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Cyclic OmpC peptidic epitope conjugated to tetanus toxoid as a potential vaccine candidate against shigellosis

Anna Jarząb [a,c], Danuta Witkowska [a,c,*], Edmund Ziomek [c], Bartosz Setner [b], Aleksandra Czajkowska [a], Małgorzata Dorot [a], Zbigniew Szewczuk [b], Andrzej Gamian [a,c,*]

[a] Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland
[b] Faculty of Chemistry, University of Wroclaw, Wroclaw, Poland
[c] Wroclaw Research Center EIT+, Wroclaw, Poland

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ABSTRACT

In earlier works we have described that mice immunized with outer membrane protein OmpC survive the challenge with live *Shigella flexnerii* 3a. We have also identified conformational epitope of this protein, that was recognized by mice antibodies. The aim of current work was to investigate whether synthetic OmpC epitope homologs can elicit immunological response sufficient in protecting mice against shigellosis. Several linear peptides containing RYDERY motif were synthesized and conjugated to poly-lysine. These conjugates appeared to be poor immunogens and to boost the immunological response an addition of the adjuvant (MPL) was required. Unfortunately, the MPL alone caused a very high immunological reaction that was masking response to peptidic epitope. Under those circumstances we used tetanus toxoid (TT) as the carrier protein for the peptides and the agent stimulating immunological response. Series of cyclic peptides, homologs of the OmpC main epitope were synthesized and conjugated to TT. The loop size in cyclic peptides varied by number of glycine residues, i.e., 1–3 residues added to the GLNRYDERYIGK motif. The linear GLNRYDERYIGC-TT was also prepared as the control. The latter conjugate gave the highest immunological response, followed by the cyclic-GGLNRYDERYIGC-TT and cyclic-GLNRYDERYIGC-TT. The third peptide, cyclic-GGGLNRYDERYIGC-TT, gave a very low response, although it was the most resistant to proteolysis. However, antibodies obtained against cyclic-GGLNRYDERYIGC-TT were more potent to recognize both OmpC and *Shigella flexnerii* 3a cells than the antibodies against linear GLNRYDERYIGC-TT. Furthermore, the monoclonal antibodies raised against linear GLNRYDERYIGC-TT showed 20-fold lower dissociation constant (KD) than the naturally occurring polyclonal antibodies from umbilical cord sera. Monoclonal antibodies also gave a weaker signal in electron microscope than mice and human polyclonal antibodies. In overall, our results point to cyclic peptides as better candidates for a vaccine development, since they are eliciting production of the higher affinity antibodies against *Shigella* cells and OmpC.

1. Introduction

Shigellosis, or bacterial dysentery, caused by rod-shaped bacteria of the *Shigella* genus is a major health problem in the developing world. The incidence of shigellosis has been estimated at 164.7 million cases annually. More than 1 million of these cases involve children under the age of 5 years old, of which majority results in death [1]. The rise of antibiotic resistance among *Shigella* strains and lack of reliable and quick diagnostics limit the treatment of shigellosis and makes development of an effective vaccine an urgent issue [2,3]. Unfortunately, there is no commercially available vaccine against shigellosis. The World Health Organization has recently given a high priority to the development of such vaccine [4]. During the last few decades various approaches, including killed whole bacterial cells, live attenuated cells and more recently subunit vaccine strategies have been investigated. So far, in clinical trials, those vaccines showed poor immunogenicity, caused side effects and the immune response was limited to particular serotypes of *Shigella* [5,6]. In view of the wide range of *Shigella* serotypes and subtypes, there is a need for a multivalent vaccine effective for all species and serotypes. In our previous study we have shown that *Shigella flexneri* 3a has a 39 kDa outer membrane protein that is a very good vaccine candidate. Furthermore, this protein is quite characteristic for the *Enterobacteriaceae* family

* Corresponding authors at: Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland (D. Witkowska).
E-mail address: witkows@iitd.pan.wroc.pl (D. Witkowska).

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and may elicit immunity not only against *Shigella flexneri* 3a, but also against other members of this bacterial family [7].

This protein was identified as the Outer Membrane Protein C (OmpC). It is located in the outer membrane of *Shigella flexneri* 3a and other members of the *Enterobacteriaceae* family [7]. This protein is composed of 352 amino acids forming a β-barrel structural motif [8]. Recently, the OmpC has been purified and characterized and its sequence was determined [Gene Bank: 24113600]. The three-dimensional model for OmpC from *S. flexnerii* 3a was built and used to predict conformational antigenic epitopes [9]. It turned out that only one of the OmpC's loops, i.e., loop V [8] that was predicted as one of five potential epitopes, was recognized by human sera. Peptide mapping of the loop V narrowed down the epitope's sequence to RYDERY [9]. The same epitope was preferentially recognized by mice sera, while a totally different epitope (loop VII), was interacting with rabbit's sera. It turns out that in mice immunized with OmpC interaction of their sera with loop V epitope can account for approx. 70% of OmpC's antigenic activity. For this reason our further studies focused on the loop V epitope.

In our earlier studies we have also shown that active immunization with purified OmpC protected mice against infection with live pathogen. Serological studies demonstrated that OmpC reacted strongly with serum from immunized mice, human normal plasma and human umbilical cord sera. The latter interaction is of special interest, considering that anti-OmpC IgGs in umbilical cord sera are transferred from mother to fetus and may play a pivotal role in protecting infants [7,10]. The protection against *S. flexneri* 3a infection observed after mice vaccination with OmpC and the fact that similar antibodies are in human plasma made plausible use of the OmpC in human vaccine development.

In this work we describe the synthesis of peptides, linear and cyclic, representing OmpC epitope and their attachment to different carriers. We have also examined the peptide-carrier conjugates for their immunogenic properties, raised antibodies and examined their protective properties while infecting mice with live *Shigella flexneri* 3a.

2. Material and methods

2.1. Bacterial strain, culture conditions

Shigella flexneri serotype 3a strain (PCM 1793) used in this study was obtained from the Polish Collection of Microorganisms (PCM) of the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). Bacteria were grown on plates with enriched Bacto Agar (Difco) and cultured for 8 h with gently shaking in liquid Brain-Heart Infusion (BHI) medium (Difco).

2.2. Animals

All experiments were carried out using 6–7 weeks old BALB/c female mice (20–25 g) purchased from the Mossakowski Institute of Experimental and Clinical Medicine of the Polish Academy of Sciences in Warsaw, Poland. Animals were held in quarantine for one week before being used in experiments. All animal studies were conducted according to the ethical guidelines of the National Ethics Committee and approved by the First Local Ethics Commission at the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (LKE 53/2009).

2.3. Sera

Human sera were obtained from healthy donors from the Military Center for Blood Donation, Terrain Station in Wroclaw. This study was approved by the Medical Ethics Committee of the Medical University of Wroclaw (KB-543-2014). Human umbilical cord sera from healthy women were obtained from the Obstetric Clinic of the Medical University of Wroclaw. The samples acquisition was approved by the Medical Ethics Committee of the Medical University of Wroclaw (KB-882–2012) and they were obtained with patients' written informed consent.

2.4. Isolation and purification of the OmpC from S. flexneri 3a

The outer membrane proteins (OMPs) fraction was extracted from dry bacterial mass with valeric acid following procedure described by Arcidiacono et al. [11]. The OmpC was purified from the OMPs crude fraction by gel filtration and ion-exchange chromatography as previously described [9,10]. The fractions containing OmpC were collected, dialyzed against 0.1 M ammonium acetate, concentrated and stored at −20 °C. Protein concentration was determined by Lowry method [12].

2.5. Peptide synthesis on polyethylene pins

Peptides were synthesized using NCP Block of 96 Hydroxypropylmethacrylate pins (MIMOTOPES, Clayton, Victoria, Australia) according to the standard protocol [13] with slight modifications as described in [9]. Briefly, each pin was submersed in 100 µl of the solution containing 60 mM F-moc amino acid and equimolar amount of diisopropylcarbodiimide and N-hydroxybenzotriazole as coupling reagents for minimum 4 h. Finally, peptides were deprotected, acetylated on N-end and used for ELISA testing after disruption.

2.6. Cyclic peptides synthesis

All F-moc amino acids, solvents and reagents were purchased from Novabiochem® and used as recommended by the manufacturer. Cyanogen bromide was obtained from Sigma Aldrich. The syntheses were performed manually on the TentaGel RAP resin (500 mg, loading: 0.28 mM/g) in a polypropylene syringe reactor (Intavis AG, Köln, Germany) equipped with polyethylene filter, according to the standard F-moc (9-fluorenylmethoxycarbonyl) solid phase synthesis procedure. 160 mg (3 equiv.) of HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate) were used as coupling reagents. 60 mg (3 equiv.) of Oxyma Pure ethyl 2-cyano-2-(hydroxyimino)acetate and 103 µl (6 equiv.) of DIPEA (*N*,*N*-diisopropylethylamine) were used as additives. DMF (*N*,*N*-dimethylformamide) was used as a solvent. Each coupling step was performed for 2 h. The end of a coupling was verified with ninhydrin test. Listed below are the steps of the synthesis:

a. The *N*-terminal amino acid of peptidyl resin (TentaGel RAP resin – 100 mg, loading: 0.28 mM/g) was succinylated using 141 mg (50 equiv.) of succinic anhydride and 259 µl of DIPEA in DMF (3 times for 10 min). The selective deprotection of Lys(Mtt) was performed by repeated washing of peptidyl resins using solution of TFA/TIS/DCM (2.5/2.5/95, v/v/v) at room temperature (3 times for 2 min then 3 times for 10 min and then again 3 times for 2 min).
b. Next each peptidyl resin was washed with DCM, 5% DIPEA in DMF (3 times) and DMF.
c. Peptides were cyclized on the resin by forming an amide bond between the *N*-terminal succinic acid residue and the ε-amine group of Lys. Each reaction was conducted in DMF at room temperature using 32 mg of HATU (3 equiv.), 12 mg of Oxyma Pure (3 equiv.) and 31 µl of DIPEA (6 equiv.). The cyclization was completed within 24 h based on the

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