Ganglioside GM1-binding peptides as adjuvants of antigens inoculated by the intranasal route

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Abstract

Forty-five GM1-binding peptides were identified using phage-displayed peptides libraries of random peptides. Most have a motif containing a hydrophobic amino acid followed by a serine (S). Based on a GM1-binding assays, two of these GM1-binding peptides (named 15 and 40) were chosen to investigate its immunostimulatory properties when chemically coupled to antigens. Mice intra-nasally (i.n.) vaccinated with some of these complexes developed a better local and systemic antibody response than mice i.n. vaccinated with the respective uncoupled antigens. The efficiency of the complex GM1-binding peptide-antigen strongly depends on the composition and structure of both of the components of the complex.

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1. Introduction

Mucosal application of antigens in general induces low immune responses; with the exception of naturally acquired or live vaccine induced active infections. A number of adjuvants have been assayed to enhance the immunogenicity of mucosally administered antigens. Among them, cholera toxin (CT) and Escherichia coli enterotoxin (LT) are the most potent mucosal adjuvants when co-administered with soluble antigens. However, their use in humans is unfeasible because of their extremely high toxicity. Many efforts have been made to isolate CT toxicity from its adjuvant ability. CT is a hetero-hexameric protein composed by an enzymatically active A subunit (CTA) and a pentameric receptor-binding B subunit (CTB). As a first step of the immunostimulatory process mediated by CT, the CTB subunit binds to the ganglioside GM1, a glycosphingolipid especially abundant on the surface of the M cells associated to the Peyer’s patches in the intestine, lung and central nervous system [1–3]. Macromolecules or macromolecular complexes bound to the apical plasma membranes of M cells are efficiently endocytosed or phagocytosed [4]. Thus, antigens that can bind selectively to M cells may be more effective in induction of mucosal immune responses. In agreement with this, the use of CTB conjugates has been reported to be effective in a variety of developments: by generating site-directed CT mutants with reduced toxicity and strong adjuvanticity [5–7], by construction of chimeric proteins containing an immunogenic peptide fused to CTB [8,9] or by conjugation of CTB with antigenic peptides using a chemical linker [10–12]. However, the size of the peptide that can be fused to CTB seems to be restricted to about 20 residues since peptides longer than this impair CTB ability to fold properly to bind the GM1 ganglioside [13]. Moreover, also CTB holotoxin are causative agent of
diarrhoea and peripheral facial nerve palsy [14]. Then, there is a demand of an effective and safer adjuvant vaccine that elicits mucosal immunity but does not require the use of CTB or LT.

In this context, and approaching an alternative adjuvant technology, we coupled an antigenic protein to peptides with affinity for the ganglioside GM1 in order to improve mucosal immune response. As a first step, we have identified small peptides with capacity for binding to the GM1 ganglioside, using the phage display technique and synthetic peptide arrays. Then, these peptides were coupled to antigens or bacterial cell wall preparations and it was found that they could act as adjuvants of intranasal vaccines that generate both, mucosal as well as systemic antibody responses. However, efficiency of the antigenic complex was strongly dependent on the structure of both the antigen and the peptide adjuvant.

2. Materials and methods

2.1. Isolation of GM1-binding peptides with a phage library

2.1.1. Reagents

Three different commercial phage random peptide libraries expressing 12-mer, 7-mer and 7-mer-cyclic peptides (New England Biolabs Inc. USA) were used. Ganglioside GM1, Bovine serum albumin (BSA) and cholera toxin, subunit B (CTB) were purchased from Sigma (USA). Microtiter plates (MaxiSorb) were purchased from Nunc Inc. (Denmark).

2.1.2. Selection of GM1-binding phages

Experimental procedures were performed according to the manufacture guidelines. Briefly, GM1 was attached to microtiter plates by incubation overnight (ON) at 4 °C in 0.1 M NaHCO₃ (pH 8.6). After this, plates were washed and blocked with 0.1 M NaHCO₃ (pH 8.6), 5 mg/ml BSA. A suspension of phage expressing 2 × 10⁹ different peptides was added. After 1 h incubation, plates were washed 10 times with a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS) and phage bound eluted with a buffer containing 0.2 M Glycine–HCl (pH 2.2) and 1 mg/ml BSA. The eluate was neutralized with 1 M Tris–HCl buffer (pH 9.1) and phage amplified by infecting bacterial cells (E. coli ER2738). Phage DNA extraction and sequencing was as previously described [15].

2.1.3. GM1-binding assay on cellulose membranes

GM1-binding peptides identified above plus three peptide sequences from literature were synthesized as an array (strawberry) by SPOT-synthesis as previously described [15]. GM1 was attached to cellulose membranes by immobilization of a buffer containing 6 M Guanidine–HCl, 100 mM Tris–HCl (pH 8.5) and extensive dialysis with 100 mM Tris buffer pH 8.5. Fluorescence specific for each peptide was evaluated as the fluorescence in the spot after incubation with labelled GM1 minus the fluorescence in the same spot before incubation.

2.1.4. Competitive displace of CTB bound to immobilized GM1 by synthetic GM1-binding peptides

Some of the peptides with high affinity characterized by the spot test were tested by competitive displace of CTB bound to immobilized GM1. Briefly, microtiter plates containing immobilized GM1 were incubated with 1% PBS-BSA for 2 h and washed. After this, horseradish peroxidase-conjugated CTB (Sigma, USA) in PBS containing 0.5% BSA was added and incubation proceeded ON at 4 °C. The next day, plates were washed and selected synthetic peptides added at different concentrations. Incubation proceeded ON at 4 °C. After this, the amount of CTB released to the media was measured by addition of the chromogenic peroxidase substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (1mg/ml in 0.1 M citrate buffer, pH 4.5), incubation for 15 min at room temperature and colour reading at 400 nm.

2.2. Selection of modified GM1-binding peptides

Two of the most effective GM1-binding peptides (15 and 40) and the most effective GM1-binding peptide described by Matsubara et al. [18] (peptide 3, renamed 46 in this study), were synthesized with modifications as described [19]. Modifications consisted in addition of a C-terminal bromacetyl group to allow labelling with 125I in order to calculate efficiency of the attachment. Amino acid composition of the modified peptides was: modified peptide 15: bromacetyl-[βA-βA-R-S-T-K-P-P-L-S-P-L-G-Y-amid, modified peptide 30: bromacetyl-[βA-βA-P-P-S-P-S-V-S-H-R-T-Y-amid and modified peptide 40: bromacetyl-[βA-βA-βA-V-W-R-L-L-A-P-P-F-S-N-R-L-L-P-Y-amid.

2.3. Antigens

Influenza viral proteins hemaglutinin (H) and neuraminidase (N) were extracted from virosomes of the Flu vaccine (Inflexal, Berna, Switzerland) by treatment with 6 M GuHCl in 100 mM Tris buffer (pH 8.5) and extensive dialysis with 100 mM Tris buffer pH 8.5. Streptococcus pneumoniae cell wall preparations were obtained as described [20].

2.4. Coupling reaction

Antigen coupling via thioether bridges was performed following the Englebreten et al. [19] procedure. Briefly, 1 ml of a buffer containing the antigen in 6 M Guanidine–HCl.
100 mM DTT, 100 mM Tris (pH 8.5) was incubated for 1 h at room temperature. After this, the DTT was eliminated by dialysis against 0.9 M Guanidine-HCl, 100 mM Tris buffer (pH 8.5). A mixture of free bromoacetylated peptide (10 μg) and radio-iodinated peptide (106 cpm) was added, and the reaction mix incubated for 2 h at room temperature. Finally, the product was dialyzed twice 15 h against 0.05 M PBS to remove unbound peptide.

The protein/peptide ratio was chosen according to the expected number of cysteines present in an average protein of a given molecular weight [21]. The molecular weight of the immunodominant protein antigen from the Streptococcus cell wall preparation was determined to be about 35 kDa by Western blot analysis developed with sera of mice intramuscularly immunized with a cell wall preparation. The number of molecules of this protein per cell wall mass was deduced from literature data. Efficiency of the coupling reaction was estimated measuring bound versus unbound radioactivity. Efficiency was usually 50–90%.

2.5. Iodination

Radio-labelling of peptides was performed as follows: 0.5 μCi of 125I (Amersham, Biosciences, USA) diluted in 20 μl of 0.05 M Phosphate buffer (PB) (pH 7.5) containing 10 μg of peptide were gently mixed with 10 μl of a solution containing 12 mg of chloramine-T in 5 ml of PB 0.05 M (pH 7.5). The reaction was developed for 1 min and stopped by addition of 300 μl of cold PB. Labelled peptide was then separated from free iodine using a Sephadex G25 (Amersham Biosciences, USA) column eluted with PB 0.05 M (pH 7.5). Fifteen fractions of 0.5 ml each were collected and incorporation of 125I was measured using a Gamma Counter (Beckman, USA). Traditionally, fractions 3–8 incorporate the labelled molecule. These fractions were combined and radio-labelled peptides were stored at 4°C until used.

2.6. Immunization

Young mice (Balb-C, 3 weeks old) were intranasally inoculated with 10 μl of the indicated vaccine preparation. Physiological solution was administrated to control mice. Inoculation was performed in a four doses scheme (days 1, 3, 7 and 9). Two weeks after the last dose, animals were anesthetized with a mixture of 2-methyl-1-butanol Teramylacetol 99% and 2,2,2-tribromoethanol 97% (Aldrich, USA) and a blood sample was collected. Bronchial alveolar lavages (BAL) were collected using a 1 ml syringe containing physiological solution. Each experimental group contained six animals. Experiments were repeated twice.

2.7. Antibody detection

IgA and IgG levels were measured by the ELISA assay in BAL and sera respectively. Briefly, 96 well Maxisorp microtiter plates (Nunc, Denmark) were coated with the antigen and blocked with 1% non fat milk in 0.1 M PBS. Different dilutions of the serum samples were poured into the wells and incubated for 2 h at 37°C. After washing, secondary antibody (rabbit anti-mouse Fcγ, anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b or anti-mouse IgG3, Zymed, San Francisco, CA, USA) was added and plates incubated for 1 h at 37°C. When BAL was assayed, rabbit anti-mouse IgG was used. After washing, HRP-conjugated anti-rabbit IgG was used as tertiary antibody. As enzymatic substrate, OPD-hydrogen peroxide was used and the resulting colour was measured at 490 nm using a microtiter reader (Cambridge technology Inc., USA). End-point titration (corresponding to OD490nm 0.1) was performed by serial dilutions.

3. Results

3.1. Selection of GM1-binding peptides from phage-displayed peptides libraries

In order to identify GM1-binding peptides three different phage libraries of random peptides, expressed as part of the M13 phage protein MIII, were subjected to three rounds of affinity selection using GM1 immobilized on polystyrene microtiter plates. Two of the assayed libraries expressed linear peptides of 7 and 12 amino acids long, respectively, and the other one expressed cyclic peptides of 7 amino acids long. The relevant region of the DNA of 45 selected phage clones was sequenced and the composition of the corresponding encoded peptides deduced from it. Using this information the identified GM1-binding peptides were classified in three groups (Table 1). Group 1, the most numerous (70%) is composed of peptides that have all in common a motif containing a hydrophobic amino acid followed by a serine (S) or, less frequently, a threonine (T) residue. Furthermore, most of these peptides (66%) have in common an extended version of this motif containing two hydrophobic amino acids followed by a serine (S). Two of the three GM1-binding peptides previously reported by Matsubara et al. [18] are included in this group (peptides 46 and 48). Group 2 is composed of only three peptides that have in common the motif QnnnW whereas Q is glutamine, n any amino acid and W tryptophan and Group 3 is a collection of peptides without any apparent homology except for the fact that more than half of them are cyclic. The three-dimensional structures of CT [22] and CTB complexed with the ganglioside GM1 are known [23] and several residues in CTB (tyrosine-12, glutamine-51, lysine-91, alanine-95, tryptophan-88, glycine-33, etc.) have been identified as essential for receptor binding by a variety of techniques [24]. Regarding this, it is interesting that one of the identified groups of GM1-binding peptides (group 2) is characterized because its members have two of these amino acids (glutamine and tryptophan) in common, separated by three other amino acids. However, peptides with the highest affinity for the GM1 ganglioside here identified have the tripeptide proline-leucine-serine (PLS) in common (Table 1,
Table 1
GM1-binding peptides from phage-displayed peptides libraries

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>O(D)TR</td>
<td>GSRT</td>
<td>F</td>
</tr>
<tr>
<td>4</td>
<td>HAG(L)A</td>
<td>AGH(L)</td>
<td>G</td>
</tr>
<tr>
<td>8</td>
<td>CSTH(T)</td>
<td>SP</td>
<td>F</td>
</tr>
<tr>
<td>9</td>
<td>CSTH(T)</td>
<td>SP</td>
<td>F</td>
</tr>
<tr>
<td>10</td>
<td>CAVQ(?)</td>
<td>SP</td>
<td>F</td>
</tr>
<tr>
<td>16</td>
<td>GDI(TM)</td>
<td>GSRT</td>
<td>F</td>
</tr>
<tr>
<td>20</td>
<td>KNP</td>
<td>SRT</td>
<td>F</td>
</tr>
<tr>
<td>25</td>
<td>TRK</td>
<td>SRT</td>
<td>F</td>
</tr>
<tr>
<td>40</td>
<td>N(P)S</td>
<td>F</td>
<td>F</td>
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<tr>
<td>15</td>
<td>RSP</td>
<td>G</td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>TPS</td>
<td>G</td>
<td>F</td>
</tr>
<tr>
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<td>TPS</td>
<td>G</td>
<td>F</td>
</tr>
<tr>
<td>35</td>
<td>TPS</td>
<td>G</td>
<td>F</td>
</tr>
</tbody>
</table>

In group 1 and 2 putative common motifs are highlighted in grey. (*) From reference [18].

3.2. GM1 binding assays of the peptides selected from phage-displayed peptides libraries

The 45 GM1-binding peptides identified in the affinity selection step were synthesized on a cellulose membrane as previously described [16,17] and their GM1-binding ability assayed by incubation with pyrene-labelled GM1 and UV light irradiation (400 nm) for spot visualization (Fig. 1). The three GM1-binding peptides previously reported by Matsubara et al. [18], here renamed 46, 47 and 48, were included in the array as reference controls. The figure documents that...
Fig. 1. Relative affinity for GM1 of GM1-binding peptides immobilized on a cellulose membrane. An array containing the 48 GM1-binding peptides identified in the affinity selection step (Table 1) was constructed on a cellulose membrane and incubated with pyrene-labelled GM1. Binding ability was determined by UV irradiation (400 nm) and spot visualization. Fluorescence specific for each peptide was calculated as described in Section 2. Results are presented as % of the fluorescence corresponding to peptide 37.

Several peptides show GM1-binding capacity even higher than the reference controls. Among them, peptides 2, 15, 37 and 40 were chosen for further studies. As a first step, an affinity assay based on the competitive displacement of CTB from a complex with GM1 immobilized on polystyrene microtiter plates was used (Fig. 2). This experiment was necessary because the screening assay performed on membranes in some cases was hampered by a high fluorescence background at 400 nm of some of the analysed peptides. Therefore, it was necessary to confirm the results using an independent affinity assay. As can be observed in Fig. 2, peptides 15 and 40 were potent competitors of CTB; however, peptides 02 and 37 were not as effective as expected from the membrane screening assay. As it can be observed, the best affinity level for peptides is about 100 times lower that the affinity of the CTB subunit. It is worth noting that peptides 15 and 40 are the only ones within group 1 that possess the PLS tripeptide as part of its sequence (Table 1). This suggests that this tripeptide sequence may be the optimum for GM1-binding within the common motif of group 1.

3.3. Antibody response to antigens coupled with selected GM1-binding peptides

In order to couple different antigens to selected GM1-binding peptides the latter were synthesized adding a bromoacetyl group to the N-terminal amino acid. This modification allows the coupling of peptides to thiol groups present in proteins antigens [19]. Additionally, a Tyrosine residue was added to the C-terminal amino acid of the peptides to allow radio labelling with $^{125}$I (see Section 2) and estimation of coupling yields. For our studies, peptides 15 and 40 (Table 1) and the most potent GM1-binding peptide previously identified by Matsubara et al. [18], here renamed peptide 46, were chosen.

3.3.1. Influenza virus proteins

The first antigenic preparation to be coupled with GM1-binding peptides was a mixture containing the Influenza virus proteins H and N. Mice were i.n. inoculated with a suspension of both proteins in saline. A four doses scheme (days 1, 3, 7 and 9) was applied. One week later, sera and BAL were collected and antibody titres determined. Fig. 3 shows that the Flu vaccine prepared with antigens coupled to peptide 40 was very effective as compared with the vaccines prepared with uncoupled antigens or antigens coupled to peptides 15 or 46.

In order to characterize the type of response generated in mice treated with antigens coupled to peptide 40, antibody subtypes were determined (Fig. 4). The increment over the basal was as follows: IgG1, 28-fold; IgG2a, 8-fold; IgG2b 3-fold and IgG3 remains equal. This strongly suggests that the H and A antigens from Influenza coupled to peptide 40 develop a predominantly Th2 immune response.

3.3.2. S. pneumoniae cell wall preparation

It has been reported that CTB immobilized on about 1 μm particles failed to adhere to the surface of M cells [25]. Therefore, it was important to investigate the performance of the GM1-binding peptides as mucosal adjuvants coupled to larger antigens like bacterial cell wall preparations. Fig. 5 shows the antibody titers in sera and BAL of mice i.n. inoculated with a $S. pneumoniae$ cell wall preparation coupled with either of the GM1-binding peptides 15, 40 or 46. In this case, peptide 15 was the most effective one in stimulating antibody response. The vaccine containing uncoupled cell walls was antigenically poor since antibody titres in mice inocu-
Fig. 3. Antibody response of mice i.n. inoculated with flu proteins coupled to GM1-binding peptides. Flu viral proteins H and N coupled with GM1-binding peptides separately were i.n. inoculated in a four doses scheme (days 1, 3, 7 and 9). One week later, sera and BAL were collected and antibody titres determined. PS: physiological solution; FP: Flu viral proteins alone; FP-Pep46: FP coupled to the reference peptide 46 [18]; FP-Pep15: FP coupled to peptide 15; FP-Pep40: FP coupled to peptide 40. Statistically significant difference compared with mice inoculated with FP (* \( p < 0.05 \)).

Fig. 4. Serum IgG subtypes of mice i.n. inoculated with flu proteins coupled to GM1-binding peptide 40. PS: physiological solution; FP: Flu viral proteins alone; FP-Pep46: FP coupled to peptide 40. Characterization of the type of immune response generated in mice treated with Flu viral proteins coupled with GM1-binding peptide 40. Antigen complex were i.n. inoculated in a four doses scheme (days 1, 3, 7 and 9) and serum antibody subtypes (IgG1, IgG2a, IgG2b, and IgG3) were determined 1 week later.

Fig. 5. Antibody response of mice i.n. inoculated with a *Streptococcus pneumoniae* cell wall preparation coupled to GM1-binding peptides. *Streptococcus* antigens were coupled with GM1-binding peptides separately and i.n. inoculated in a four doses scheme (days 1, 3, 7 and 9). One week later, sera and BAL were collected and antibody titres determined by ELISA. PS: physiological solution; St.: *Streptococcus* cell wall alone; St-Pep46: *Streptococcus* cell wall coupled to control peptide 46 (taken from reference 18); St-Pep15: *Streptococcus* cell wall coupled to peptide 15; St-Pep40: *Streptococcus* cell wall coupled to peptide 40. Statistically significant difference compared with mice inoculated with St (* \( p < 0.05 \)).

4. Discussion

It is well recognized that oral vaccines would be easier to produce and administer. On the other hand, immunization with oral vaccines also presents several advantages, such as reduced costs, and the potential to induce immunity at the mucosal surfaces. The mucosal immune system plays a crucial role in protecting against respiratory and enteric infections. Therefore, the development of effective mucosal vaccines is of utmost importance.

Here, the adjuvant effect of peptide 15 was quite modest (only about three times higher than the non-conjugated reference) as compared with the adjuvant effect of the most effective peptide (peptide 40) in the case of the vaccine prepared with influenza antigens (about 40 times higher than the background). Therefore, the size and/or the overall composition of the antigen seems to be very important for the antigenic properties of complexes containing GM1-binding peptides. It is also worth noting that the most effective peptide adjuvant was not the same for both of the antigenic preparations assayed, a fact that suggests that in order to obtain an effective mucosal vaccine the coupling with several GM1-binding peptides should be assayed.
tion against many infections that take place at the mucosal surfaces of the aero-digestive tract usually requires mucosal vaccine application. However, it has proven to be difficult to stimulate IgA responses using non-replicating antigens and experience with soluble protein antigens has been in general disappointing. Regarding this, an exception is the CT [1], the most potent mucosal adjuvant, and its closely related LT. Much effort and significant progress have recently been made in order to avoid toxicity. Among these progresses is the cloning and expression of the gene of CTB. Recombinant CTB is currently a specific protective antigen component of a widely registered oral cholera vaccine as well as a promising vector for either giving rise to mucosal anti-infective immunity or inducing peripheral anti-inflammatory tolerance to chemically or genetically linked foreign antigens mucosally administered. CT and CTB have been also recently used as combined vectors and adjuvants for markedly promoting ex vivo dendritic cell (DC) vaccination with different antigens as well as for steering the immune response to the in vivo-reinforced DCs towards either broad Th1 + Th2 + CTL immunity (CT) or Th2 or tolerance (CTB) [26]. The strong immunogenicity of this protein can to large extent be explained by its ability to bind to the GM1 receptor on the surface of mucosal cells [4].

In this study, we demonstrated for the first time that small peptides with GM1-binding capacity chemically coupled to proteins or large sub cellular fractions such as bacterial cell wall preparations could significantly increase their antigenic properties when inoculated as mucosal vaccines by the i.n. route. Forty-five GM1-binding peptides were identified by affinity enrichment from several phage libraries of random peptide sequences expressed as part of the M13 phage protein PIII. It was observed that many of these peptides have a common motif containing two hydrophobic amino acid followed by a serine or less frequently a threonine residue. Using a competition assay it was found that the best GM1-binding peptide previously identified with its components. Therefore, careful studies should be performed in order to identify the optimal combination in each case.

GM1-binding peptides have been proved to be useful to increase the efficiency of intranasal administered antigens. However, extensive preclinical studies should be performed in order to establish whether GM1-binding peptides are safe enough to be used in clinical trials in humans. These studies should specially address the possibility of antigen targeting to the CNS as previously reported for some intranasal vaccines adjuvanted by the cholera toxin [3]. In this context, the present study should be considered just as a first step in the long way to clinical application.

References


