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Biopanning of endotoxin-specific phage displayed peptides☆

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Abstract

Systemic bacterial infections frequently lead to a plethora of symptoms termed "endotoxic shock" or "sepsis." Characterized by hypotension, coagulation abnormalities, and multiple organ failure, treatment of sepsis still remains mostly supportive. Of the various experimental therapeutic interventional strategies, neutralization of endotoxin by peptides or proteins is becoming popular recently. Hence, design of endotoxin binding peptides is gaining currency as their structural complexity and mode of recognition of endotoxin precludes mounting of resistance against them by the susceptible bacteria by genetic recombination, mutation, etc. Earlier work from our laboratory had shown that the amphiphilic cationic peptides are good ligands for endotoxin binding. In this study, we report the results of studies with the 12 selected lipid A binding phage displayed peptides by biopanning of a repertoire of a random pentadecapeptide library displayed on the filamentous M-13 phage. A comparison of the sequences revealed no consensus sequence between the 12 selected peptides suggesting that the lipid A binding motif is not sequence specific which is in accord with the sequence variation seen with the naturally occurring anti-microbial and/or endotoxin binding peptides. Thus, the flexibility of the peptides coupled with their plasticity in recognizing the lipid A moiety, explains their tight binding to endotoxin. At a structural level, asymmetric distribution of the charged polar residues on one face of the helix and non-polar residues on the opposite face appears to correlate with their activity.

Keywords: Lipopolysaccharide; Lipid A; Endotoxin; Surface plasmon resonance; Biopanning

Appearance in circulation of lipopolysaccharide (LPS, endotoxin), the invariant structural component of Gram-negative bacterial outer membranes, as a consequence of systemic bacterial infections frequently leads to a plethora of symptoms termed "endotoxic shock" or "sepsis" [1]. Endotoxic shock is characterized by hypotension, coagulation abnormalities, and multiple organ failure, treatment of which remains non-specific and supportive. Hence, it is associated with poor outcome (40–60% mortality) in human beings [2]. Nonetheless, rapid strides in our understanding of the mechanism by which endotoxin activates the target cells is opening avenues towards several experimental strategies for treating sepsis [3]. These include neutralization of endotoxin by peptides or anti-LPS antibodies, use of its non-toxic analogs that prevent its binding to the susceptible cells or the molecules that compromise the cascade of events that cause the production of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1, interleukin-6, etc. [4–9]. Of these interventional modalities, neutralization of LPS by peptides or proteins is quite an attractive one [4,9]. In fact, a wide variety of organisms, from invertebrates to mammals, elaborate a large number of cationic peptides and proteins that bind endotoxin (cecropins, magainins, and defensins) for defending themselves from microbial onslaughts [3,10–13]. Antibacterial peptides are thought to kill bacteria by perturbing their membranes [12,13]. While their exact mode of membrane disruption remains

☆ *Abbreviations:* LPS, lipopolysaccharide; TNF, tumor necrosis factor; SPR, surface plasmon resonance; TBS, Tris buffer saline; DMPC, dimyristoylphosphatidyl choline; BSA, bovine serum albumin.

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underexplored, they are proposed to act through "pore" formation and "carpet" mechanisms [14–16].

Design of LPS binding peptides is gaining currency as their structural complexity and mode of recognition of LPS/bacteria precludes development of resistance against them by genetic recombination, mutation, etc. In this context, earlier work from our laboratory had shown that the amphiphilic cationic peptides are good ligands for LPS binding [12,17]. These studies have shown that while some of the peptides may be unstructured intrinsically, their acquired helicity upon interactions with the negatively charged surfaces on lipid A accounts for their high specificity as well as their antibacterial activity [12,16]. The latter property of antibacterial peptides also spares the zwitterionic mammalian cell membranes [18,19].

Gram-negative bacterial lipopolysaccharides are comprised of an outermost highly variable "O-antigenic" oligosaccharide region, relatively less variable middle core oligosaccharide region, and the most invariant innermost hydrophobic portion "lipid A" [1,20]. Lipid A accounts for all the toxic effects of endotoxin [1,20]. In this study, we report the results of obtaining lipid A binding peptides by biopanning of a repertoire of a random pentadecapeptide library displayed on the filamentous M-13 phage [21–23]. These studies show that flexibility of the peptides coupled with their plasticity in recognizing the lipid A moiety, explains their tight binding to endotoxin. Asymmetric distribution of the charged polar residues on one face of the helix and nonpolar residues on the opposite face is observed to a considerable extent to correlate with their activity.

Selection procedure

Library used for selection. The fUSE5/15 mer library was obtained from G.P. Smith, Division of Biological Sciences, University of Missouri at Columbia, MO, USA. The complexity of the library used was 1.1×10^{12}.

Panning. The process of selection and panning was carried out as given by Parmley and Smith with minor modifications [21,22]. Lipid A (1 mg) in water was vortexed and heated at 60 °C for 10 min. The lipid A solution was then sonicated for 5 min with a needle probe. This solution (2.0 ml) was then added to polystyrene petri dishes and incubated at 60 °C. Subsequently, the dishes were washed five times with TBS (50 mM Tris–HCl, pH 7.5, and 150 mM NaCl) and blocked with 5 mg/ml of BSA for 2 h. Phages (2×10^{11}) were pipetted into each coated and blocked dish and were kept for 2 h at 4 °C. Unbound phages were washed off with TBS and the bound phages were eluted with 0.1 M glycine HCl buffer (pH 2.2) containing 1 mg/ml BSA and neutralized with 0.1 M Tris–HCl, pH 9.0. The eluate was titered and amplified for use in subsequent round of panning, which were carried out in similarly coated wells of a 96-well microtitre plate. The unbound phages of this round were used as the control phages [ET-(0) clone].

Panning was repeated for an additional three rounds wherein the bound phages eluted successively with TBS containing 0.1, 0.2, and 0.5% Tween 20. Twelve clones from the last eluate, designated as ET-(1–12), were used for further characterization and DNA prepared for sequencing.

Surface plasmon resonance

Hybrid bilayers containing 20 mol% lipid A in DMPC on HPA chip were formed as described earlier [17]. Typically 850–900 response units (RU) of lipid A in DMPC were layered on each of the flow cell.

Conditions for the specific binding of the phages were determined initially using phages eluted with TBS alone, ET-(0), in the second panning reaction (non-binding phage clones) as the control. Binding reactions with ET-(1–12) clones were then studied at different pH (5.5–8.2). Phosphate buffer (10 mM) was used between pH 5.5–6.5 and Tris–HCl (10 mM) in pH 7.0–8.2 region. The apparent strength of binding of the phages was evaluated by injecting the phages (1.5–24 nM; 1–16×10^{11} pfu/ml) at a flow rate of 25 μl/min for 110 s followed by sodium phosphate buffer (10 mM), pH 6.3, containing 150 mM NaCl for 890 s. These data were subtracted against those obtained with non-binding phage clone ET-(0). Binding surfaces were regenerated by the 20 s pulses of 50 mM NaOH followed by washing with PBS [12,17,24]. The rate constants were determined by the nonlinear least squares fitting of the primary sensogram data using BiaEvaluation, Version 3.0 software.

Prediction of the α-helical content and physicochemical parameters of the peptides

Amino acid composition and p*I* of the peptides were calculated online with the help of Compute pI/Mw tool at ExPASy (http://tw.expasy.org/tools/pi_tool.html), while their helix content at pH 6.2, 278 K, and ionic strength of 0.15 was predicted with Agadir algorithm [25]; http://www.embl-heidelberg.de/services/serrano/agadir/agadir.start.html.

Mean hydrophobic moments, which represent the average values of the whole sequence in either α-helical or β-sheet conformation, and the mean hydrophobicities were calculated as described in [http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html] [26–28].

Results and discussion

Our studies reported here exploit phage display to identify lipid A binding peptide sequences. A number of studies in the past have shown that lipid A, the invariant component of LPS, is responsible for all the toxic properties of endotoxin [1–3,20]. Hence, we have used lipid A for the selection of specific phages that bind during biopanning. After five rounds of panning and amplification cycles, 12 phage clones against the immobilized lipid A were randomly selected. The clones thus selected have been designated as ET-(1–12) and characterized further for their lipid A binding propensities by surface plasmon resonance (SPR), which in turn are correlated with the helicity, hydrophobicity, and the amphiphilicity of the phage-displayed peptides.

SPR offers several advantages for the study of macromolecule-ligand interaction, as it relies solely on mass changes during the reaction [12,16,29]. Hence, use of fluorophores or chromophores, etc. that can sometime perturb the nature of the reaction can be avoided. Moreover, from a single experimental run it is possible to obtain both the association and dissociation rate constants, k_1 and k_{-1}, respectively, as well as the association constant ($K_a = k_1/k_{-1}$). Finally, SPR allows the study of surface/membrane-associated phenomena due

to the possibility of incorporating one of the reactants (receptor) in a model membrane system, such as a monolayer or a bilayer [16,17]. In these studies, we have incorporated lipid A in L-α-dimyristoylphosphatidyl choline on an HPA chip and studied the kinetics of its binding to the selected phage clones.

Initially, the conditions for the specific adsorption of phages to lipid A immobilized on HPA chip were standardized, as shown in Figs. 1A and B, with ET-(5) clone as a representative example, between pH 5.5 and 8.2 (Fig. 1A) and as a function of NaCl concentration (Fig. 1B). As can be seen from Fig. 1B maximum specific adsorption of an ET phage to the immobilized lipid in DMPC monolayers is observed at 150 mM NaCl and between pH 6.0 and 6.5. Like ET-(5), most phages show highest activity between pH 6.0 and 6.5 (10 mM phosphate) and with 150 mM NaCl. Hence, most of the SPR experiments were performed in 20 mM phosphate buffer

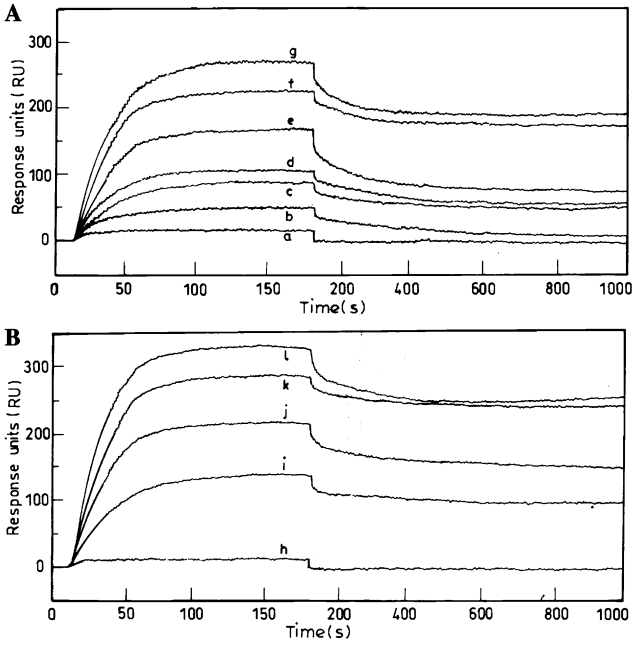

Fig. 1. Effect of pH and salt on the binding of phage displayed peptide [Et-(5)] to the immobilized lipid A at 20 °C. (A) Sensogram for the binding and dissociation of ET-(5) phage to lipid A immobilized on HPA chip at pH 8.2 (b), 7.5 (c), 7 (d), 5.5 (e), 6.5 (f), and 6.0 (g) with 18 nM of ET-(5) phage was studied. In the pH range of 7.0–8.2 Tris-buffered saline (10 mM Tris and 150 mM NaCl) and from pH 5.5–6.5 in Na phosphate buffered (10 mM) saline, were used. Binding phase of the reaction was studied by flowing 18.0 nM of the phage displayed peptide at a rate of 25 μl/min for 3 min in the appropriate buffer followed by the dissociation phase of the reaction where the same buffer only was passed over the immobilized lipid A. Control experiment (a) consisted of repeating the above experiment with ET-(0) clone in phosphate buffer (pH 6.3, 10 mM) containing 150 mM NaCl. (B) Binding and dissociation of ET-(5) phage displayed peptide was studied in 10 mM phosphate buffer (pH 6.3) without NaCl (i), with 100 (j), 150 (l), 250 (k), and 500 mM NaCl (h). All the other conditions are same as for (A).

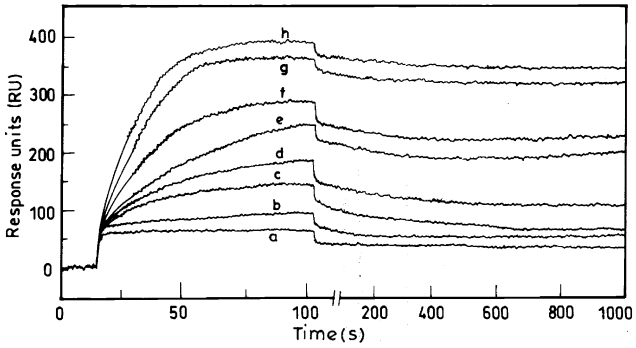

Fig. 2. Determination of the association (k_1) and dissociation rates ($k-1$) for the binding of phage displayed peptide [ET-(5)]. Binding and reactions were carried out in phosphate-buffered saline (10 mM, pH 6.3, containing 150 mM NaCl) at 20 °C. Concentrations of phage displayed peptide used were 1.5 nM (b), 3.0 nM (c), 6.0 nM (d), 9.0 nM (e), 12.0 nM (f), 18.0 nM (g), and 24.0 nM (h). ET-(0), 24 nM under identical conditions was used as the control.

(pH 6.3) containing 150 mM NaCl. The fact that ET phages bind to immobilized lipid A at relatively high ionic strength supports the notion that the hydrophobic interactions contribute significantly to the stability of interactions between the side chains of the displayed peptides and lipid A and that neutralization of electrostatic repulsion explains their heightened interactions at moderate ionic strengths. However, abrogation of these interactions at still higher ionic strength indicates the significance of electrostatic interactions in these reactions as well. ET-(5), shown as a representative example, exhibits association and dissociation rates and association constant of $8.2 \times 10^4\ M^{-1}\ s^{-1}$, $0.9 \times 10^{-2}\ s^{-1}$, and $9 \times 10^6\ M^{-1}\ s^{-1}$, respectively, for interacting with endotoxin (Fig. 2). K_a values for the other displayed peptides are in the range of $0.1–1.2 \times 10^7\ M^{-1}\ s^{-1}$ (Table 1).

Selected pentadecapeptide display cationicity and amphiphilicity

The deduced amino acid sequences of phage-displayed peptides, determined from the DNA sequence inserted in the gpIII coat protein are shown in Table 1 from which the abundance of positively charged and non-polar amino acid residues becomes obvious. The observed cationicity and amphiphilicity is consistent with the features expected in lipid A binding peptides. Basic amino acids impart cationicity to the peptides, which allows their strong and specific binding to bacterial surface membrane. It may, however, be noted that of the three cationic residues arginine, lysine, and histidine, the latter occurs most frequently and the first type being the least abundant. Though both arginine and histidine are basic amino acids, arginine in phage displayed-peptides tends to preclude the secretion of the corresponding viral clones explaining its low abundance as compared to histidine in the ET-clones described here

Table 1
[Helicity]: predicted helical content (%) at pH 7, 278 K, ionic strength of 0.15 M NaCl

Peptide		[H]	MH	MHM/a	MHM/b	p*I*	k_{+1}	k_{-1}	K_a
1	KALLHHLALLALHLA	1.27	1.31	1.00	0.76	8.77	2.1	0.45	0.46
2	WIALHHLLHLAAHWI	0.33	0.99	1.19	0.81	7.10	1.1	0.05	4.2
3	WALAHKALHALAHKP	0.70	−0.04	0.96	0.31	10.00	6.4	0.12	5.3
4	KWLAKHAAGLALHAL	0.41	0.49	1.23	0.68	10.00	5.1	1.1	4.6
5	VLALHHALALAHKKA	0.92	0.65	0.84	0.08	10.00	7.3	0.9	9.0
6	PAALHHALALAHHLW	0.44	0.59	1.09	0.82	7.49	7.2	0.6	12.0
7	WMHKHQALAAMHAHR	0.71	−0.76	0.41	0.16	11.00	0.45	3.7	0.12
8	RQAHTHALHHLALWC	0.14	−0.20	0.74	0.91	8.28	0.23	1.8	0.13
9	WRLHHRHFLALALKR	0.76	−0.45	0.24	0.91	12.30	2.1	9.8	0.21
10	TPHLHMFHAHKLAPR	0.11	−0.65	0.47	0.64	11.00	1.4	8.4	0.16
11	TPHAHMWHAHKRNPK	0.25	−1.94	0.44	0.55	11.17	5.6	1.1	0.56
12	PVHHVHLTAHHAVGC	0.11	0.25	1.00	0.88	7.49	2.4	1.58	1.5

http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html; MH, mean hydrophobicity, MHM/a: mean hydrophobicity moment as a α-helix (projection angle = 100). MHM/b: mean hydrophobicity moment as a β-sheet (projection angle = 180) http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html; p*I* calculated according to the method of Bjellqvist et al. [25] http://us.expasy.org/tools/pi_ tool.html. k_{+1}: Forward rate (Unit $\times 10^4$ M^{-1} s^{-1}), k_{-1}: backward rate (Unit $\times 10^{-2}$ s^{-1}), and K_a: association rate (Unit $\times 10^6$ M^{-1}).

A

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1                -KALLH-HLALLALHLA- 15
2                -WIALH-HLLHLAAHWI- 15
3                -WALAHKALHALAHKP-- 15
4                KWLAKHAAGLALHAL--- 15
5                -VLALH-HALALAHKKA- 15
6                -PAALH-HALALAHHLW- 15
7                -WMHKHQALAAMHAHR-- 15
8                --RQAHTHALHHLALWC- 15
9                -WRLHHRHFLALALKR-- 15
10               ---TPHLHMFHAHKLAPR 15
11               ---TPHAHMWHAHKRNPK 15
12               --PVHHVHLTAHHAVGC- 15
Consensus/80%    ...h.H.hhhhhhhb...
Consensus/70%    ...h.H.Hhhhhhhb...
Consensus/60%    ...hhH.Hhhhhhh+...
Consensus/51%    ..hshH.HhhALhh+b..
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B

Small Polar				Large Polar			Small Non-Polar			
D	E	N	Q	K	R	H	S	T	A	G
0	0	0.55	1.11	6.67	3.89	23.89	0	2.22	23.33	1.11

Large Non-Polar								
L	I	V	M	F	Y	W	C	P
20.55	1.11	2.22	2.22	1.11	0	5	1.11	3.89

Fig. 3. (A) Multiple sequence alignment of peptides and the consensus sequences. h, denotes hydrophobic; +, positive; b, big; and s, denotes small amino acid. (B). Percent amino acid composition of peptides.

[30]. Unpaired cysteines on the other hand are deleterious for phage infectivity and hence are least abundant of the amino acids in these clones [30].

Among the bulkier hydrophobic residues i.e. tryptophan, tyrosine, and phenylalanine, the former was relatively more abundant. Tryptophan is known to position itself near the membrane interface. Tryptophan residues thus help in the partitioning of the amphiphilic peptides in the membrane and are found in abundance in some antibacterial peptides [31–33]. Among the uncharged polar residues proline and threonine are also seen which may be related to their ability to facilitate partitioning into membranes and consequently are relatively abundant in some of the naturally occurring antibacterial peptides [31] (Fig. 3).

Contiguity of basic residues on one face of the amphiphilic helix

A comparison of the sequences using multiple sequence alignment program. CLUSTAL W [34] revealed no consensus sequence between the 12 selected peptides, suggesting that the lipid A binding motif is not sequence specific which is in accord with the sequence variation seen with the naturally occurring anti-microbial peptides [10,13–16,31]. However, around 51% consensus of two histidines (at positions 4 and 6), an alanine and a leucine residue appear to occur most frequently at positions 9 and 10, respectively, in the displayed peptides. Thus, a relatively specific distribution of cationic and hydrophobic residues in the displayed peptides can be noted. Also, these peptides do not seem to form a very defined secondary structural element, Table 1). Nonetheless, in contact with membranes, especially, those of bacteria, they may exhibit α-helical structures. Hence, we analyzed their lipid A binding activity versus their helicity using a helical wheel projection. In an earlier study, we surmised that in the helical wheel representation of the designed 23-residue synthetic anti-microbial peptides, which had cationic residues, clustered on one face of the helix and the hydrophobic residues on the other, were the most potent endotoxin binders [12]. Moreover, peptides, which had a disruption in the contiguity of the basic residues, were poor in recognizing lipid A. These earlier insights are supported by studies on the phage-displayed peptides reported here. For example, peptides 2, 3, 5, and 6, which show a marked asymmetric distribution of the basic and the non-polar residues on the opposite sides of the amphipathic helix, show the highest

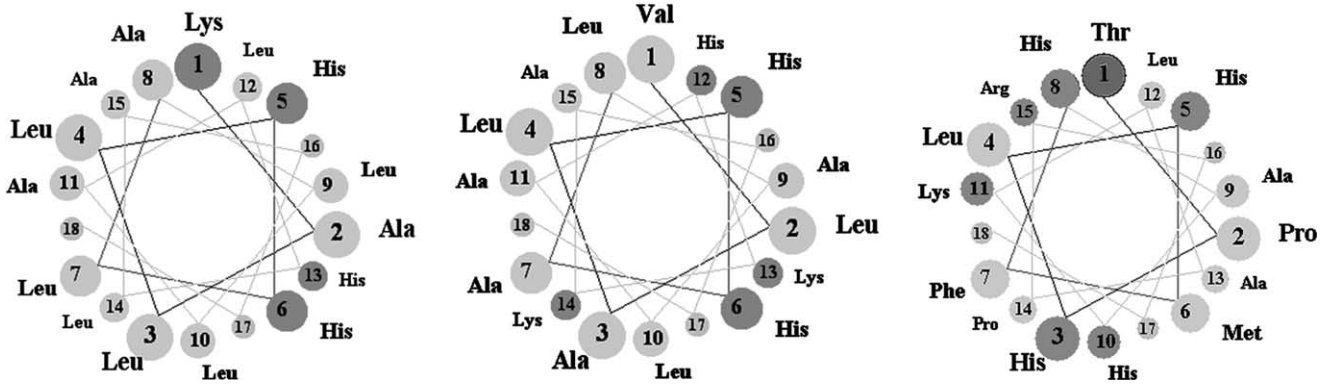

Fig. 4. Helical wheel representation of a moderate [ET- (1)], a strong [ET- (5)], and a poor endotoxin binding peptide [ET- (10)]. Shading scheme: light circles (non-polar), dark circles (basic) and closed dark circle (popular-uncharged). Helical wheels were generated by using an applet available at http://cti.itc.virginia.edu~cmg/Demo/wheel/wheelApp.html.

affinity for lipid A as compared to their poor binding counterparts, such as peptides 7, 8, 9, and 10, whereas the rest of the peptides had intermediate potency (Fig. 4).

In conclusion, our studies show that biopanning of a 15-mer random phage display library shows a propensity for the selection of sequences that exhibit pronounced cationicity and amphipathicity. Selection with constrained peptides should lead to more potent endotoxin recognizing peptides as has been noted earlier with synthetic peptides [35].

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