisorp, Nunc). After blocking the plates with blocking buffer (PBS containing 1% BSA with or without 0.02% triton X-100), antibody-containing samples were added to each well. After 2 h incubation, each plate was washed and subjected to 1 h incubation with second antibodies bearing HRP. Visualization was done with tetramethyl benzidine (TMB) microwell peroxidase substrate (KPL, Gaithersburg, MA). Absorbance was measured at 450 and 620 nm with an ELISA reader.

2.6. FACS

Supernatants of the hybridoma cells were incubated with human melanoma cell line, SK MEL-28 cells at 4 °C for 30 min. After washing the cells, FITC-labeled second antibodies were incubated at 4 °C for 30 min. Fluorescence intensity of cell surface was analyzed by FACS. FITC-labeled anti-mouse IgG_3 or FITC-labeled anti-mouse IgG_1 were used as second antibodies and mouse IgG_1 and mouse IgG_3 were used as negative controls.

2.7. Immunohistochemistry

Frozen, serial tissue sections (5 μm) of unfixed human melanoma were collected on glass slides and fixed with acetone at −20 °C for 10 min. After which, the slides were blocked with 5% goat serum containing PBS for 15 min. The slides were incubated with primary antibodies for 45 min at room temperature. After washing with PBS, the slide was incubated with biotinylated second antibodies (anti-mouse IgG) for 15 min at room temperature, and then was incubated with HRP-conjugated streptavidin. Each tissue section was stained with DAKO immunoperoxidase kit. Hematoxylin was used as counter staining. A Mab (KM48) against keratinocyte was used as negative control.

3. Results

3.1. Selection of GD3-replica peptides

Preparation of peptides that bind to anti-GD3 Mab, 4F6, was performed using a phage library. Recovery of the phage increased to 0.58% in third round, as compared with the recovery rates in the first round (0.0003%) and in the second round (0.04%). Thirty randomly picked up clones from the third round were subjected to DNA sequences and the deduced amino acids are shown in Table 1. A total number of 27 peptides were obtained and divided into 4 groups (GD3P1, GD3P2, GD3P3, and GD3P4).

There were two consensus alignments as follows, Leu-Ala-Pro-Pro-$(X)_8$-Leu-Ser in GD3P1 and GD3P3, and Ala-$(X)_4$-Ala-Glu-$(X)_2$-Phe-Leu-X-Ser in GD3P3 and GD3P4. Whereas no conclusive consensus sequence was observed in GD3P2 and the other three peptides, except for Glu-Leu-Z (Z: Val or Leu) between GD3P2 and GD3P1.

3.2. Binding of the GD3-replica peptides to Mab 4F6

Binding between GD3-replica peptides and 4F6 is shown in Fig. 1A. Binding of 4F6 to GD3P1, GD3P3, and GD3P4 was observed, but not to GD3P2. Dose-dependent binding activities of phage to 4F6 were in the increasing order GD3P4 > GD3P3 > GD3P1.

Binding activity of 4F6 to MAPs by ELISA is shown in Fig. 1B. 4F6 bound to GD3P3 and GD3P4, however, the binding to GD3P1 was weaker compared with those to the phage clones, GD3P3 and GD3P4 (Fig. 1A). The binding profiles of 4F6 to the MAPs of GD3P3 and GD3P4 immobilized on the plastic wells were dose-dependent and the binding of 4F6 to GD3P4 was slightly stronger than to GD3P3. On the other hand, none of these peptides bound to a Mab (IgG_3) recognizing both GD2 and GD3 (data not shown). Moreover, neither an anti-GD3 Mab (IgM) nor an anti-*O*-acetyl GD3 Mab (IgG_3) bound to the peptides (data not shown). These results suggested that the obtained peptides bound to 4F6 specifically and closely mimic a part of GD3 structure.

3.3. Inhibition of GD3-replica peptides on the binding between 4F6 and GD3

Inhibitory effect of GD3-replica peptides on the binding of 4F6 to GD3 was examined. 4F6 was incubated with GD3 or GD3-replica peptides, which were immobilized on 96 well plates in advance. After which, each supernatant was transferred into a GD3-coated 96 well plate then subjected to ELISA to see the residual 4F6 (Fig. 1C). The 4F6 binding to the GD3-coated plate decreased in a dose-dependent manner, and the inhibition potencies found for GD3P3 and GD3P4 were similar to that of GD3.

When 4F6 and various concentrations of GD3 or GD3-replica peptides were added without pre-incubation into a GD3-coated 96 well, the binding of 4F6 to GD3 was inhibited by

Table 1
Amino acid alignments of the selected four peptides

Peptides	Amino acid alignments	Frequency (total 27)
GD3P1	LAPPRPRSELVFLSV	16/27
GD3P2	PHFDSLLYPCELLGC	7/27
GD3P3	GLAPPDYAERFFLLS	3/27
GD3P4	RHAYRSMAEWGFLYS	1/27