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

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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.**
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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).

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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

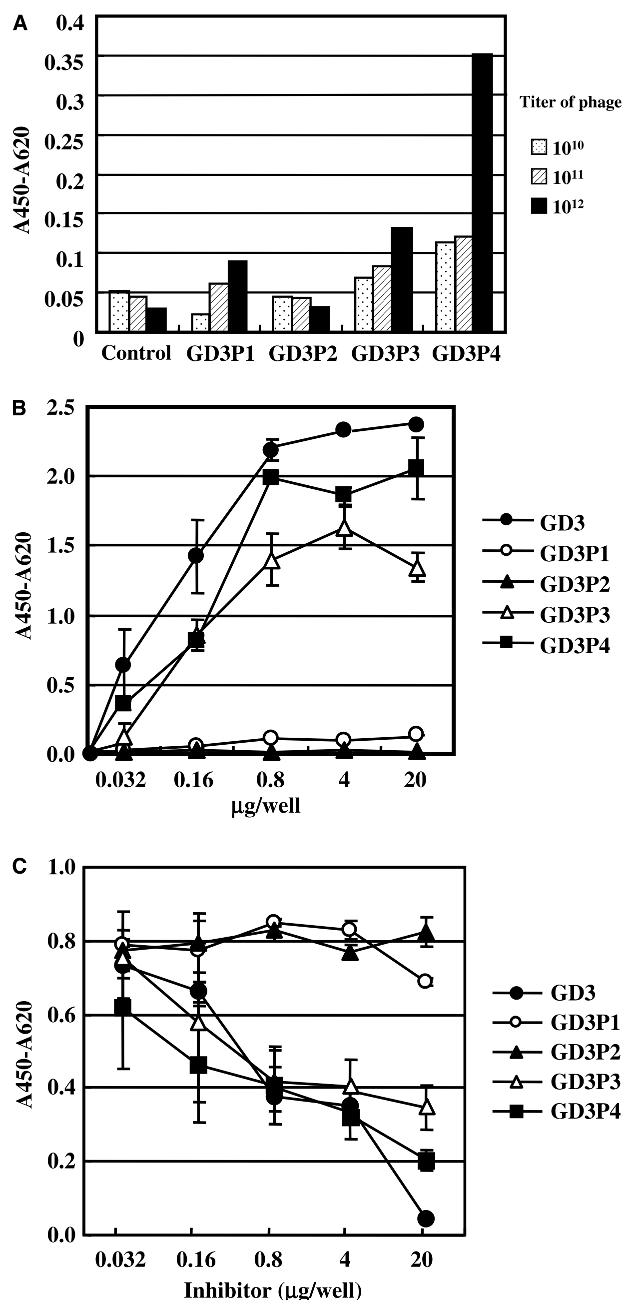

Fig. 1. Binding to 4F6 of the GD3-replica peptides. (A) Phage ELISA. Each phage clones were amplified and concentrated. Various concentrations (10^{10}/well to 10^{12}/well) of phage clones were fixed and subjected to ELISA as described in Section 2. Wild type of phage clone was used as control. The experiment was done in triplicate and each column shows the average. (B) Dose dependency of the binding of 4F6 to GD3-replica peptides. Various concentrations of the individual MAP was fixed and subjected to binding of 4F6. (C) Inhibitory effect of GD3-replica peptides on the binding of 4F6 to GD3. Various concentrations of GD3 or the MAP were fixed on the 96 well plates and incubated with 4F6 for 2 h in advance. Each supernatant containing the non-absorbed 4F6 was transferred into GD3-coated another plates and subjected to ELISA. The experiments were done in triplicate and values and error bars show averages and S.D.s, respectively.

GD3 at 4 μg/well, but more than 20 μg/well of GD3-replica peptides were required to obtain a significant level of inhibition (data not shown).

3.4. Anti-GD3 Mabs produced by GD3P4

In order to confirm that the obtained peptides truly mimic a part of GD3, whether the peptides can induce antibodies against GD3 was investigated by immunizing mice with the peptides. MAP for each peptide was used as antigen and the antibody producing activity against GD3 was determined. GD3P3 and GD3P4 were more effective than GD3P1 and GD3P2 in terms of antibody response in mice (data not shown). In order to produce Mabs recognizing GD3 by immunizing with peptides, MAP of GD3P4 was selected as the antigen. Finally, two Mabs (3D2 and 2C6) were obtained. The isotype of 3D2 was IgG_1 with λ light chain, 2C6 was an IgM, whereas 4F6 was an IgG_3 with λ light chain (data not shown).

Binding of the two Mabs was assessed by immunostaining on thin-layer chromatography plate (ITLC) using a ganglioside fraction purified from human melanoma tumors (Fig. 2A). Mabs 3D2 and 4F6 bound specifically to GD3 and not to any other tested ganglioside, whereas no ganglioside was recognized by Mab 2C6 using this technique. Binding specificity of 3D2 was also investigated using bovine brain ganglioside fraction (Fig. 2B). 3D2 slightly bound to GT1b (arrow), suggesting that 3D2 recognizes NeuAcα2-8NeuAcα2-3Gal structure. 4F6 reacted with only GD3.

3.5. FACS and immunohistochemistry

Experiments were carried out to determine whether the obtained Mabs recognize GD3 on human melanoma cell surface (Fig. 2C). Both Mabs 4F6 and 3D2 bound the human melanoma SK MEL-28 cells and approximately 82% of the cells were recognized by 4F6, whereas 3D2 stained 13.7% of the melanoma cells. The Mab 3D2 shows binding to the cell surfaces of the melanoma cell line but the binding activity is rather weaker compared to that of 4F6.

Immunostaining of tumor tissue with 3D2 was performed (Fig. 2D). The melanoma was strongly reactive with 4F6 and less reactive with 3D2, whereas control staining with Mab KM48 was negative. A clear-cut both peripheral and cytoplasmic staining of the tumor cells is observed with 4F6 antibody, whereas the staining with 3D2 seems to be on the membranes of tumor cells.

3.6. DNA sequence of heavy chain and light chain of 4F6 and 3D2

To clarify the relationship between these similarities at the molecular level, DNA alignments of variable regions (heavy chain variable region, V_H; light chain variable region, V_L) were investigated (Figs. 3 and 4). Thereafter, the full length of the heavy chain was amplified, using oligonucleotides from each V_H region as sense primers, and completed. From the full-length sequences of two Mabs, it was confirmed that the isotypes of 4F6 and 3D2 were, respectively, IgG_3 and IgG_1.

By comparing the respective V_H of Mabs 4F6 and 3D2, it can be seen that only 18 point mutations were detectable at the nucleotide level. Interestingly, only six amino acid substitutions (3K → Q, 19K → M, 37V → I, 58T → I, 75A → S, 77G → S, and 81L → M) could be observed. When the complementarity-determining regions (CDR) were compared, there were one, two, and three point mutations in CDR1, CDR2, and CDR3, respectively. Five of the six mutation sites were silent and only one mutation resulted in amino acid substitution (58T → I) in CDR2. Furthermore, there were high similarities in V–D–J segments between 4F6 and 3D2.

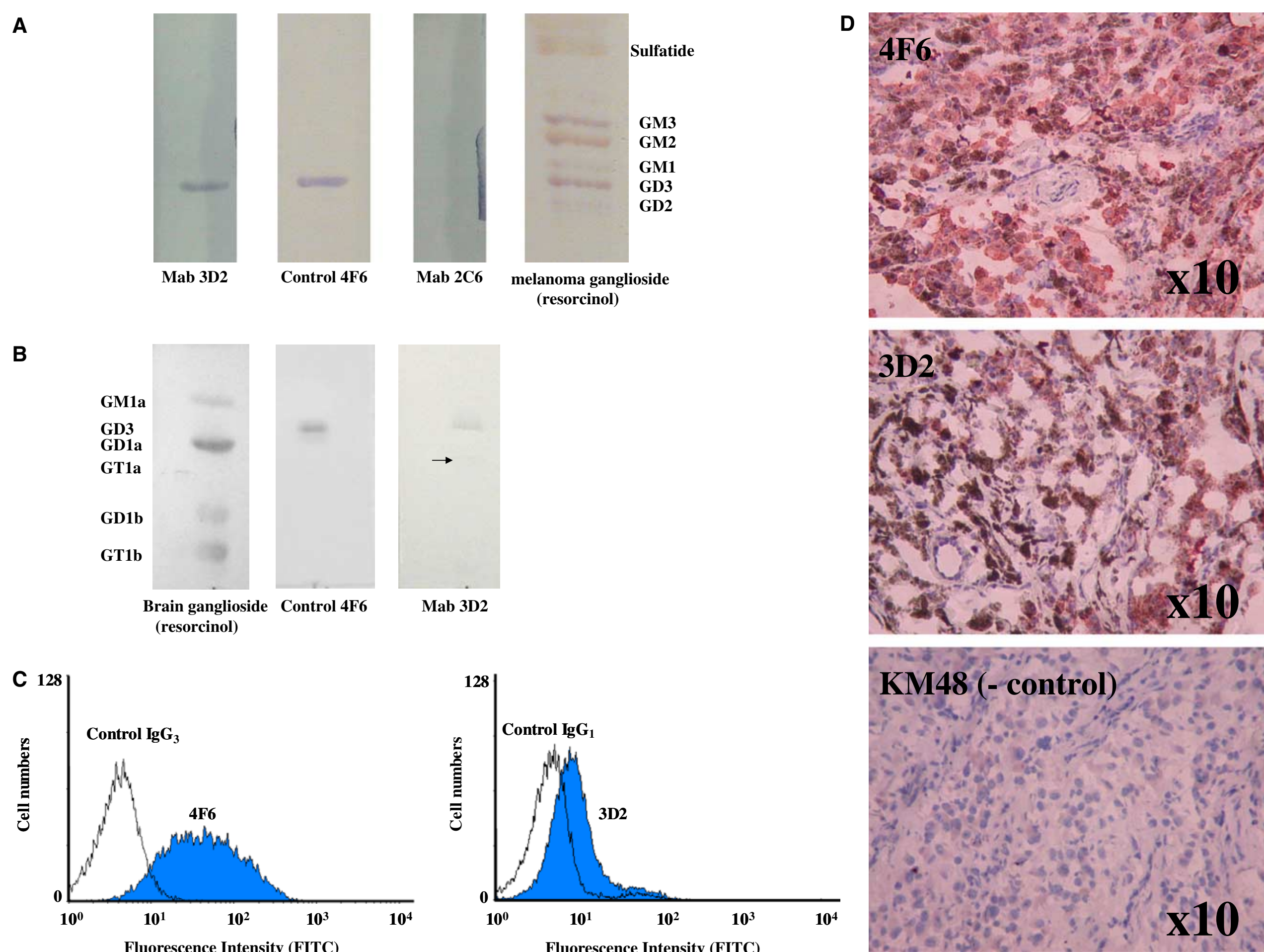

Fig. 2. Production of anti-GD3 Mabs by immunization of GD3P4. (A) ITLC. Ganglioside fraction extracted from human melanoma cells were developed on a high-performance thin-layer chromatography plate. All ganglioside bands were detected with resorcinol-HCl reagent and GD3 was detected by the individual Mabs shown in the figure. (B) Binding specificity of 3D2. Bovine brain ganglioside fraction was used. (C) FACS analysis. Human melanoma cell line, SK-MEL 28 cells were used for binding study of 3D2 and 4F6 for FACS analysis. (D) Immunohistochemistry with 4F6 and 3D2. Immunohistochemistry was carried out described in Section 2. Antibody-labelled tumors were colored in red. The blue coloration of nuclei is due to hematoxylin as counterstaining. Pictures at 10× magnification allow the comparison between the antibody-labelled tumor cells and the unstained portions of the melanoma tumor.

Variable region of both lights chains (V_L) were also determined (Fig. 4). By comparing the DNA and amino acid sequences, the mutation sites were two in CDR1 and one in CDR3. Their deduced amino acid residues (30S, 35A, and 95T) in 4F6 were substituted to Thr, Val, and Ser in 3D2, respectively. These individual substitutions occur among quite similar amino acids indicating that the substitutions seem not serious enough to alter the protein structure critical for the antigen-binding property. The V–J segments in 3D2 were similar to those in 4F6, except for cytidine to guanine at 284.

4. Discussion

Since Jerne advocated the idiotype network hypothesis [20], the fact that variable regions in some anti-Id antibodies include the internal images of the antigens has been documented [21–23]. Many anti-Id antibodies are used in place of the original antigens to induce antigen-specific immune responses. However, administration of the whole anti-Id antibodies in vivo often caused unexpected allergic responses. Therefore, an unwanted production of excessive amounts of irrelevant antibodies should be considered. Replacing the internal images with oligopeptides could represent an alternative approach.

Here we succeeded in obtaining GD3-replica peptides from a phage peptide library using an anti-GD3 Mab, 4F6. There were some consensus sequences between GD3P3 and GD3P4 (Ala-$(X)_4$-Ala-Glu-$(X)_2$-Phe-Leu-X-Ser). A carboxyl (–COOH) group of Glu may mimic that of sialic acid, and phenyl group of Phe and hydroxyl (–OH) group of Ser may mimic the bone structure of the carbohydrates. Moreover, there were Trp and Tyr in GD3P4. Amino group and indole group also seem to mimic the partial structure of carbohydrates [16,19,24]. However, any deduction about which corresponding amino acid is accountable for which part of GD3 structure is impossible from the present data.

The consensus sequence between GD3P3 and GD3P4 is mainly present at the C-terminus side (Ala-Glu-X-X-Phe-Leu-X-Ser). The nonamer peptide at C-terminus (GD3P4-C9) was thought to be enough to bind to 4F6. However, GD3P4-C9 neither bound to 4F6 nor inhibited the binding between 4F6 and GD3 (data not shown), indicating that the

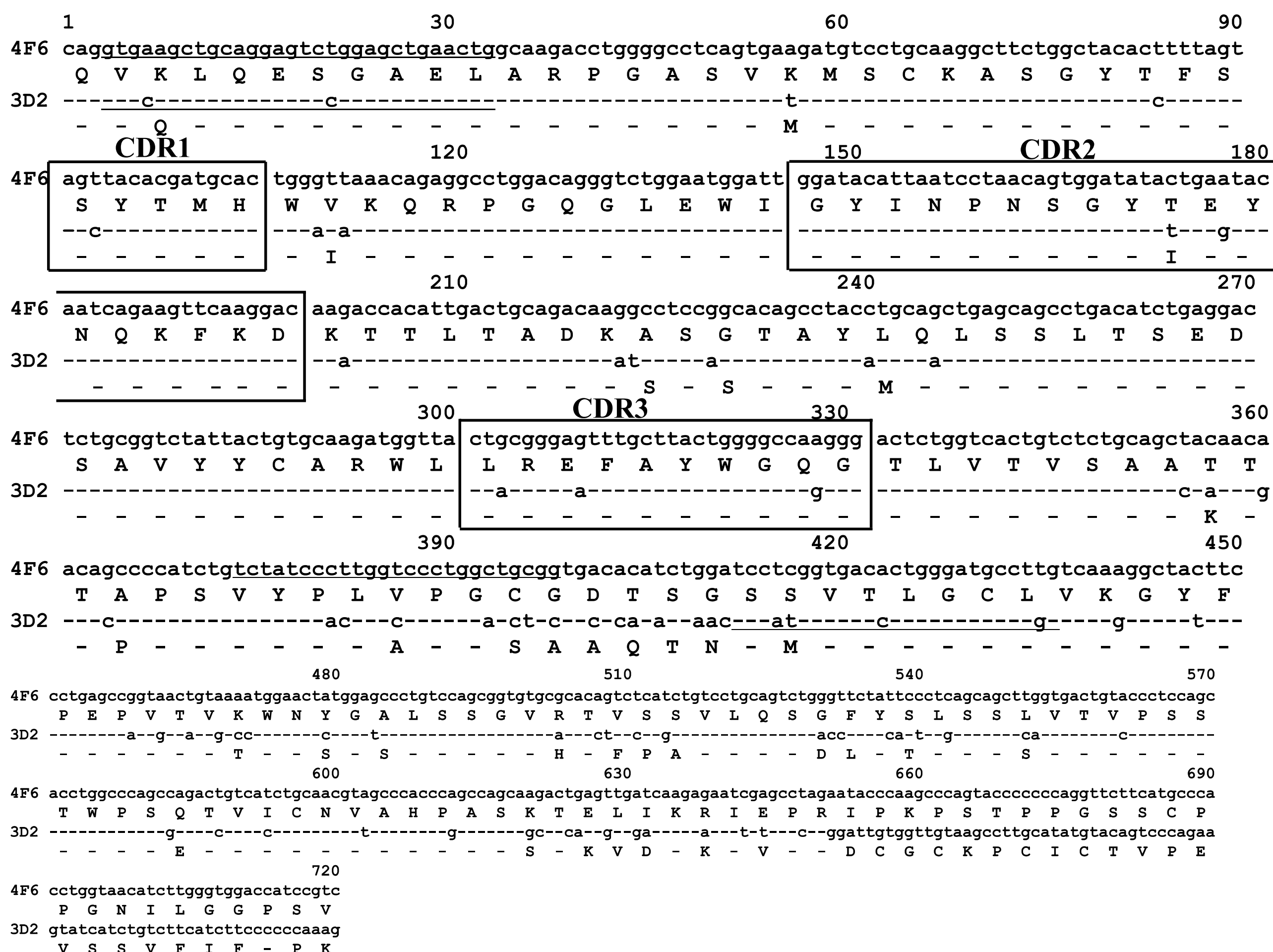

Fig. 3. Comparison of sequence of heavy chains between 4F6 and 3D2. Both heavy chains were amplified by RT-PCR, inserted into a cloning vector, and transformed into *E. coli* DH5α. Each 5 individual bacterial colonies were randomly picked up and subjected to DNA sequence. Oligonucleotides used for sequence were underlined. Amino acid sequences were deduced from the DNA sequences. Both 5 clones had the same sequences. 4F6 and 3D2 were, respectively, confirmed as IgG_3 and IgG_1. Possible positions corresponding to CDR1, CDR2, and CDR3 were shown as square. These sequence data are available from GenBank under Accession No. AY498569.

other part of the sequence at the N-terminus is also required for binding to 4F6 to form the stereo structure of GD3.

Willers et al. reported the selection of four GD3-replica peptides by using a phage peptide library and anti-GD3 Mab [25]. By comparing those sequences with the four peptides obtained in the present study, no significant common sequences were seen, suggesting that the individual Mabs used in the respective study may recognize different parts of the GD3 structure.

The immunogenicity of carbohydrates is generally low and the immune response is known to be T cell-independent. It will be of major importance to determine whether the immunization with carbohydrate-mimicking peptides (in terms of anti-carbohydrate antibody binding peptides) can convert the immune response from T-independent to T cell-dependent for the production of carbohydrate recognizing antibodies. The obtained Mab 3D2 was IgG_1 whereas 4F6 was IgG_3. The present results may support the expectations described above, even if further detailed studies are necessary to confirm these findings.

Although anti-carbohydrate antibodies induced in animals by immunization with carbohydrate-mimicking peptides were already described [26,27], the previous reports showed the production of polyclonal antibodies. The present study is the first to prove that Mabs can be successfully obtained through an immune response to glyco-replica peptides.

We have also given evidence that the variable regions of the obtained Mab produced by a peptide antigen (GD3P4) are quite similar with those of the original Mab produced by the carbohydrate antigen (GD3). Interestingly, V–(D)–J segments in both heavy and light chains of two Mabs are almost identical, except for a few point mutations which may be located in the position of joint between D–J or V–J segment. Both Mabs have lambda light chain, nevertheless it is rare to produce lambda light chain-containing antibodies in mice [28]. Taken together, we can speculate that there may be present the dominantly used gene for constructing antibodies when the antigens are exposed on antigen-presenting cells (APCs) with certain specific conformations depending of whether the antigen is a peptide or a carbohydrate.

These results support a mechanism of initiation of some autoimmune diseases that are caused after infectious diseases [29–32]. For example, regarding the three-dimensional structure, we can say that the shape of a protein antigen or metabolized fragment antigen derived from microorganisms is

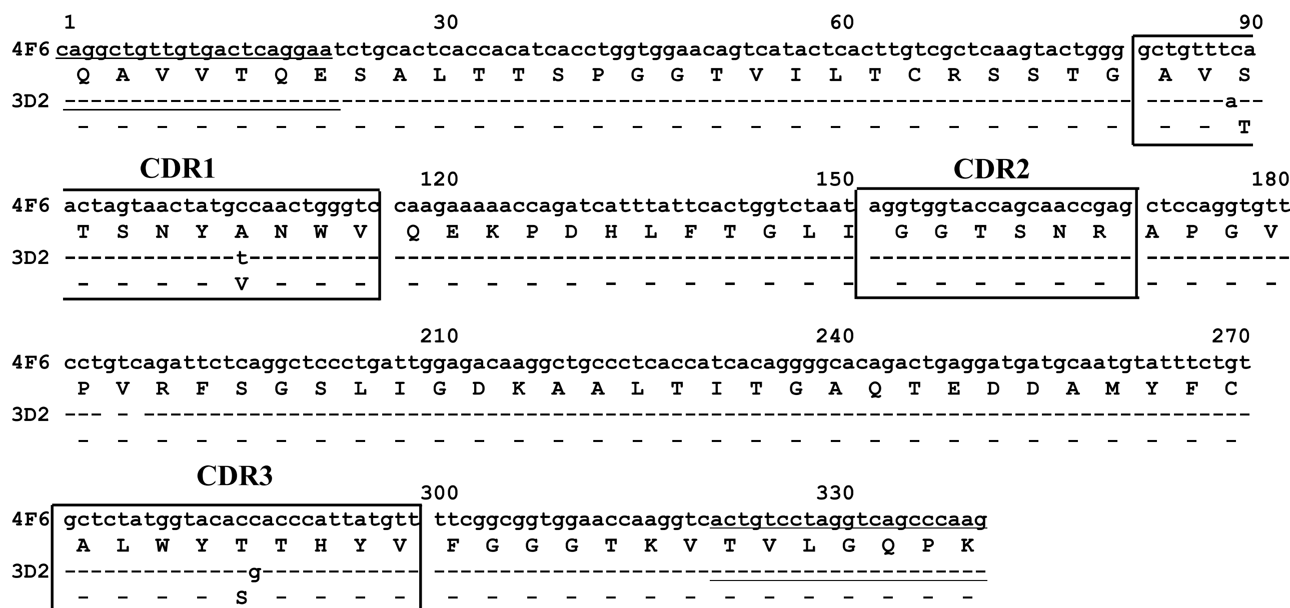

Fig. 4. Comparison of sequence of light chains between 4F6 and 3D2. Variable regions of both light chains were amplified by RT-PCR using oligonucleotides as primers (underlined) and the DNA sequences were analyzed as same methods for heavy chains (Fig. 3). Oligonucleotides used as primers underlined in the figure are for amplification of lambda chain and no bands were amplified by many oligonucleotides for kappa chain. Lambda chain was detected by Isotyping Kit in both Mabs (data not shown). Together with these results, both light chains consist from lambda chain. Possible positions corresponding to CDR1, CDR2, and CDR3 were shown as square. These sequence data are available from GenBank under Accession No. AY498568.

similar to the shape of a glycoconjugate of the host tissues and the production of antibodies against this antigen(s) may lead to the onset of an autoimmune disease.

Present report indicates that certain oligopeptides can expose the internal image of the variable region in anti-Id antibodies. It remains to show how GD3P4 should be modified to expose it on the APCs with keeping GD3-mimicking form in order to produce anti-GD3 antibodies more effectively. When this question is answered, mimicking peptide vaccines or peptide-coding DNA vaccines will be available for therapy.

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