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Antibody-Binding Motif of Mimetic Peptides to *V. cholerae* O139 Lipopolysaccharide

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ABSTRACT

This study explores deduced amino acid sequences of mimetic peptides of *Vibrio cholerae* O139 epitopes in order to design specific antigens for use in diagnostic method. Mimetic peptides expressed on *E. coli* flagella were selected from a FliTrx random peptide library via the interaction with purified monoclonal antibody to *V. cholerae* O139. Inserted nucleotides encoding bound peptides were determined by PCR. Peptides from clones giving positive results were confirmed by Western blot analysis. Sixty-two positive *E. coli* colonies were obtained and nucleotide-sequenced. Inserted nucleotides were translated into amino acids. Fifty-six patterns of deduced amino acid sequences were obtained without a consensus sequence. Most sequences of mimetic peptides have amino acid motif as RXXR with approximate molecular weight of 1,700 to 2,000. Arginine and glycine occupy the highest percentage of amino acid composition.

Key words: *Vibrio cholerae* O139, lipopolysaccharide, random peptide library, FliTrx random peptide

INTRODUCTION

Vibrio cholerae serogroup O139 is one of the two causative agents of severe diarrheal disease or cholera which transmits via fecal-oral route. It can cause pandemic and still remains one of the major health problems in developing countries (Murray *et al*., 2002). The O-polysaccharide antigen, part of lipopolysaccharide (LPS) on the outer cell membrane (Kaper *et al*., 1995), is a major antigen inducing immune responses, which has potentially used in diagnosis and vaccine development (Cunto-Amesty *et al*., 2001). However, the extraction of O-polysaccharide is laborious, time-consuming and provides low yield. This study, therefore, aimed to explore mimetic peptides to the O-polysaccharide in order to study the potential use as an antigen in diagnosis.

Selection of mimetic peptides from phage display random library has a number of limitations, for example, the requirement of bacteriophage to infect bacterial cells and the time consumption on the culture process. An alternative display, bacterial flagella display described by Lu *et al*., (1995) may overcome these problems since the display does not require bateriophage and the culture of bacteria consume lesser time.

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Moreover, fusion peptides of the flagella display are expressed 4,000-8,000 copies on flagella, which is much more than those of phage display, (expressed only 3-5 copies on protein phage III) (Westerlund-Wikstrˆm, 2000). In addition, proteins on flagella are strong immunogens (Kuwajima, 1988; He *et al*., 1994).

Flagella display relies on genetic in-frame fusion of random peptides that are constrained to co-express directly on *E. coli* flagella. The library is composed of 10^8 clones of dodecapeptides of which 36 random nucleotides are inserted into pFliTrx at active site loop of thioredoxin gene (*TrxA*). The *TrxA* is inserted in *FliC* encoding flagellin, a major bacterial flagellar protein. The expression of thioredoxin-peptides, fusion proteins, is controlled under a P_L promoter to co-display on the surface of *E. coli* flagella at the active site loop of thioredoxin. This method has been used extensively for many purposes, eg. for mapping of antibody epitopes, for identifying protein-protein binding sites and also for producing peptides mimicking non-peptide antigens (Westerlund-Wilkström, 2000; Adda *et al*., 2002). Currently, a few mimetic peptides to protein and non-protein antigen have been investigated, eg. peptides binding to tumor-derived endothelial cells (Brown *et al*., 2000), to HBS-preS (Xin *et al*., 2003), and to glycoaminoglycan hyalaluronan (Amemiya *et al*., 2005).

In this study, mimetic peptides of *V. cholerae* O139 lipopolysaccharide were selected from a FliTrx peptide library via the interaction with specific monoclonal antibody. Deduced amino acid sequences of mimetic peptides were analyzed and their antibody-binding motifs were determined.

MATERIALS AND METHODS

Purification of monoclonal antibody to *V. cholerae* O139

Monoclonal antibodies to *Vibrio cholerae* O139 were produced by hybridoma technique (Köhler and Milstein, 1975) and characterized against 135 strains of enteric bacteria including 18 strains of *V. cholerae* O1 serotype Ogawa, 11 strains of *V. cholerae* serotype Inaba, 19 strains of *V. cholerae* O139, 17 strains of *V. cholerae* nonO1/nonO139, and 70 strains of other enteric bacteria by indirect ELISA. Characterization of immunoglobulin isotype was performed by using mouse Immunoglobulin Isotyping kit (Zymed, USA). Monoclonal antibody giving the highest antibody titer to *Vibrio cholerae* O139 without cross-reaction against other enteric bacteria was subjected to purification by Protein G affinity chromatography. Antibody fractions giving activities against *V. cholerae* O139 tested by ELISA were pooled and then concentrated by centrifugation using ultra-filter (Centricon, Amicon, USA). Protein level of concentrated antibody was measured by Lowry's method (Lowry *et al*., 1951).

Panning method

Mimetic peptides were selected from a FliTrx random peptide library (Invitrogen, USA) by panning method. Briefly, peptides were induced to be expressed by adding 100 μg/ml tryptophan to *E. coli* (GI826) culture grown in IMC medium (1x M9 salts, 0.2% casamino acid, 0.5% glucose, 1mM $MgCl_2$) containing 100 μg/ml ampicillin. One and a half milliliter of 20 μg/ml purified NHV4 specific to *V. cholerae* O139 was immobilized on 60-mm petri-dishes and then blocked with 1% non-fat dry milk, 150 mM NaCl, 1%∝-methyl mannoside. The induced *E. coli* cells were placed onto petri-dishes to interact with immobilized NHV4 for 5 rounds. After the fifth round of panning, bound clones were plated on RMG (1xM9 salts, 2% casamino acid, 0.5% glucose, 1mM $MgCl_2$, 1.5% agar) containing 100 μg/ml ampicillin. Selected colonies were randomly picked up and tested by PCR and Western blot analysis.

Polymerase chain reaction (PCR)

Plasmids were extracted from 2 ml of cells grown in RM medium (1x M9 salts, 2% casamino acids, 1% glycerol, 1mM $MgCl_2$) containing 100 μg/ml ampicillin by using miniprep kit, (QIAgen, Germany). Purified plasmids were used as templates for PCR, 5'-ATTCACC TGACTGACGAC-3' as a forward primer, and 5'-GCCCTGATATTCGTCAGCGAT-3' as a reverse primer. The amplified products were electrophoretically run on 2% agarose gel. The DNA bands were stained with ethidium bromide.

Western blot analysis

Each selected colony of *E. coli* was individually grown in 4 ml of RM medium containing 100 μmg/ml ampicillin for 12-18 h and 20 μl of cells were then inoculated into 2 ml of inducing medium until cells reached a log phase with optical density at 600 nm of about 0.5. One and a half milliliter of cells of each colony was transferred into micro-centrifuge tubes and centrifuged at 17,900xg for 10 min at room temperature. Fifty microliters of sample buffer was added to cell pellets and boiled at 100°C for 5 min. Five microliters of each sample was loaded onto 12% polyacrylamide gel. The protein bands were separated by electrophoresis. The gel was subjected to Western blot analysis by trans-blotting separated protein bands onto nitrocellulose membranes. The unbound sites of membranes were blocked with 3% non-fat dry milk in Tris-buffered saline (TBS: 20 mM Tris-HCl, 500 mM NaCl) pH 7.5, and incubated at 37°C for 1 h, washed 3 times with TBS-T (TBS, 0.05%,w/v Tween-20) pH 7.5, then probed with an appropriate concentration of monoclonal antibody to *V. cholerae* 139, incubated at 37°C for 1 h. After washing 3 times as described above, the membranes were probed with AP-anti mouse immunoglobulin and incubated at 37°C for 1 h and then washed 3 times with washing buffer. The color was developed by placing BCIP/NBT substrate onto the membranes. The membranes were rinsed with water after the color was fully developed.

Nucleotide and deduced amino acid sequencing

Clones giving positive results by both Western blot analysis and PCR were subjected to automated nucleotide sequencing using Big Dye Terminator v3.1 (Applied Biosystems, USA). Oligonucleotide 5'-GGTGCTACCACC ACAAA CAA-3' was used as a primer. Deduced amino acids were translated from inserted 36 nucleotides.

Computerized analysis of molecular properties of mimetic peptides

Amino acid sequence alignment was performed by using program CLUSTAL W (http://expasy.org). Isoelectric point (pI) and molecular weight (Mw) of mimetic peptides were predicted by program compute pI/Mw (http://expasy.org).

RESULTS AND DISCUSSION

Four hybridoma clones, secreting monoclonal antibodies against *V. cholerae* O139, were obtained. Supernatant from a hybridoma clone, named NHV4, gave the highest antibody titer, 1:4,000, without cross-reaction against 116 strains of related enteric pathogens other than *V. cholerae* O139. The monoclonal antibody from NHV4 belongs to IgG3, k isotype and is specific to lipopolysaccharide of *V. cholerae* O139. It was used, in this study, to select peptides that mimic the binding ability of lipopolysaccharide antigen.

Screening of peptides via interaction between random peptides and immobilized monoclonal antibody performed by panning method revealed that not all colonies of *E. coli* provided the exact size of 180 bp of amplified PCR product (Figure 1). Confirmation by Western blot analysis, positive clones showed an intense band at 63 kDa, the size of thioredoxin (Figure 2). This may predict that monoclonal antibody binds to

peptides that are expressed on thioredoxin protein.

Alignment comparison of 56 patterns of deduced amino acid sequences using multiple sequence alignment program, CLUSTAL W, showed no consensus sequences, suggesting that binding site is not sequence-specific in accordance with the sequence of bacterial lipid A (Thomas *et al.*, 2003). Of all amino acid compositions, arginine and glycine occupy the highest percentage,14.3 and 12.3, respectively. The peptides confer the positive charge due to the presence of arginine.

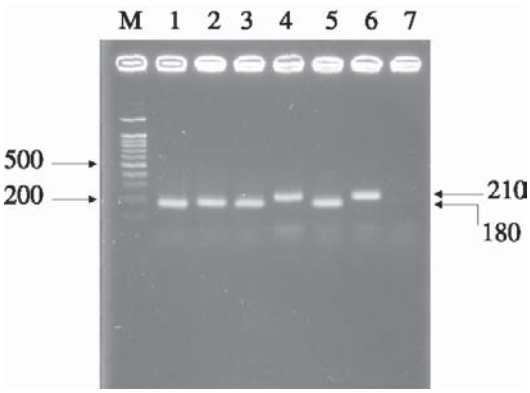

Figure 1 PCR products of positive clones on 2% agarose gel Lane M: 100 bp ladder plus Lane 1-6: clone # 3,4, 8, 20, 21, 55 Lane 7: reagent control.

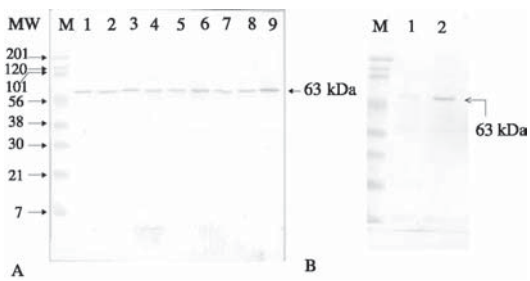

Figure 2 Western blot analysis of thioredoxin fusion peptides displayed on *E. coli* flagella

A. probed with MAb NHV4
Lane M: molecular weight markers
Lane 1-9: plasmid # 3, 4, 8, 12, 13, 30, 42, 57, 63

B. probed with anti-thioredoxin
Lane M: molecular weight markers
Lane 1-2: plasmid # 3, 4

Composition of amino acid sequences are classified into 3 groups, 26 sequences of RR-group,15 sequences of RG-rich group, and 21 sequences of unclassified group. Samples of sequences of RR- group are shown in Table 1. RR-sequences consist of the three most frequently-found amino acids, 25.8% arginine, 8.6% glycine and 8.0% alanine, RG-rich sequences are composed of 10.3% arginine, 21.1% glycine and 10.9% alanine, and an unclassified group contains 10.5% glycine, 9.7% alanine and 8.9% valine.

As shown in Table 1, isoelectric points of mimetic peptides of RR-group ranged from 4.56 to 12.0. and molecular weights were from 1713.96 to 2097.40. Deduced amino acid motifs of RR group may be RR, RXR, RXXR, and RXXXR, (X is any amino acid). Of all 26 sequences, motif RXXR was frequently found in 13 sequences. However, the RX_7R sequence has been shown to have significant binding to hyaluronan in which R-R was shown to play this role (Amemiya *et al*., 2005).

CONCLUSION

Deduced amino acid sequences of mimetic peptides are rich in arginine and glycine. Amino acid motif of most peptides is RXXR which may be a binding site. No consensus sequence according to CLUSTAL W sequence alignment was revealed. It is strongly proven that binding sites of protein-protein interaction is not sequence-specific.

Though monoclonal antibody reacted with mimetic peptides at thioredoxin site where inserted peptides were constrained to co-express, binding abilities of both molecules need to be further confirmed. The binding ability of the

Table 1 Deduced amino acid sequences and pI / Mw of mimetic peptides in RR- group.

Clone names and deduced amino acids				pI / Mw*
Clone 3	---------- TTLII	*RRAR*	GG------- --- (2 times)	5.98/ 1713.96
Clone 4	---------- QPTIA	*RGAR*	ADM ----------	8.07/ 1801.09
Clone 11	---------- P	*RKKR*	LG VSW ----------	11.56/ 2053.48
Clone 8	----------	*RGTR*	TTFSK ---------- (2)	10.79/ 1723.99
Clone 12	----------	*RRKR*	TTVAL ----------	12.0/ 2083.51
Clone 17	---------- PTI	*RMPR*	LAHL ----------	10.41 / 1975.42
Clone 30	---------- ARI	*RAHR*	PDEM ----------	8.08 / 1866.16
Clone 13	---------- QEYL	*RARR*	AA ----------	11.56/2053.48
Clone 43	---------- APR	*RECR*	QHKRG ----------	9.80/ 2008.33
Clone 63	----------	*RLSR*	TKPAHTR ----------	10.79/ 1837.15
Clone 20	---------- CVLI	*RSFR*	WQS ----------	8.73 / 1909.27
Clone 60	----------	*RSR*	QDSGVKTRS-(2) ---	9.69 / 1891.11
Clone 25	---------- VCD	*RHR*	LSKGRG ----------	9.22 / 1898.21
Clone 19	---------- V	*RHR*	YIAPGIW ----------	8.96 / 1882.23
Clone 72	---------- AGDETADG	*RNR*	N ----------	4.56 / 1789.8
Clone 10	---------- GGEV	*RQYVR*	AFA ---------- (2)	8.06/ 1867.13
Clone 48	---------- TSVLL	*RTRYR*	A ----------	9.69/ 1850.19
Clone 56	---------- GV	*RVGLF R*	R MYG ----------	9.69/ 1925.32
Clone 57	---------- FE	*RRR*	LPGAKGR ----------	10.72/ 1957.30
Clone 42	---------- DELTLEKI	*RR*	MA ----------	6.17 / 1989.35
Clone 75	---------- AQPT	*RR*	PSLHRY ----------	9.69/ 1996.30
Clone 50	---------- GNIDRYWLF	*RR*	----------	8.96/ 2097.40
			Range	4.56-12.0/ 1713.96-2097.40

-Amino acid motifs are shown in *italics*

-*Calculated from sequences having CGP at the first and the last of inserted amino acid residues

peptides will be compared with native antigen and evaluated for possible use as an antigen to *V. cholerae* O139.

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