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MINI-REVIEW

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Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins

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Abstract Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin are well-studied prototypes of pore-forming bacterial cytotoxins. Each is produced as a water-soluble single-chain polypeptide that inserts into target membranes to form aqueous transmembrane pores. This review will compare properties of the three toxin prototypes, highlighting the similarities and also the differences in their structure, mode of binding, mechanism of pore formation, and the responses they elicit in target cells. Pore-forming toxins represent the most potent and versatile weapons with which invading microbes damage the host macroorganism.

Key words Alpha-toxin · Streptolysin-O · *Escherichia coli* hemolysin · Transmembrane pores · Microbial pathogenesis

Abbreviations *HlyA Escherichia coli* hemolysin · *ICE* Interleukin-converting enzyme · *RTX-family* Repeats in toxin family · *SLO* Streptolysin-O

The concept of transmembrane pore formation by bacterial protein toxins was first forwarded 15 years ago to explain the mechanism of action of staphylococcal alpha-toxin (Füssle et al. 1981). Since then, the list of bacterial pore-forming toxins has been growing steadily, and it is today apparent that the majority of medically relevant pathogens produce pore-forming proteins (Bhakdi and Tranum-Jensen 1988; Bhakdi et al. 1994). Many of these toxins have been designated hemolysins because of their lytic action on red cells. However, it is now recognized that their biological significance derives from their action on nucleated cells and platelets. The toxins exert detrimental local effects by destroying tissue cells and also immune cells involved in first-line defense. They thus directly promote bacterial infections. Furthermore, pore-forming toxins provoke a wide spectrum of secondary reactions on target cells that in turn elicit short- and long-range effects in the macroorganism, thus contributing to the pathogenesis of bacterial infections (Bhakdi et al. 1994). Our laboratories have been engaged in the study of staphylococcal alpha-toxin, streptolysin-O (SLO), and *Escherichia coli* hemolysin (HlyA) on a molecular and functional level. In this review, we will compare the properties of these three toxin prototypes and discuss how the basic principle of pore formation can be varied to yield a surprisingly complex theme. Properties and some basic features of the three toxin prototypes are summarized in Table 1.

Structure and functional domains

All three toxins are single-chain polypeptides, but their structural similarity ends here. Alpha-toxin is a 34-kDa protein consisting of 293 amino acid residues and lacking cysteine (Gray and Kehoe 1984). The molecule contains no extended stretch of hydrophobic amino acids. CD measurements indicate an abundance of β-sheet structure in the native monomer and in the pore-forming oligomer (Tobkes et al. 1985). Native alpha-toxin is perfectly water-soluble, and it is present in monomeric form in aqueous buffers. However, it has a tendency to aggregate slowly in solution to form the same ring-shaped oligomers that form rapidly in lipid bilayers. The rate of oligomer formation in solution is markedly accelerated by deoxycholate (Bhakdi et al. 1981). The toxin complexes formed

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Table 1 Properties and basic features of the toxin prototypes alpha-toxin, streptolysin-0, and *Escherichia coli* hemolysin

	Alpha-Toxin	Streptolysin-O	E. *coli* Hemolysin
Molecular weight	33,400	61,500	110,000
Amino acid residues	297 (no cysteine)	538 (one cysteine)	1024 (no cysteine)
Oligomer size	Heptamer	Heterogeneous: estimated 25–80 monomers	Unknown/existence uncertain
Membrane-binding domain	Probably composite, formed from regions in N- and C-terminal halves	C-Terminal domain; central domain possibly also involved	Repeat, Ca^{2+}-binding domain implicated; hydrophobic domain possibly also involved
Pore-forming domain	Central amino acid sequence (residues 118–140)	Unknown	Unknown
Specific features	Primary binding to unidentified receptor; small pore size (estimated 6–10 Å);	Prototype of cholesterol-binding toxin family (includes pneumolysin, perfringolysin, listerolysin); very large pore size (up to 30 nm diameter); N-terminal 67 amino acid residues not required for binding or pore formation	Prototype of RTX family (at least 14 members); activity dependent on the presence of Ca^{2+}; mimics chemokines and triggers G-protein-dependent processes; fatty acylation required neither for binding nor for pseudo-chemokine activity, but necessary for efficient pore formation; pore size 10–15 Å

in the presence of this detergent have been crystallized, and the available data indicate that they represent heptamers (Gouaux et al. 1994). Thus, the alpha-toxin pore is probably a heptamer and not a hexamer, as was long believed on the basis of earlier biochemical and physicochemical studies (Bhakdi and Tranum-Jensen 1991).

Indirect evidence indicates that the N- and the C-terminal regions are involved in forming a composite, membrane-binding epitope (Walker and Bayley 1995; U. Weller et al., unpublished results). The central molecular domain encompassing residues 118–140 was recently identified as the major and perhaps sole membrane-inserting, pore-forming domain (A. Valeva et al., in press).

Secreted SLO is a 61-kDa polypeptide containing 541 amino acid residues (Kehoe et al. 1987). It is the prototype of a large family of cholesterol-binding cytolysins that are produced by gram-positive organisms (Alouf and Geoffrey 1991). The C-terminal region harbors a highly conserved stretch of hydrophobic amino acids. The single cysteine residue of the protein is located within the hydrophobic region. The hydrophobic C-terminus of SLO endows the molecule with an amphiphilic nature; the native monomer binds the non-denaturing detergent Triton X-100 (U. Weller et al., unpublished results). Nevertheless, secreted SLO does not form large aggregates in solution. The N-terminal region of SLO is, surprisingly, superfluous for its pore-forming function. The N-terminal 70 residues of the secreted protein, representing over one-eighth of the molecule, can be removed without loss of specific activity. The C-terminal region of the molecule is involved in membrane binding; other functional domains have not been identified (U. Weller et al., unpublished results).

E. coli hemolysin (HlyA) is a 107-kDa protein of 1,024 amino acid residues devoid of cysteine (Felmlee et al. 1985). The polypeptide requires post-translational fatty acylation of two lysine residues to obtain pore-forming activity (Issartel et al. 1991; Stanley et al. 1994). It represents the prototype of the RTX family of cytolysins that are produced by a large number of gram-negative organisms (Welch 1991). The protein harbors a hydrophobic domain between residues 177 and 411. The C-terminal half of the molecule contains 12 repeats of the consensus nonapeptide sequence X-Leu-X-Gly-Gly X-X-Gly-Asn/Asp-Asp. This repeat sequence represents a calcium-binding domain and is essential for function (Felmlee and Welch 1988). Withdrawal of Ca^{2+} by chelating agents leads to perturbation of the molecular conformation required for binding of the toxin to lipid bilayers (Ludwig et al. 1988; Boehm et al. 1990). In contrast to alpha-toxin and SLO, HlyA displays a strong tendency to aggregate spontaneously in solution, this being accompanied by loss of „bindability" (binding capability).

Membrane binding

Binding studies conducted with all three toxins have illustrated their different modes of interaction with target cells. Alpha-toxin binds to rabbit erythrocytes via two distinct interactions (Hildebrand et al. 1991). At low concentrations (< 50 nM), the toxin binds exclusively via a high affinity interaction with an as yet unidentified cellular receptor. Binding is saturable and displays an optimum at 22–26°C. At higher concentrations (> 200 nM), the toxin will absorb nonspecifically to lipid bilayers. This accounts for its permeabilizing action on cells lacking the receptor (e.g., human erythrocytes) and on protein-free lipid bilayers (liposomes and planar lipid membranes). Binding displays no ionic requirements and occurs at low temperature. Since specific binding sites for alpha-toxin are limited, this toxin never quantitatively binds to target cells.

SLO interacts primarily with cholesterol molecules in target bilayers (Alouf and Geoffrey 1991). Binding studies conducted with perfringolysin, a related toxin of this family, indicate that binding sites with different affinities may exist depending on the lipid micro-environment (Ohno-Iwashita et al. 1988). Binding of monomeric SLO occurs at low and ambient temperature and is rapidly reversible. The C-terminus plays a pivotal role in binding; truncation of only two residues at the C-terminus causes loss of bindability (U. Weller et al., unpublished results). The importance of the C-terminus for toxin-binding has also been shown for perfringolysin (Iwamoto et al. 1990) and pneumolysin (Owen et al. 1994). Modification of the single sulfhydryl group also leads to loss of bindability. However, the cysteine residue is per se not essential, and it can be replaced with alanine without loss of functional activity (Pinkney et al. 1988). The alanine-substituted SLO is then no longer prone to inactivation by sulfhydryl-reactive reagents or by oxidation. Since SLO binds to cholesterol, cell binding displays no saturability, and the toxin is rapidly and quantitatively incorporated into the plasma membrane of target cells.

In contrast to alpha-toxin and SLO, HlyA does not appear to interact with any specific acceptor molecule in target membranes. Binding is non-saturable and is independent of the presence of proteins or cholesterol in the membranes (S. Bhakdi et al., unpublished results). Binding does not per se require the presence of the covalently bound fatty acids; however, it is possible that the fatty acids stabilize the membrane interaction (S. Bhakdi et al., unpublished results). Functional domains of HlyA have not been clearly identified. The Ca^{2+}-binding, repeat domain is required for the toxin to acquire binding properties. However, whether this domain directly constitutes the binding epitope is not known. In analogy to SLO, we speculate that the hydrophobic sequence in HlyA forms at least part of the primary binding site. Possibly this domain can spontaneously insert into lipid bilayers without the requirement of any specific acceptor molecules.

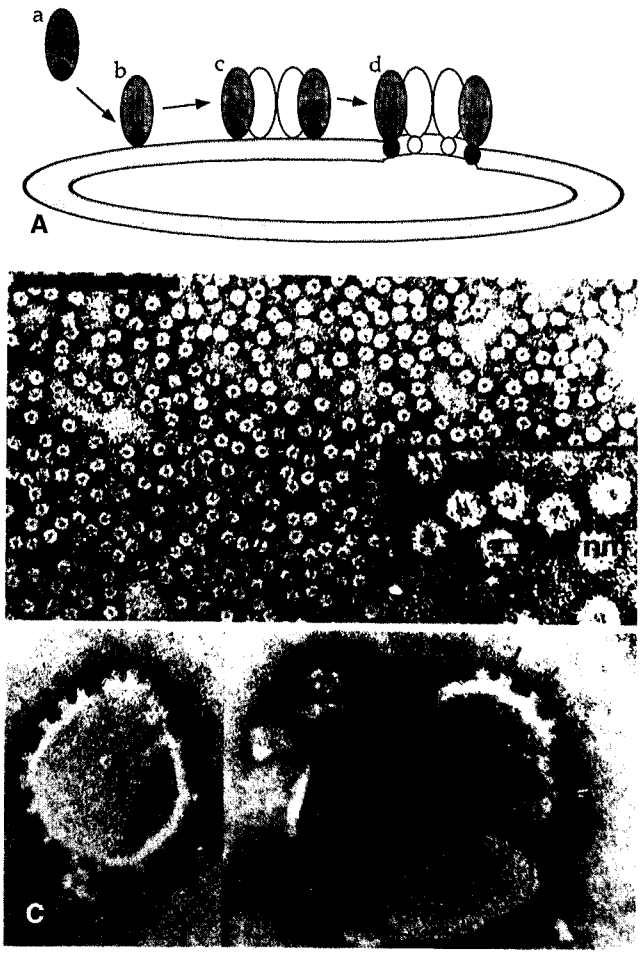

Fig. 1 **A** Assembly model for alpha-toxin in lipid bilayers. Water-soluble native monomers (*a*) bind to and orient themselves on lipid bilayers (*b*). Membrane-bound monomers collide via lateral diffusion in the membrane plane to form pre-pore complexes (*c*). Oligomerization provides the driving force for insertion of the central molecular domain into the bilayer; a hydrophilic transmembrane pore traverses the center of the circularized, heptameric protein complex (*d*). **B** Isolated toxin heptamers in detergent solution. **C** Lecithin liposomes carrying reincorporated alpha-toxin heptamers. The heptamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (*arrows*). *Bar* 100 nm (in **B** and **C**). (From Bhakdi and Tranum-Jensen 1988; with permission from Karger Verlag)

Pore formation

In order for aqueous transmembrane pores to be created, lipid molecules must be laterally displaced. The process of pore formation is always ATP-independent; therefore, the energy required for creating the pores must derive from conformational changes in the toxin molecules themselves. The most easily envisaged underlying mechanism is the oligomerization of toxin protomers to form partially or fully circularized protein complexes. This mechanism is definitely operative in the case of alpha-toxin (Füssle et al. 1981) and SLO (Bhakdi et al. 1985). That HlyA also forms oligomers in membranes has been proposed (Benz et al. 1989), but the supporting data are indirect.

Pore formation by alpha-toxin has been studied in the greatest detail. The available data on the steps underlying pore assembly can be accommodated in the following model that envisages four states for the toxin to exist (Walker et al. 1992). The first state is the water-soluble, native monomer (Fig. 1A, a). The second state is the membrane-bound monomer, which can be produced by incubating membranes with low concentrations of alpha-toxin (Fig. 1A, b). The third state is represented by a nascent oligomer or a pre-pore complex, in which membrane-bound toxin molecules have entered into tight non-covalent interaction with each other, and the pore-forming domain is on the verge of being forced into the bilayer (Fig. 1A, c) (Walker et al. 1995). It is possible to arrest the assembly mechanism at this stage with the use of toxin mutants that show faulty oligomerization. The fourth and

final state is the pore configuration itself (Fig. 1A, d). Once formed, these protein pores assume properties akin to those of integral membrane proteins and remain irreversibly anchored in the bilayer.

Delineating the principle of pore assembly by alpha-toxin necessitated the introduction of experimental approaches that combined molecular engineering with spectroscopic, biochemical, and functional studies. An important approach was the use of cysteine-substitution mutants. By attaching the polarity-sensitive probe acrylodan to the sulfhydryl group contained in the different single cysteine mutants, it became possible to probe the environment of different amino acid residues throughout the entire molecule (A. Valeva et al., in press). These studies disclosed that of the 293 amino acid residues in the molecule, probably only the central domain encompassing residues 118–140 are involved in forming the lipid-inserted protein cylinder. Residues 118–124 thereby form part of the surface that is involved in protein-protein interaction. Other as yet unidentified domains, possibly including those flanking the pore-forming central region, provide further contact sites whose interaction is thought to release the energy required to drive the pore-forming domain into the bilayer. Residue 121 lines the mouth of the pore, and residue 130 inserts deeply into the bilayer, probably to reach the cytoplasmic membrane face (Palmer et al. 1993a; Ward et al. 1994; A. Valeva et al., unpublished results).

The contention that the central region in the alpha-toxin molecule is directly involved in forming the pore is supported by several other independent lines of evidence. Proteolytic nicking in the central region alters the functional properties of the toxin without affecting bindability (Palmer et al. 1993b). Toxin mutants engineered to yield overlaps, nicks, and gaps in this region also exhibit altered pore-forming activities (Walker et al. 1992, 1993; Walker and Bayley 1994). Furthermore, toxin mutants carrying histidine residues in the central region form pores that can be closed by Zn^{2+} (Walker et al. 1994). All these data point to a pivotal role of the central domain in forming the pore.

The size of the pore created by alpha-toxin exhibits slight variation. In planar lipid membranes and erythro-

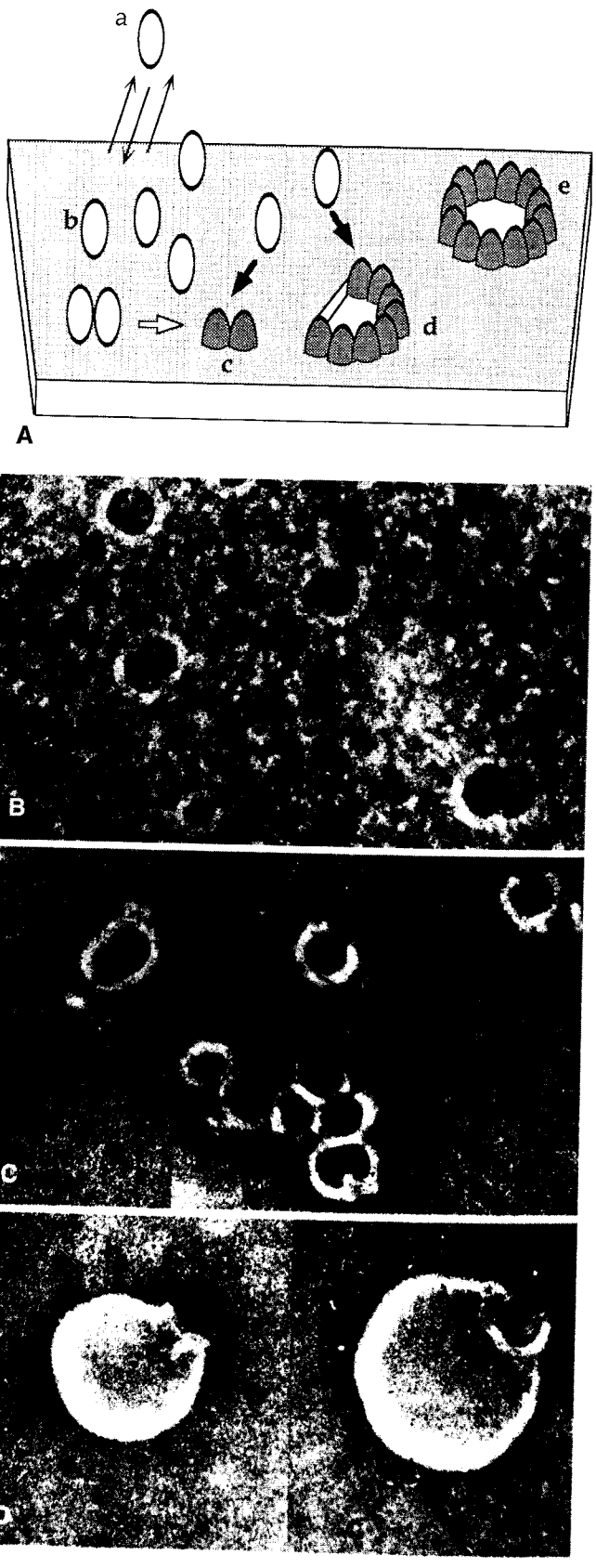

Fig. 2 **A** Assembly model for streptolysin-O pores in lipid bilayers. Monomers in solution (*a*) first bind reversibly to the membrane. Two membrane-bound monomers (*b*) then react to form a membrane-inserted dimer (*c*), which by rapid association of further monomers is extended into (*d*) arc-shaped and finally (*e*) ring-shaped complexes constituting transmembrane pores. Along with oligomer growth, the membrane is disrupted in a stepwise fashion. **B** Negatively stained erythrocyte membrane lysed by streptolysin-O (SLO) showing numerous curved rods 25–100 nm long and approximately 7.5 nm wide with an inner radius of curvature of 13–16 nm. **C** Negative staining of isolated SLO polymers, showing numerous curved rod structures identical to those found in toxin-treated membranes. **D** SLO polymers reincorporated into lecithin liposomes. The toxin oligomers form holes in the liposomes; *p* indicates a lesion seen in profile. *Bar* 100 nm (in **B–D**). (From Bhakdi and Tranum-Jensen 1988; with permission from Karger Verlag)

cytes, the pore has been sized to 1–1.5 nm in diameter (Füssle et al. 1981; Menestrina 1986). In nucleated cells harboring the acceptor sites, however, the pores appear to be smaller, exhibiting a selectivity for monovalent ions (Walev et al. 1993; Jonas et al. 1994). The cause for this small, but significant variation in pore size is essentially unknown.

SLO also binds as a monomer to membranes. A rate-limiting nucleation process, probably involving the formation of dimers, then follows. Thereafter, the putative dimers function as crystallization points for the rapid successive attachment of other monomers. The SLO complexes grow into arcs and rings that represent the transmembrane pores (Palmer et al. 1995) (Fig. 2). The contact sites involved in protein-protein interactions and the membrane inserting domains are currently being studied by using cysteine-substitution mutants in experiments analogous to those performed with alpha-toxin. The pores created by SLO are the largest known to date and measure 30–35 nm in diameter (Buckingham and Duncan 1983; Bhakdi et al. 1985). The pores are stable and can be isolated in non-denaturing detergents, but they are dissociated in SDS (Bhakdi et al. 1985).

The mechanisms underlying pore formation by HlyA remain largely unknown. Data derived from experiments with planar lipid membranes have been interpreted to indicate that pore formation involves assembly of oligomers (Benz et al. 1989). However, these have not been directly detected.

Reactions of cells under attack by pore formers

Widespread assumptions are that bacterial cytolysins act primarily by killing host cells and that their cytocidal action is the main or even sole factor underlying their significance in the context of microbial pathogenesis. This is unfortunate since secondary reactions mounted by cells under toxin attack are diverse and often provoke profound, long-range effects in the infected host organism (Bhakdi et al. 1994). A brief discussion of the cellular reactions elicited by the three cytolysin prototypes is useful because it serves to illustrate how diverse such reactions can be depending on the size of the transmembrane pores generated.

The very small pore produced by alpha-toxin in nucleated cells permits selective flux of monovalent ions, but does not permit rapid flux of Ca^{2+}. This has surprising consequences. The absence of overwhelming Ca^{2+}-influx saves the cells from the immediate toxic action of this divalent ion. Furthermore, Ca^{2+}-dependent secondary reactions are not elicited. Instead, cells may react in unexpected manners as a consequence of perturbation of Na^+-K^+ homeostasis. Two possibly related phenomena have been discovered. First, K^+-depletion in monocytes leads to activation of interleukin-converting enzyme (ICE); therefore, rapid and massive release of mature IL-1β occurs in cells containing the IL-1β precursor (Walev et al. 1995). Secondly, T-lymphocytes that leak K^+ undergo programmed cell death (apoptosis) (Jonas et al. 1994). Both IL-1β conversion and apoptosis can be prevented if cells are permeabilized with alpha-toxin in medium containing high K^+ concentrations. Since apoptosis is in some unclarified manner under the control of an ICE-related protease, it is tempting to speculate that the major intracellular cation is directly or indirectly involved in controlling the function of vitally important proteases. It is noteworthy that apoptosis does not occur if cells are permeabilized with very high toxin doses such that Ca^{2+}-permissive pores are generated. Thus, rapid flooding of cells with Ca^{2+} probably suppresses the chain of reactions leading to DNA degradation (Jonas et al. 1994).

Ca^{2+}-permissive pores can be produced by alpha-toxin in certain cells (e.g., platelets) and in all nucleated cells by HlyA. In this case, rapid influx of Ca^{2+} triggers an array of cellular reactions. This is possible because proteins egress from toxin-damaged cells at a much slower rate; therefore, machineries for various Ca^{2+}-dependent reactions remain intact for a limited time span. Such Ca^{2+}-dependent reactions include the production of lipid mediators derived from the arachidonic acid cascade, which in turn elicit short- and long-range effects on neighboring cells and which can thus provoke organ dysfunction, secretion of granular constituents such as elastase from granulocytes or procoagulatory substances from platelets, contractile dysfunction that will dramatically enhance the permeability of endothelial cell monolayers and thus promote edema formation, and the stimulation of NO production (reviewed in detail in Bhakdi et al. 1994). All these processes are indeed encountered in patients suffering from infections with cytotoxin-producing bacteria.

The effects of toxins such as SLO that produce very large pores have been studied less extensively. Local cell damage may dominate, but a number of unexpected aspects have recently arisen. Listeriolysin is employed by *Listeria monocytogenes* to escape from endosomes, forming an essential step in the establishment of intracellular infection (Michel et al. 1990; Portnoy et al. 1992). SLO (and probably pneumolysin) is able to provoke such severe damage to multilayered membranes that they become permeable to ions and even macromolecules. This effect has been demonstrated on round window membranes that separate the inner and middle ear. Flux of ions and macromolecules between middle and inner ear could explain the sensineural hearing loss that often accompanies acute otitis media (Engel et al. 1995).

Resistance and repair mechanisms

Different cells species can display highly varying susceptibilities towards the action of different pore-forming toxins, whereby the underlying mechanisms remain largely unclarified. Lack of specific binding sites is the most obvious explanation in the case of alpha-toxin. However, this argument cannot hold for SLO since all mammalian cells contain cholesterol. In a similar context, HlyA apparently binds nondiscriminatingly to lipid bilayers;

therefore, reasons for varying sensitivities of different cells are difficult to envisage. Formation of non-functional oligomers is a second possibility that, indeed, has been documented for both alpha-toxin (Walev et al. 1994) and SLO (Walev et al 1995). The reason for nonfunctionality of the toxin oligomers is not understood. A third mechanism involves repair of the lesions. This has been most clearly demonstrated for alpha-toxin. Thus, fibroblasts that are initially permeabilized by the toxin exhibit reduction in K^+ and ATP levels; however, the cells are able to recuperate from the initial permeabilizing event and to replenish their K^+ and ATP content over a period of 16 h (Walev et al. 1994). The repair mechanism remains to be elucidated. The presently available evidence indicates that it is due to closure of toxin pores in the bilayer rather than to endocytic uptake (Walev et al. 1994). Shedding of both alpha-toxin and SLO oligomers from cells has been detected and represents another possible mechanism for membrane repair (Walev et al. 1994, 1995).

Conclusions

In contrast to endotoxins and to superantigens, pore-forming bacterial exotoxins still receive relatively little attention as contributors to microbial pathogenesis. This is inappropriate since they are produced by the majority of important bacterial pathogens and their toxic action has been extensively documented in many cell systems, in isolated organs, and in animal models. Mutant bacterial strains that have lost their capacity to produce important pore formers such as alpha-toxin, *E. coli* hemolysin, and pneumolysin, also exhibit marked reduction in virulence in animal experiments (reviewed in Bhakdi et al. 1994). Antibodies against alpha-toxin have been shown to protect animals against various infections with *S. aureus* significantly (K. Hungerer, personal communication). Thus, there is every reason to assume that pore-forming cytotoxins are important determinants of microbial pathogenicity. Quite apart from their biological significance, pore formers represent unique models to study the insertion of initially water-soluble proteins into lipid membranes, an event that is frequently linked to oligomerization, wherein the interacting protomers "instruct" one another to undergo conformational changes, leading to their hydrophilic/amphiphilic transition and to the generation of water-insoluble aggregates that assume properties akin to those of integral membrane proteins. Finally, the study of pore formation in membranes is generating novel insights into the regulation of many cellular processes. Indeed, pore-forming toxins are increasingly being used as tools in cell biology to study the molecular composition and the regulation of diverse cellular machineries (Bhakdi et al. 1993; Bayley 1994). The field of pore-forming cytotoxins is thus steadily growing in breadth and in depth, and an ever increasing number of investigators from different fields in molecular and cell biology, biochemistry, biophysics, and medicine are now sharing the excitement generated by the study of these fascinating proteins.

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