Structural basis of endotoxin recognition by natural polypeptides

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At the end of the 1970s, it became evident that the biological reactions induced by bacterial endotoxin, or lipopolysaccharide (LPS), resulted from the peculiar features of lipid A, the biologically active region of LPS, which is conserved among LPS originating from different Gram-negative bacteria[1]. This was unequivocally confirmed when lipid A was chemically synthesized as *N*,*O*-acyl-β-1,6-diglucosamine-1,4'-bisphosphate, and found to be capable of reproducing all the major biological effects of natural LPS (Ref. 2). Since then, a large body of literature has reported on the variety of biological effects that are induced by LPS in mammals. Much of this work has focused on the interactions between LPS and the receptor proteins of immunocompetent cells, which result in the induction and release of cytokines, such as tumor necrosis factor, interleukins 1, 6 and 8 and gamma-interferon[3–5], all of which are co-mediators and markers of septic shock.

The target receptors and the mechanism by which LPS interacts, via lipid A, with cell and serum proteins are gradually becoming clear. For example, the 55 kDa glycoprotein CD14 is now recognized as the receptor for LPS on mononuclear phagocytes[6]. The size and amino acid sequence of the lipid-A-binding site have been mapped recently from the structures of synthetic peptides that mimic the primary and secondary structures of the antibiotic polymyxin B (Ref. 7). Polymyxin B is known to bind stoichiometrically to and detoxify lipid A (Ref. 8). This finding prompted the search for similar amino acid sequences in the primary structures of five proteins from different species that are known to have a high specificity for the lipid A moiety of heterologous LPS. The proteins studied were the 55 kDa receptor protein CD14 of humans and mice, the 60 kDa plasma glycoprotein LPS-binding protein (LBP) of humans and rabbits, the 55 kDa bactericidal/permeability-increasing protein (BPI) of humans, the 15 kDa polypeptide *Limulus* anti-LPS factor (LALF) and the 12 kDa polypeptide *Limulus* endotoxin-binding protein-protease inhibitor, both of the horseshoe crab *Limulus polyphemus*. In each of these natural polypeptides, at least one sequence of 8–13 amino acids was found with physicochemical characteristics similar to those reported for the lipid-A-binding site[7]. The corresponding synthetic peptides competitively inhibit the toxicity of heterologous LPS, *in vitro* and *in vivo*[9].

Among the peptides defined as described above is that corresponding to amino acids 41–51 of LALF. This peptide is cationic and lies in the amphipathic loop (amino acids 32–50) of LALF. In the recent work by Hoess *et al.*[10], this sequence was proposed to be the lipid-A-binding region of LALF, based on studies of the crystal structure of recombinant LALF, as well as on the sequence similarity with polymyxin B. These authors also speculate, on the basis of sequence similarities, that the mammalian proteins LBP and BPI should bind lipid A somewhere within the region of amino acids 85–104. Synthetic peptides corresponding to amino acids 92–100 of LBP and amino acids 90–99 of BPI do bind and detoxify lipid A (Ref. 9). Thus, different laboratories using different experimental approaches are coming to the consensus that the lipid A moiety of LPS is recognized by short sequences from natural polypeptides that have cationic and amphipathic characteristics.

Is it possible to speculate about the minimal and optimal sequence requirements of natural polypeptides for binding to lipid A? A tentative answer may come from studies using linear and cyclic peptides synthesized in our laboratory with $(AB)_n$ or $(ABC)_n$ motifs, where A amino acids are aliphatic and cationic, with solvent parameter values $\geq +1.5$ kcal mol^{-1} (lysine and arginine), and B, C amino acids are hydrophobic, with solvent parameter values ≥ -1.5 kcal mol^{-1} (tryptophan, phenylalanine, tyrosine, leucine, isoleucine and valine). The solvent parameter values used to select the amino acids were those assigned by Levitt[11], and each peptide synthesized was characterized by the value of Rc/h, which expresses the content of aliphatic cationic versus hydrophobic amino acids present in the sequence[9]. Based on binding competition for lipid A and on inhibition of the lipid-A-induced clotting of *Limulus* amebocyte lysate, the minimal amino acid sequence that binds and detoxifies lipid A has six (cyclic) or seven (linear) residues containing a minimum of three cationic amino acids with Rc/h values ≥ 0.5. High-affinity binding occurs with sequences of ten amino acids having Rc/h values ≥ 1.0. The results obtained in our laboratory with a variety of synthetic peptides similar to those defined from primary sequences, with and without restricted secondary rearrangement[9], suggest that these features may be similar to the general target of lipid A in the secondary structure of proteins.

How do cationic and amphipathic sequences bind to the structure of lipid A? From the synthetic peptide model, the binding mechanism appears to involve preliminary

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interactions with the anionic phosphate groups of lipid A, since lysine and arginine residues are essential. However, the stability of the complex requires hydrophobic interactions, possibly including those between the fatty acid residues of lipid A and hydrophobic amino acids and/or the alkyl chains of lysine and arginine residues. Indeed, similar peptides containing histidine do not bind to lipid A of heterologous LPS, the reactivity of the ε-amino groups of lysine residues to picrylsulfonic acid is retained after complex formation and the complex is stable at extreme pH values and high ionic strength[7]. Similar conclusions have been reported for LPS interacting with tubulin, the globular protein that forms microtubules in the cytoskeleton[12].

What is the significance of these studies? Being able to explain the molecular mechanism by which LPS binds to tubulins might result in a deeper understanding of the process by which LPS injures the blood–brain barrier. Such injury may, for example, allow otherwise noninvasive viruses to penetrate the central nervous system and cause encephalitis[13]. The wider significance of these studies, however, lies in the efficiency of the recognition systems involving natural polypeptides, which perform different biological functions in various species, but which bind to the conserved lipid-A-binding site of heterologous LPS via the conserved cationic and amphipathic amino acid sequences. Furthermore, the dimensions estimated for the lipid-A-binding site as derived from structurally defined peptides[7] agree well with those reported from structural studies of lipid A (Ref. 14). They are also comparable with the size of epitopes recognized by monospecific antibodies[15,16] and by human major histocompatibility complex class I molecules[17]. In this respect, the recognition mechanism used by proteins that bind to LPS shows some similarities with immunorecognition. Finally, this work provides the opportunity to exploit a novel generation of peptide-based molecules that specifically bind to lipid A for the development of drugs against LPS-mediated diseases.

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