Progress in Allergy

Cover illustration

Negatively stained fragment of rabbit erythrocyte lysed with staphylococcal α-toxin. Numerous 10 nm ring-shaped craters are seen over the membrane, identical to the channel structures generated by complement and also by cytolytic peptides released from activated killer lymphocytes.

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Printed in Switzerland by Boehm-Hutter AG, Reinach BL

ISBN 3-8055-4636-X

Prog. Allergy, vol. 40, pp. 1–43 (Karger, Basel 1988)

Damage to Cell Membranes by Pore-Forming Bacterial Cytolysins

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Introduction

Mammalian cell membranes consist of single bilayers of lipid with integral and surface-associated membrane proteins. These membranes measure only 7–9 nm, yet they hold together cells whose diameters may reach 10–15,000 nm. Ion pumps in the membrane maintain the characteristic intracellular ionic composition that is essential for diverse cellular functions. Any perturbation of the membrane permeability barrier represents an immediate threat to the existence of a cell; if lesions are not repairable, a leakage site of critical size will be lethal. Nucleated cells are generally able to remove a limited number of permeabilized sites from their surface, and a certain number of transmembrane pores may therefore be generated without causing cell death. However, sublethal dose may elicit pathophysiological events in the attacked cells and in their environment.

An important and very widespread mechanism for perturbation of membrane integrity is the formation of transmembrane pores by the insertion of alien proteins into the lipid bilayer. Following the work on gramicidin A, a microbial pentadecapeptide that was shown to form dimeric channels in membrane targets [15, 63, 68, 100, 135], the first proposal that high molecular weight proteins may also enter the bilayer to form pores was made by Mayer [90] in 1972 to account for the mechanism of complement cytolysis. In the original doughnut theory, terminal C5-C9 plasma complement components were proposed to come together to form the ring structures known as the classical complement 'lesions', leakage occurring through the protein-lined pores. Today, it is increasingly being recognized that although many protein pores do indeed display this basic feature,

some pores do not possess completely circularized protein 'walls' and are instead partially lined by an edge of free lipid membrane. The unifying feature of all protein pores is the presence of membrane-inserted regions of which one face interacts hydrophobically with membrane lipid, whilst the other is hydrophilic and allows the transmembrane passage of ions and small molecules. The apolar regions of the proteins avidly associate with membrane lipid, generally without causing marked perturbation of neighboring lipid-lipid interactions; hence, the inserted proteins remain firmly anchored in the bilayer and there is no evidence that 'leaky patches' within lipidic membrane areas arise as a consequence of pore insertion.

Following the verification of the pore concept of complement action, the recognition quickly followed that the same principle was operative in many other biological systems. Moreover, it is becoming apparent that several pore-formers can act as monomers, i.e. without mutual association in the membrane to form circular or semicircular structures. In this review, general features of pore-forming proteins will first be outlined. Thereafter, salient features of three prototypes of pore-forming bacterial cytolysins, i.e. staphylococcal α-toxin, streptolysin-O (SLO) and *E. coli* hemolysin will be summarized. In the last section, examples of secondary effects elicited by toxin-membrane interactions will be discussed. The ultimate aim of this review is to document the similarities, but also to point out basic differences, in the mode of action of pore-forming cytolysins, and to illuminate the biological significance of the interactions of these proteins with the target cells.

General Features of Pore Formation

Types of Protein Pores

Pore sizes can differ widely. To date, the smallest protein pores forming in mammalian target membranes have been sized to around 1–2 nm effective diameter. Representatives of such pores are staphylococcal α-toxin [24, 49, 94, 126, 127], *E. coli* hemolysin [27] and the cytolysins of *Pseudomonas aeruginosa* [86] and *Aeromonas hydrophila* [69]. Complement pores are generally larger (5–10 nm) [13, 16, 50, 96, 109]. The largest presently recognized pores are formed by the sulfhydryl-activated cytolysins (e.g. SLO); their diameters may approach 30–35 nm [26, 31].

Pores may display a characteristic ultrastructure, or they may be undetectable by currently established electron microscopic techniques. In the former case, pores are seen as partially or fully circularized structures partially buried within the lipid bilayer. They represent stable, macromolecular oligomers of the native, monomeric components, and they can be isolated as such after solubilization of membranes with mild, nondenaturing detergents. In no instance have membrane constituents themselves been found to be incorporated into the oligomeric protein structures. Pores devoid of recognizable structure have generally been less well characterized with respect to composition. It currently appears that some pores may form solely through insertion of protein monomers into the bilayer (e.g. *E. coli* hemolysin) [27]. It is conceivable that other pores without visible ultrastructure arise through metastable dimerization of membrane-inserted polypeptides, possibly giving rise to fluctuating pores as appears to be the case for gramicidin A.

Pores may be homogeneous or heterogeneous with respect to composition. Homogeneous, structured pores are formed when a constant number of protomers constitutes each lesion, as is probably the case for the majority of staphylococcal α-toxin pores, which represent hexamers of the molecule [22, 49]. Homogeneity would also be expected for lesions induced by toxin monomers.

Structured, oligomeric pores are more often heterogeneous due to the presence of varying numbers of protomers that form the individual lesions. Typical examples are the lesions formed by sulfhydryl-activated cytolysins (e.g. SLO) and complement [17–19]. These lesions are characteristically seen in the electron microscope as partially or fully circularized ring structures with varying diameters, and functional heterogeneity of pore size can be detected with the use of appropriate assays.

Mode of Pore Formation

The events that induce binding, oligomerization and membrane insertion of pore-forming proteins, as well as those responsible for cessation of the processes, are not clearly understood. With regard to primary membrane attachment, there are proteins which require the presence of specific lipids in the membrane (e.g. cholesterol in the case of sulfhydryl-activated toxins) [2, 3, 14, 120], whereas specific membrane lipid substrates have not been identified for many pore-formers, including the complement C5b–9 complex, staphylococcal α-toxin and *E. coli* hemolysin. Oligomerization can be envisaged to follow one of two modes. First, pro-

tein monomers bind to the membrane. Lateral movement of these molecules in the membrane then leads to their collision and this in turn might trigger the conformational changes responsible for exposure of hydrophobic domains (e.g. through unfolding of the molecules). This type of oligomeric pore formation has been proposed for SLO [72] and for staphylococcal α-toxin [61]. A second mechanism for initiation of the oligomerization process may involve primary attachment of a first molecule to a membrane site, with the concomitant exposure of a novel binding site for attachment of the next protomer to the initially bound protein. This mechanism is probably operative in the case of the complement lesion [21, 118].

Membrane insertion by oligomerizing protein pores appears to follow spontaneously after exposure of apolar regions on the protein complexes, i.e. because of the energetically favored hydrophobic interactions of these areas with membrane lipids. Thus, no specific requirement, such as membrane potential, has been identified [13, 94, 96]. In contrast, membrane insertion by monomeric pore-formers may follow a different pattern. For example, *E. coli* hemolysin forms pores in planar lipid membranes only in the presence of a correct transmembrane electrical potential [Menestrina et al., submitted]. In this respect, this hemolysin displays highly interesting similarities to pore-forming colicins [36, 113] and to alamethicin [85].

The fate of membrane constituents during the process of pore formations has not been clarified in detail. One possibility is that pore-forming proteins may expell lipid (and integral membrane proteins) at their sites of membrane insertion. Some evidence for this mode of pore formation has been advanced in the complement field [76, 84, 117]. Alternatively, the hydrophilic surfaces of the inserted polypeptide might just laterally repell lipids and membrane proteins. In this case, expulsion of membrane constituents into the extramembranous environment would not be required, but insertion of a sufficient number of pores would be expected to cause nonosmotic membrane expansion and an increase in membrane surface area. Nonosmotic cellular swelling has indeed been observed following formation of the complement lesions in erythrocyte membranes [136]. Furthermore, we have been unable to detect the expulsion of significant amounts of membrane lipid into the fluid-phase despite extensive pore formation in erythrocyte membranes by SLO, staphylococcal α-toxin, or complement [unpublished data]. Therefore, we currently favor the second mode of pore formation over the first possibility.

Membrane Orientation and Stability of Pores

Membrane-bound protein pores have always been found to expose part of their surface at the external membrane surface. We suspect that partial penetration of a relatively large water-filled pore, inserted for example into the external membrane monolayer, would probably already suffice to induce a transmembrane leak. Hence, pore-forming proteins probably need not span the entire thickness of the bilayer. The most detailed analyses on the depth of bilayer penetration have been made on the complement pore. Here, it appears that the C9 complement component spans the entire bilayer [99, 147] and an epitope appears to have been detected at the cytoplasmic membrane surface [99].

A general feature of possible biological significance is the marked stability of oligomeric membrane-bound pores. All pores studied to date remain antigenically, structurally and functionally intact despite extensive proteolytic attack at neutral pH. Hence, oligomeric pore-formers in their membrane-bound (but not native) state represent remarkably stable molecules whose elimination may require total removal of the afflicted membrane areas (e.g. by endocytosis or phagocytosis).

Molecular Basis for Pore Formation

The primary structures of three pore-formers, namely staphylococcal α-toxin, *E. coli* hemolysin, and the C9 complement component, have been elucidated by molecular cloning [37, 44, 53, 123], and the primary structures of others will probably follow in the near future. Nevertheless, a molecular model for the formation of the intramembranous domains has not been worked out for any single pore-former. One surface of the pore interacting with lipid necessarily must be hydrophobic; the other surface that permits passage of ions and water must be hydrophilic. Therefore, a stretch of apolar amino acids as found for integral membrane proteins is hardly to be expected, nor need the intramembranous domains exhibit α-helical structure. Elucidation of the 3-dimensional structure of pores will clearly require extensive biophysical analyses in the future.

Staphylococcal α-Toxin

Biological Significance

α-toxin is produced by most strains of *S. aureus* and is regarded as an important factor in staphylococcal pathogenicity [60, 77, 92]. The toxin at-

tacks all mammalian cells studied to date, albeit with greatly varying efficacy. It is hemolytic, cytotoxic and lethal when injected in large doses; the mechanisms of its lethal action have not been defined, but are likely to be due to the cumulative primary and secondary effects of the toxin deriving from its pore-forming action on several cell types. Antitoxin antibodies are found in plasma of all healthy individuals; hence, the toxin is produced by staphylococci in the human host organism. Staphylococcal strains that do not produce α-toxin have been reported to be less virulent in animal experiments [79, 105]. In a recent study [105], site-directed mutagenesis was employed to selectively inhibit gene expression, and the resulting mutant was found to be less virulent in a mouse peritonitis model than the toxin-producing isogenic strain. The general consensus that α-toxin represents a significant factor of staphylococcal pathogenicity is thus well supported.

Properties of Native Toxin

The toxin is secreted as a water-soluble, single-chain polypeptide of M_r 34,000 devoid of carbohydrate [14, 22, 53, 92]. The sedimentation coefficient is 3.4 S; the isoelectric point is 8.6. The primary structure deduced from cDNA sequence is unremarkable. CD spectra indicate that the toxin exhibits 6% α-helical and 68% β-sheet structure [129]. The toxin can be isolated from overnight bacterial culture supernatants in mg amounts by conventional protein separations. The native toxin is cleaved and destroyed by proteases.

In 1967, Arbuthnott et al. [8] noted that heating of α-toxin to 60°C caused the protein to spontaneously aggregate into what they regarded as hexameric ring structures. Morphologically identical structures were detected by the same investigators on toxin-lysed target lipid bilayers. These findings led Arbuthnott and co-workers [8, 9, 46, 47] to conclude that formation of ring-structured toxin hexamers in a membrane was probably causally related to its membrane-damaging properties. Retrospectively, it is today apparent that their data represented the first documentation of a phenomenon that is probably common to most if not all oligomeric pore-formers. Spontaneous oligomerization in solution has subsequently been described both for sulfhydryl-activated cytolysins [35, 98, 110] and for complement component C9 [132, 133].

Native α-toxin can be stored with little loss of activity at −20°C or lower, or in lyophilized form. In solution, the toxin will continuously form oligomeric aggregates. This process is irreversible in the absence of denaturing agents. The oligomers are water-insoluble and noncytolytic, due

to aggregation and covering up of the apolar protein domains, and toxin solutions therefore gradually become opaque and lose their cytolytic activity. Native 3 S toxin can easily be separated from 11 S aggregates by molecular sieve column chromatography. The active toxin elutes in a sharp, symmetrical peak and yields a single band of M_r 30,000 upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Formation of Transmembrane Pores by α-Toxin

What factors govern the primary binding of α-toxin to target bilayers? Do certain cells contain specific binder molecules that facilitate attachment of 3 S toxin monomers, and does hexamer formation then follow in a second step by lateral aggregation and collision of the membrane-bound monomers? Or does α-toxin represent the prototype of a 'receptorless' pore-former that will attack lipid bilayers with little discrimination? Consensus has not been reached for any of these questions at present. Since they are of basic importance to the understanding of the action of this and related pore-forming proteins, a critical evaluation of presently available data appears warranted here.

A major and unresolved puzzle is the widely varying susceptibilities of different target cells towards toxin action. For example, rabbit erythrocytes are approximately 100 times more sensitive towards hemolytic toxin action than human erythrocytes. However, liposomes that are prepared from lipids of either erythrocyte species are equally sensitive towards the toxin, as assessed by marker-release experiments [92]. Hence, surface membrane proteins or glycoproteins must in some way markedly influence the net susceptibility of a cell. It is important to note that α-toxin will attack and perturb protein-free lipid monolayers [30] as well as planar lipid bilayers that consist of lecithin [94]; therefore, the process of membrane binding and pore formation cannot be entirely dependent on the presence of 'receptors'. However, this does not exclude that surface membrane molecules (proteins) may augment and facilitate toxin attack on cells.

There are basically two ways through which this may occur. First, a membrane 'receptor' or specific binder molecule could be present on highly susceptible cells, such as rabbit erythrocytes, to enable toxin binding to the cells at low concentrations. Second, toxin binding could be prevented or facilitated more unspecifically because of peculiarities in the mosaic structure of the cell surface.

Earlier investigations favored the first possibility. Utilizing radioiodinated toxin, Cassidy et al. [32] reported the presence of a low number of

specific receptor molecules for α-toxin on rabbit erythrocytes. Receptors were not detected on human erythrocytes. These investigators tentatively concluded that such receptors were responsible for the susceptibility of rabbit cells towards concentrations of toxin that were ineffective on human cells. Since higher toxin concentrations lysed all types of erythrocytes and hemolysis then was accompanied by the appearance of membrane-bound ring structures, the following conclusions were tentatively drawn. First, the biological effects of α-toxin may be due primarily to its action on receptor-containing cells. Second, the toxin effects on such cell membranes may not be based on the same molecular mechanisms as the effects on receptorless membranes that are evoked at high toxin concentrations. In particular, it has been suggested that the appearance of ultrastructural ring lesions may be a phenomenon that is encountered only at high toxin concentrations, and biological effects may be elicited at low concentrations without hexamer formation. Support for this contention initially appeared to derive from the observation that cells treated with low concentrations of toxin carried no detectable ring structures.

Several points are worthy of note here. The existence or absence of membrane 'receptors' for α-toxin can be established only when methods are available for detecting and quantifying binding of functionally intact, 3 S toxin monomers to the membrane, and binding of these moieties must exhibit reversibility, saturability and high affinity. The early receptor studies were all performed with the use of radioiodinated toxin. However, iodination is essentially hazardous because it alters the biological properties of the toxin to the point of ultimately destroying its hemolytic capacities. Second, reliable quantitative assays for membrane-bound toxin have not been available. It has been impossible to quantitatively differentiate between monomeric and oligomeric forms of the toxin on a membrane, and this naturally places constraints on the concept that toxin monomers can already perturb the bilayer integrity. Absence of ultrastructurally visible rings need not indicate absence of oligomers because (a) oligomers consisting of fewer than 6 monomers may conceivably create pores that exhibit no visible ultrastructure, and (b) the number of ultrastructurally visible hexamers may simply be too low to be detectable.

To gain better insight into the process of pore formation by α-toxin in erythrocyte membranes, we have recently developed a very sensitive immunoassay based on the use of a monoclonal antibody that permits differentiation and quantitation of monomeric and oligomeric, membrane-bound toxin. This assay led to the following observations. First, there is no

binding of toxin to either rabbit or human red cells at subcytolytic concentrations: when 10^9 rabbit erythrocytes are incubated with 3 ng/ml toxin (10^{-10} *M*) no toxin binds to the cells at all. Obviously, this excludes the presence of high-affinity binding sites for native toxin on rabbit erythrocytes. Second, cell lysis can occur both at 4°C and at elevated temperature (37°C), and lysis is always paralleled by the formation of toxin oligomers on the membrane. Third, within a narrow range of concentrations, rabbit cells can be treated with toxin at low temperature without ensuing lysis. Under these conditions, toxin is bound exclusively in the 3 S form, and in low numbers to the cells. When these cells are washed in the cold and resuspended at 37°C, lysis occurs and this again is paralleled by the formation of oligomers. Fourth, binding of toxin to both rabbit and human erythrocytes increases in a nonlinear fashion suggestive of cooperative effects when toxin doses are increased to more than 10 μg/ml. Overall, the results indicate that binding of α-toxin to cell membranes will occur independent of the presence of specific membrane binder molecules whenever a critical threshold concentration of the toxin is reached. This threshold is different for various cell types and may be due to differences in the mosaic architecture of the membrane surface. The evidence clearly indicates that toxin monomers first bind to the membrane, and oligomers form in a second stage through lateral aggregation of the molecules. This process is slowed down at low temperature. However, oligomerization can basically take place at 4°C, and the process of pore formation with membrane insertion is thus not strictly temperature-dependent. An essentially similar mode of pore formation has been found for SLO (see below).

The overall binding of α-toxin to cells is rather ineffective. At relatively lower concentrations (e.g. 1–10 μg/ml), only about 5% of the toxin becomes bound to rabbit erythrocytes. At high concentrations (e.g. 100 μg/ml), binding levels approach 30–35% with both rabbit and human cell targets. These results, obtained with the sensitive enzyme-linked immunosorbent assay (ELISA), corroborate the estimates obtained through more indirect titration methods in our laboratory [24], and are also basically in accord with results of Phimister and Free [107], who measured binding of radiolabelled and hemolytically active toxin to the cells. If 10^9 rabbit erythrocytes in 1 ml are incubated with the toxin at a concentration of 60 ng/ml (approximately 10^{12} molecules), approximately 70 toxin molecules become generated on an average per cell and this causes approximately 60% hemolysis, i.e. one functional hit per cell. This is compatible with the concept that a single hexamer suffices to lyse a cell. The situation

with more resistant cells (e.g. human erythrocytes) is different. Here, either there will be no toxin bound at all (subcytolytic toxin levels), or a multitude of hexamers will be generated at the minimal hemolytic levels (approximately 10 μg/ml). With regard to rabbit cells, it is unsurprising that hexamers cannot be detected in the electron microscope when cells are lysed at such low toxin levels.

Although the major population of toxin molecules is represented by ring-structured hexamers, biochemical and physicochemical analyses indicate that oligomers containing fewer toxin molecules may also form on a membrane [12, 129]. It is possible that such oligomers produce (smaller?) transmembrane pores that cannot be visualized in the electron microscope.

In summary, we presently envisage pore formation by α-toxin to involve the following steps. First, monomers bind to the membrane via absorptive processes that do not involve high-affinity receptors, but may be enhanced or restricted by membrane surface factors such as distribution of surface charges. Second, monomers collide through lateral aggregation; again, this step may be influenced by membrane composition. The major population of oligomers forming on a bilayer are hexamers of M_r 200,000, whereas the initially critical step that induces exposure of apolar domains and membrane insertion may be the formation of dimers [12]. The hexamers can be isolated from detergent-solubilized membranes and reincorporated into liposomal membranes composed of phosphatidylcholine. The functional consequence of oligomer formation is the generation of discrete, transmembrane pores that display no marked ion selectivity and allow the transmembrane passage of molecules whose effective diameters do not exceed 1.5–2.0 nm. These effects have been observed in erythrocytes [24, 49], nucleated cells [1, 10, 124, 126, 127], and they have also been studied in detail in planar lipid bilayers [12, 94].

The Ultrastructure of the α-Toxin Lesion

The electron microscopical identification of the α-toxin lesion and its structural identity with the hexameric form of the toxin was first reported by Freer et al. [46, 47]. Erythrocyte membrane targets exposed to the toxin exhibit small annular structures, harboring a central, stain-filled pit of about 2.5 nm diameter (fig. 1a). The outer diameter of the rings vary with the stain applied. The diameter is 10 nm in stainings with uranyl acetate, whereas stainings with silicotungstate result in outer diameters of about 8.5 nm. The same unexplained difference is observed with the iso-

lated and delipidated toxin hexamer [22]. Along the bent edges of lysed membranes, the toxin pores are seen as small cylindrical stubs projecting approximately 4 nm from the membrane surface. If lysed erythrocytes are kept 3–5 h at 25 °C, the lesions accumulate in small blebs that eventually may detach from the membrane. Identical ring structures are observed on reconstituted liposomes [49], as well as on liposomes and low-density lipoprotein (LDL) particles that are exposed as targets for toxin binding (fig. 2) [23]. With appropriately thin negative stainings, a faint stain deposit may be distinguished along the axis of the hexamers seen in profile [22]. Assuming a uniform 2.5-nm diameter of the central pore/pit through the 4 nm length of the stub, a molecular weight of 160,000–200,000 daltons can be calculated for the ultrastructurally visible toxin pore [19, 22]. Since the total molecular mass of the toxin hexamer is approximately 200,000 daltons [22], the intramembranous domain of the toxin complex must be comparatively small, unless the toxin hexamer can shift between a state of deep membrane insertion and partial extrusion. Such a process has certainly not been excluded and may be related to the flickering between open and closed states of the pores that are suggested by conductance measurements in planar lipid bilayers [94].

Neutralization of α-Toxin

Neutralization of α-toxin can occur via the binding of specific antibodies which are present in sera of healthy human adults. A murine monoclonal antibody has recently been generated that neutralizes α-toxin by inhibiting its binding to target membranes [Hugo et al., submitted]. α-Toxin also binds spontaneously to plasma LDL, and this process is accompanied by hexamer formation on the lipoprotein particle [23]. Since the lipoprotein as well as membrane-bound hexamers do not dissociate to attack bystander target cells, this process results in toxin inactivation. Nothing is known on the fate of antibody- or lipoprotein-bound toxin in the organism and there have been no studies on possible repair or removal of toxin lesions on membranes of nucleated cells.

Calcium ions at relatively high concentrations appear to be able to 'close' toxin pores in membranes. This phenomenon has been documented in the erythrocyte model and in planar lipid bilayers [11, 12, 61]. In the latter studies, hexamer pores were first generated in the bilayers, and they appeared to close upon contact with high calcium levels. It thus appears that the inhibitory effect of calcium is due to its action on formed pores rather than on inhibition of lateral aggregation of toxin monomers in the

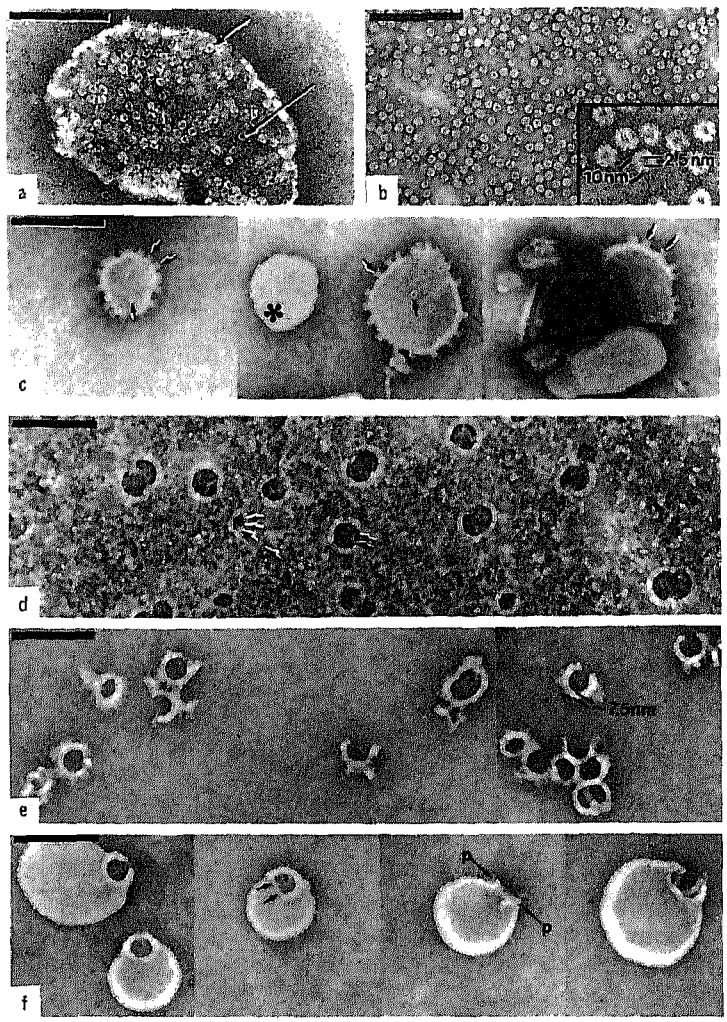

Fig. 1. a Negatively stained fragment of rabbit erythrocyte lysed with staphylococcal α-toxin. Numerous 10 nm ring-shaped structures are seen over the membrane (arrows). *b* Isolated toxin hexamers in detergent solution. *c* Lecithin liposomes carrying reincorporated α-toxin hexamers. The hexamers are seen as stubs along the edge of the lipsomal membrane and as rings over the membrane (arrows). Characteristically, liposomes that escape incorporation of the toxin are impermeable to the stain. *d* Negatively stainced erythrocyte membrane lysed by SLO showing numerous 25–100 nm long and approximately 7.5 nm broad, curved rods of 13–16 nm inner radius of curvature. Most rods are approximately semicircular, often joined in pairs at their ends. Dense accumulations of stain are seen at the concave side of the

bilayer. How calcium exerts its pore-closing effects is unknown. It is thinkable that the effect is due to an interaction with lipid molecules that immediately surround the channel. In this context, it should be reiterated that, although photolabelling studies confirm the presence of toxin within the lipid bilayer [128], there is no evidence that the toxin actually spans the membrane. Entry of a water-filled protein pore half way through the lipid bilayer, for example, would still be expected to generate a functional transmembrane channel. If this were the case for the α-toxin pore, calcium ions might act to stabilize the lipid molecules that are in the immediate vicinity of the channels.

α-Toxin as a Membrane-Permeabilizing Agent

Since α-toxin generates relatively small transmembrane pores, it can be used to selectively permeabilize membranes of various mammalian cells. This approach is eminently valuable in studies of exocytotic pathways and can be used, for example, to examine minimal requirements for exocytosis. First reports in this area have been promising [1, 10, 93]. The attractiveness of α-toxin as a permeabilizing agent over previously used substances (e.g. digitonin) lies in the fact that pores are formed exclusively in the plasma membrane, i.e. since the pores are too small to allow influx of the toxin into the cell, intracellular membranes are left intact. Furthermore, the toxin pores are apparently very stable and permit transmembrane flux of small molecules and ions over a prolonged period, whereby macromolecules are retained in the cell cytoplasm and the exocytotic machinery remains intact. In toxin-permeabilized chromaffin cells, it was found that micromolar levels of free Ca^{2+} sufficed to trigger exocytosis, and catecholamine release was shown to occur in the absence of lactate dehydrogenase liberation [1, 10]. In contrast, cells permeabilized with digitonin released catecholamines without strict dependence on extracellular

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rods. When these do not form closed profiles, the stain deposit is partly bordered by a 'free' edge of the erythrocyte membrane (arrows). *e* Negative staining of isolated SLO oligomers, showing numerous curved rod structures identical to those found on toxin-treated membranes. *f* Purified SLO complexes reincorporated into cholesterol-free lecithin liposomes. The toxin oligomers form holes in the liposomes (unlabelled arrows); p indicates a lesion seen in profile. Scale bars indicate 100 nm in all frames. Sodium silicotungstate was used as negative stain in *b–f*. Uranylacetate was used in *a*. Reproduced from Bhakdi and Tranum-Jensen [19].

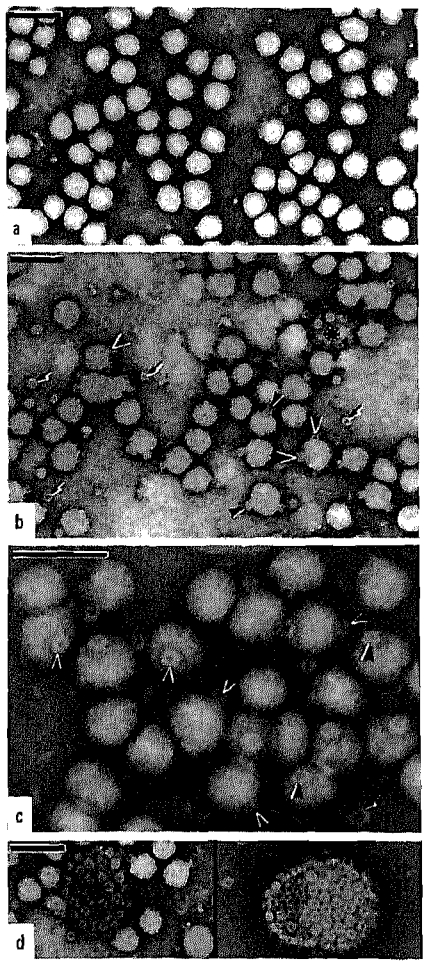

Fig. 2. Binding of *S. aureus* α-toxin to human plasma LDL. *a* Control preparation of human LDL showing a uniform population of smooth and round, approximately 25-nm particles. *b*, *c* Toxin-treated LDL. Typical ring and stub profiles of 11S α-toxin hexamers are seen attached to the LDL particles (arrowheads). 'Free' 11S toxin complexes are indicated by small arrows; toxin detachment from LDL possibly occurred during the negative staining procedure. A minor fraction of the LDL particles carry markedly more toxin structures than others (asterisk). *d* A small fraction of large 100- to 150-nm particles, possibly of a composition different from the 25-nm particles, are heavily loaded with 11S toxin structures. Sodium silicotungstate-negative staining. Scale bars indicate 50 nm. From Bhakdi et al. [23].

Ca^{2+}, and with parallel loss of cellular lactate dehydrogenase. It thus appears that α-toxin is far superior to digitonin as a permeabilizing agent in this cell system. Further studies using α-toxin as a permeabilizing agent may lead to new recognitions on the mechanisms involved in exocytotic processes.

Myelin Disruption by α-Toxin

Due to its affinity for diverse types of lipids and apolar substances, α-toxin may exert biological effects through hydrophobic interactions with lipidic or apolar substrates. Disruption of myelin may be one such process that is biologically relevant [62]. Early studies with radioiodinated toxin indeed suggested that intravenously applied toxin may accumulate in the central nervous system [77]. However, it is difficult to envisage how a hydrophilic molecule with the size of α-toxin could permeate the blood-brain barrier, and further studies in this area are warranted. At present, although α-toxin doubtlessly can disrupt myelin in vitro, it may be too early to conclude that this process is actually of pathophysiological importance for elicitation of toxin effects in vivo.

Streptolysin-O

Biological Significance

SLO is the prototype of sulfhydryl-activated bacterial cytolysins, a group comprising at least 15 exotoxins that share several properties in common [2, 14, 15, 34, 120]. All these toxins are produced and secreted as single polypeptide chains with molecular weights ranging from 40,000 to 80,000. They are inactivated through oxidation, and reactivation generally occurs in the presence of reducing agents such as dithiothreitol. The toxins bind only to membranes that contain cholesterol and hence do not attack bacterial cells. In contrast, mammalian cells are all susceptible to damage by these toxins, and marked differences in susceptibilities of erythrocytes towards hemolysis by SLO have not been noted.

SLO is produced by β-hemolytic group A streptococci [66, 67, 130], which are the major human pathogens of the genus streptococcus. Antitoxin antibodies are present in plasma of all healthy adults, reflecting the fact that the toxin must be produced and is an excellent immunogen in the human host. High antibody titers are found in patients suffering from nonsuppurative sequelae following streptococcal infections, and they are in-

variably observed in all cases of rheumatic fever. The cause of these high antibody titers is unclear, nor is it known whether they play a role in the pathogenesis of the disease, which is generally assumed to be mediated by complement-activating immune complexes containing as yet unidentified bacterial antigens.

Microgram quantities of SLO are lethal when injected intravenously into mice, rats, guinea pigs and rabbits [for review, see Alouf, ref. 2]. Death is probably due to cumulative primary and secondary effects of the toxin on blood cells, heart function [56–58], and the central nervous system [54]. The toxin efficiently attacks all mammalian cells with little discrimination [2], and it is difficult and perhaps not very relevant to surmise on the possible causes of animal death. The role of the toxin in bacterial infections is definitively more local and will vary according to the site of infection. Antibodies to the toxin possess neutralizing capacity, but immune complexes formed between the toxin and host antibody may still be potentially harmful due to their complement-activating properties. The toxin may induce more long-range effects in the human organism via such immunopathological mechanisms.

SLO may also exert effects on cells at subcytolytic doses. Inhibition of leukocyte, lymphocyte and macrophage function have been reported [6, 7, 104, 141, 148], and these properties may add to the biological significance of the toxin. Subcytolytic doses of SLO appear to cause transient impairment of membrane transport systems [40] in mammalian cells. The mechanisms responsible for these effects have not been delineated. It may also be important to note that toxin preparations used in most of the cited studies were only partially purified.

Properties of Native Streptolysin-O

The toxin is secreted as a single-chain polypeptide of M_r 69,000 devoid of carbohydrate. Milligram amounts can be isolated from bacterial culture supernatants provided that care is taken to inhibit proteases, and preparations are carried out fairly rapidly in the cold. Addition of dithiothreitol to all isolation buffers greatly improves recovery of the toxin [20]. The isolated protein retains activity for months in the presence of dithiothreitol when stored at −20 °C or lower.

Several methods for toxin isolation have been described [4, 25, 120, 139, 140]. There were some discrepancies regarding the molecular weight and isoelectric point of SLO in the earlier reports, but a consensus may now have been reached based on the recent observation that two forms of

active toxin exist. Native SLO was shown to have a molecular weight of 69,000 and an isoelectric point of 6–6.4; this molecule may be partially degraded to a molecule of M_r 57,000 with an isoelectric point of 7–7.5. The latter retains full hemolytic activity and forms ultrastructural lesions identical with native SLO [25]. The biological significance of the removable 12,000-dalton polypeptide is unknown. Native SLO is otherwise susceptible to cleavage and destruction by proteases.

SLO is one of the most potent cytolysins, and the presence of approximately 100 molecules toxin/erythrocyte generates one functional lesion [25]. As discussed below, this number is compatible with the contention that a single toxin oligomer will cause lysis of an erythrocyte. Molecular cloning of the toxin gene in *E. coli* has been reported [83], but the nucleotide sequence is not yet available.

Interaction of Streptolysin-O with Cholesterol

It appears certain that SLO (and other sulfhydryl-activated cytolysins) bind to target membranes via an interaction with cholesterol. Evidence for this derives from several findings. Addition of cholesterol dispersions to the toxin results in toxin inactivation [2, 14, 41, 70, 78, 120]. That inactivation is due to avid interaction of the protein with the sterol can be inferred from experiments showing precipitation of SLO in agarose gels containing sterol [2]. More direct evidence for binding of radiolabelled cholesterol by SLO was obtained by Johnson et al. [78]. These investigators incubated SLO with cholesterol solutions at concentrations in which the sterol was present in a soluble, micellar form. They directly demonstrated that SLO bound micellar cholesterol and showed that the ensuing toxin-sterol complexes were hemolytically inactive, and of high molecular weight [78].

Other experiments have shown that the complexing of SLO with cholesterol dispersions results in the formation of arc and ring structures that are visible in the electron microscope, and these structures appear identical to those found on target membranes after toxin attack. Based on these findings, it was originally believed that the arc and ring structures represented toxin molecules in complex with cholesterol [2, 40]. Accordingly, it was hypothesized that the functional lesions in cell membranes might arise due to cholesterol sequestration from the bilayer, due to such complex formation. In accord with the general concept of toxin-sterol interaction, it was early established that SLO was unable to attack lipid bilayers that lacked cholesterol [reviewed in ref. 2].

Some information exists on the stereospecificity of sterol binding by SLO [2, 3, 108, 144]. In general, the structural requirements appear to be similar to those found for polyene antibiotics [103]. In addition to a 3β-OH group on ring A of the cyclopentanoperhydrophenanthrene nucleus, there is a requirement for a lateral aliphatic side chain at C17, and for an intact B ring. It is still unclear how the toxin interacts with cholesterol in a lipid bilayer, however. Difficulties in envisioning such an interaction become apparent when one considers that the only hydrophilic group on the sterol molecule that is presumably exposed at the water surface is the 3β-OH group. The B ring and aliphatic side chain at C17 are buried in the apolar region of the bilayer; any interaction of SLO with these regions would have to occur subsequent to membrane penetration by the protein, i.e. after the primary binding process has taken place. One possibility may be that the hydrophobic domains of the cholesterol molecule periodically become exposed at the membrane surface to then bind the toxin. However, this remains very hypothetical at present. In any event, further studies are certainly required to clarify the nature of the primary interaction of SLO with membrane cholesterol.

Functional Consequences of Toxin Attack on Cells

Functional studies by Duncan [39] and associates have shown that lesions induced by SLO in erythrocytes must be very large. Hence, macromolecules, such as albumin, in the extracellular medium cannot protect cells from lysis. In this respect, SLO lesions differ from complement and α-toxin lesions in erythrocyte membranes. In the latter cases, pore formation causes osmotic swelling because the lesions do not permit direct egress of hemoglobin [17, 24, 91]. Escape of the latter is due to subsequent colloid osmotic rupture of the bilayer. In contrast, SLO lesions are larger than hemoglobin and therefore probably permit direct passage of the intracellular macromolecule through the membrane. According to an estimate by Buckingham and Duncan [31], the functional diameter of the largest SLO pores must exceed 15 nm in erythrocyte membranes; this is in accord with the dimensions of the ultrastructural lesions visualized by electron microscopy (see below). Studies on differential release of variously sized markers from resealed ghosts have indicated that smaller pores form at lower toxin concentrations [39]. This finding again is compatible with the concept of heterogeneous pores forming in the membrane [26]. Hemolytic titrations with highly purified toxin indicate that 70–120 molecules per erythrocytes are sufficient to generate one hemolytic lesion

[25]. The functional consequences of SLO attack on membranes of nucleated cells has not been studied in detail.

Ultrastructure of SLO-Lesions

Dourmashkin and Rosse [38] first observed that erythrocytes lysed with SLO carry typical arc- and ring-shaped structures as visualized by negative staining electron microscopy. Similar structures were observed on membranes treated with some preparations of phospholipase C from *Cl. perfringens* [97, 98, 121]. Subsequently, a contaminant of the phospholipase preparations was identified as the cause for this observation. The contaminant, termed perfringolysin-O, is another member of the family of oxygen-labile cytolysins [4, 15, 120]. In a detailed study, Duncan and Schlegel [41] showed that formation of the ultrastructural SLO lesions on erythrocytes and artificial lipid bilayers was dependent on the presence of membrane cholesterol, and formation of the lesions was not accompanied by detectable degradation of lipids. Ultrastructural studies on SLO [26]] and other members of this toxin family, i.e. cereolysin [35], tetanolysin [5, 110] and listeriolysin [106], have shown that all form very similar or identical lesions on target membranes. Moreover, arc- and ring-shaped structures may spontaneously arise in concentrated solutions in the absence of lipids [98, 110]. The latter finding led to the suggestion that the observed structures may represent polymers of the toxins rather than complexes of monomeric toxin with cholesterol, as had been previously assumed.

Clear evidence for the correctness of this proposal came with the isolation of the toxin lesions from detergent-solubilized target membranes in delipidated form [26]. The method used to isolate membrane-bound toxin was identical to that developed for the isolation of terminal C5b-9 complexes from erythrocyte ghosts, and involved quantitative solubilization of membranes with a high concentration of deoxycholate followed by a single ultracentrifugation step in linear sucrose density gradients [17, 26] (fig. 3). The isolated SLO lesions are seen as 7–8 nm broad, curved rods with a 13- to 16-nm inner radius of curvature, and lengths ranging from 25 to 100 nm. The latter correspond to fully circularized structures with an internal diameter of 26 nm (fig. 1d–f). In addition, circularized structures with smaller diameters are also observed. The presented ultrastructures fully conformed to the spectrum of arc and ring structures observed on SLO-lysed erythrocyte membranes. Lipids were not detected in preparations of purified toxin oligomers. In particular, cholesterol analyses at a sensitivity that would detect 1–1.5% by mass relative to protein were negative [26].

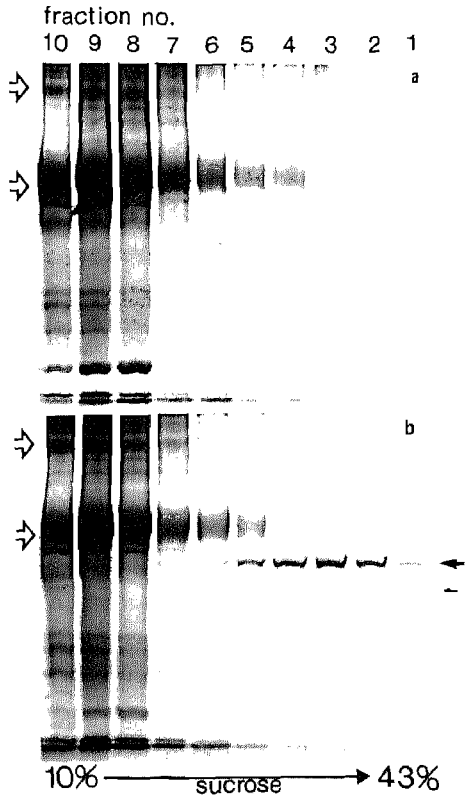

Fig. 3. Isolation of SLO-oligomers from rabbit erythrocyte membranes. Control or toxin-treated membranes were solubilized in 250 m*M* deoxycholate and centrifuged through linear, detergent-containing sucrose density gradients (direction of sedimentation: left to right). Ten equal fractions were collected, and samples were analyzed by SDS-PAGE. *a* Control membranes. *b* SLO-treated membranes. The SLO bands (solid arrows to the right) are observed in the high molecular weight regions (fractions 1–5 and 6) separated from the bulk of contaminating erythrocyte membrane proteins. Open arrows point to the spectrins and band 3 protein. From Bhakdi et al. [26].

Therefore, cholesterol cannot significantly contribute towards formation of these structures, which appear to be composed solely of noncovalently associated oligomers of the protein. The oligomers dissociate fully to yield monomeric toxin in the presence of SDS (and absence of reducing agents). Proteolytic treatment does not destroy the oligomeric structures, although some cleavage of the toxin is demonstrable after dissociation and electrophoresis in SDS [26].

The oligomers sediment over a wide region in sucrose density gradients, corresponding to approximately 20–40 S and a molecular weight range of one to several millions. On the basis of molecular volumes derived from electron micrographs, we have estimated a molecular weight of approximately 5×10^6 for fully circularized toxin oligomers, corresponding to 70–75 protomers of M_r 69,000 [26]. It is conceivable that smaller oligomers also form on target membranes that could create lesions of smaller functional size. However, these have not yet been separated from native membrane proteins in a form that would permit their ultrastructural analysis. Neither has the minimal number of protomers required for the visualization of a toxin oligomer by negative staining electron microscopy been determined.

Isolated toxin oligomers can be incorporated into liposomal membranes of phosphatidylcholine in the absence of cholesterol to produce membrane-bound lesions that are identical to those seen on erythrocytes. The analysis of such lesions indicate that the toxin pores span the thickness of the liposomal membrane. Freeze-fracture analyses of SLO lesions in erythrocyte membranes reveal arc- and ring-shaped structures on the fracture EF face, as well as on the true outer face of the membrane (ES face), decovered by etching (fig. 4). Dimensionally corresponding, sharply contoured defects in the inner lipid monolayer of the PF face can be demonstrated in etched and rotary shadowed preparations (fig. 4) [26]. Other freeze-fracture studies on SLO lesions [102] as well as similar studies on cereolysin lesions [35] and perfringolysin-O [97] have failed to demonstrate penetration of the lesions to the inner lipid monolayer. This discrepancy is probably explainable by the improved resolution obtained by rotary shadowing, as was the case in similar studies on the complement lesion [131]. Although penetration of the toxin polymer into the hydrophobic membrane core is evident from reconstitution experiments and from electron microscopical analysis, attempts to label membrane-bound perfringolysin-O with an apolar photoactivable hydrophobic probe have, interestingly, been negative [128].

Of particular interest has been the ultrastructural analysis of nonfully circularized toxin lesions following their incorporation into liposomal bilayers. These studies indicate that the residual circumference of the pore is represented by a straight, free edge of the membrane, the smallest lesions having the appearance of a small slit along the concave side of the oligomer (fig. 1f). Similar conelusions were reached through a close scrutiny of toxin pores formed in erythrocyte membranes. The possibility

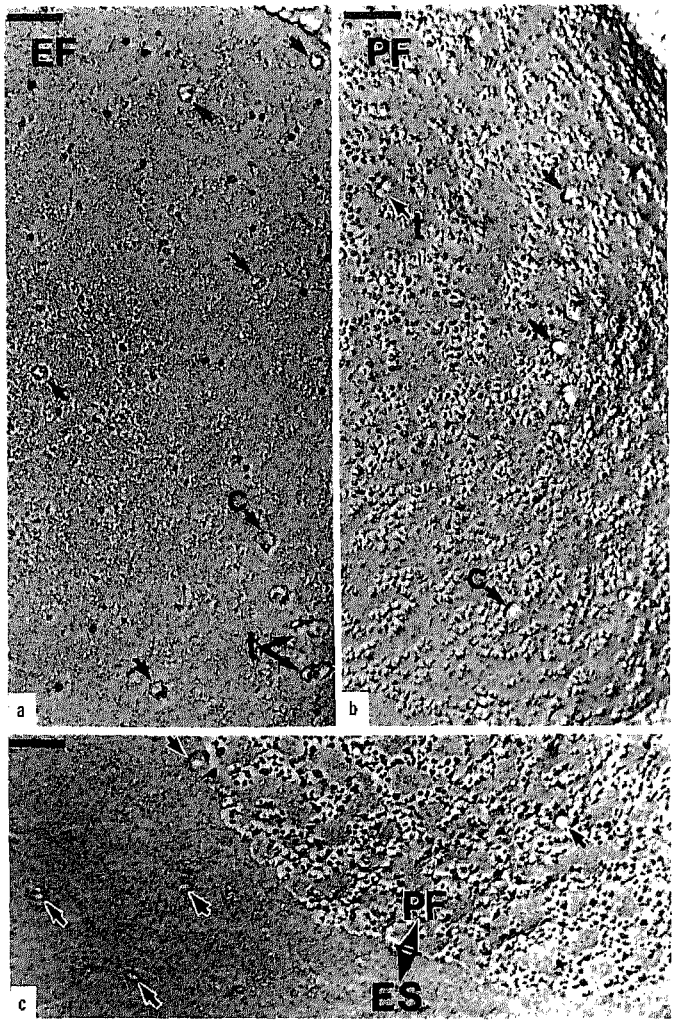

Fig. 4. Rotary-shadowed freeze-fracture replicas of erythrocytes lysed by SLO. *a*, *b* derive from membranes, fixed in glutaraldehyde and cryoprotected in glycerol, whereas the membrane in *c* was unfixed and frozen in 5 m*M* phosphate buffer. Fracture E-faces (*a*) exhibit rings and semicircular (c) structures, elevated relative to the lipid plateau. Some rings are twinned (t). Fracture P-faces (B and C) exhibit distinct defects in the lipid plateau. Some of these appear sharply and steeply contoured at one side, while fading at the opposite side (e.g. lesions labeled c). Some defects occur in pairs (t). ES-faces (*c*) exhibit circular and semicircular structures slightly elevated relative to the membrane surface. Bars, 100 nm. From Bhakdi et al. [26].

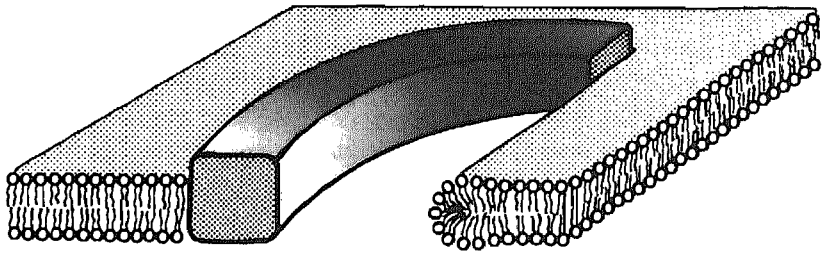

Fig. 5. Diagrammatic presentation of the gross, principle features proposed for the SLO-induced membrane lesion generated by incompletely circularized toxin oligomers. From Bhakdi et al. [26].

that transmembrane pores may be generated through insertion of noncircularized protein structures into the bilayer, with membrane lipid alone completing the circumference as deduced from reconstitution experiments with SLO, is intriguing. As depicted in figure 5, we envisage the hydrophilic sides of the protein to repell lipid molecules, leading to formation of a pore. The hydrophobic face of the protein pore thereby remains in close association with the membrane to ensure its anchorage within the bilayer. If this hypothesis is confirmed, the model could be extended to account for pore formation by monomeric protein molecules, e.g. as proposed for *E. coli* hemolysin (see below), and by other protein oligomers that do not present ultrastructurally visible lesions [e.g. C5b–9 complement complexes carrying few C9 molecules, ref. 18].

Mode of Pore Formation

SLO binds to erythrocytes at 0°C but hemolysis does not ensue at low temperature unless the number of bound toxin molecules exceeds 5,000–10,000/cell [72]. When cells are treated with subhemolytic doses at 0°C, washed, and resuspended in toxin-free buffer at 37°C, lysis occurs. These experiments indicate that toxin-binding to the membrane can be partially dissociated from the process of transmembrane pore formation [2, 72]. In an extension of these studies, a neutralizing monoclonal antibody was used to probe the mode of transmembrane pore formation by SLO. It was found that this antibody did not prevent toxin-binding. However, the membrane-bound toxin was present exclusively in monomer form [72]. Hence, direct evidence was obtained that membrane damage would only ensue if toxin oligomerization took place in the membrane. In further sup-

port of this contention, cells to which toxin + monoclonal antibody had bound were washed and resuspended at 37°C. The monoclonal antibody dissociated with time and, concomitantly, toxin oligomers formed and the cells lysed [unpublished data]. From these results, it is clear that pore formation by SLO is similar to pore generation by α-toxin. The toxin first binds to the membrane. By lateral aggregation, toxin monomers collide with each other, and this process apparently leads to unfolding of the molecule with exposure of hydrophobic domains that enter the membrane and ultimately create the transmembrane channels. In contrast to α-toxin, SLO (and other sulfhydryl-activated toxins) bind very effectively to cell membranes containing cholesterol; almost quantitative binding takes place when a surplus of target cells is present.

The convex side of the oligomeric rod structure carries a strongly apolar surface that anchors the toxin complex within the membrane through tight association with membrane lipids; cholesterol probably is no longer required for this interaction once the oligomer is formed. The concave side of the rod is hydrophilic and accordingly repels membrane lipid molecules. If toxin circularization proceeds to completion, the lipids will be fully forced aside and excluded from the pore structure. If circularization is incomplete, a straight, free edge of lipid membrane will remain between the two ends of the curved rod to complete the circumference of the pore. How the lipid molecules are oriented in these domains is not known, and figure 9 depicts a purely hypothetical possibility. An essentially similar mode of pore formation in the case of the cytolytic C5b-9 complement complex has been proposed [21, 118] and may also be operative with lymphocyte cytolysins [65]. The factors responsible for termination of the circularization process are unknown.

E. coli *Hemolysin*

Biological Significance

The hemolysin of *E. coli* has attracted much attention in the past for two main reasons. First, it represents a significant pathogenetic factor of the bacteria. This conclusion is based on several independent findings: toxin-producing *E. coli* strains exhibit greater virulence than nonproducers in animal models [33, 48, 55, 71, 137, 138, 142, 145, 146], and the majority of strains causing pyelonephritis and septicemia in the newborn also produce the hemolysin. Second, this is the only protein that is

genuinely secreted by *E. coli*, and it is therefore a useful and interesting model to study protein transport across the cell wall of the gram-negative bacteria.

E. coli hemolysin is probably always responsible for the zones of β-hemolysis observed around colonies of bacteria that are cultivated on blood agar. The hemolysin does not appear to be produced by other hemolytic, gram-negative enterobacteriaceae [73]. That the hemolysin is produced in the human organism is apparent from the finding that antitoxin antibodies are absent in most infants aged 8–24 months, whereas antibodies are invariably detected in sera of healthy adults [73]. It is not known whether neutralizing human antibodies exert a protective influence against infection and disease by hemolytic *E. coli*.

Secretion of Hemolysin by E. coli

This area has been reviewed recently [89] and will not be dealt with in detail here. There is consensus that the hemolysin determinant is composed of four genes. The structural gene is termed hlyA and encodes for the 107,000 – to 110,000-dalton hemolysin polypeptide. A neighboring gene, hlyC, encodes a 20,000-dalton polypeptide that promotes post-translational modification of the 107,000-dalton hemolysin. The nature of the intracellular post-translational modification is unknown at present, but it is certainly not due to proteolytic processing because mutants lacking the hlyC gene still secrete a hemolytically inactive 107,000-dalton polypeptide. Two further genes, hlyB and hlyD, are required for the hemolysin to be secreted into the medium. The protein products of these genes are probably located in the inner bacterial membrane; the mechanism by which translocation of the polypeptide across both inner and outer membrane of *E. coli* B is effected is not known. Recently, it has been shown that the information required for transmural transport of the protein resides in the C-terminal fragment of the molecule [89].

Properties of Native Toxin

Despite the fairly extensive knowledge that has accumulated on the structure of *E. coli* hemolysin at a molecular genetic level [44, 45, 51, 88, 89, 101, 122, 143], little has been known on the properties of the native protein and on the mechanism of membrane damage by this cytolysin. There are several reasons for this discrepancy. Only a few selected and genetically engineered strains secrete hemolysin that can be recovered in the active form as a 107,000-dalton polypeptide in appreciable amounts

from cell culture supernatants [52, 88]. Hemolysin secreted by other strains apparently very rapidly loses activity in solution. Whilst proteolytic degradation may partially account for this observation, other as yet undefined factors must also participate in toxin inactivation. Thus, inactivation becomes apparent before protein cleavage is observed in SDS-PAGE. It has been reported that native hemolysin may be associated with bacterial lipopolysaccharide [28] and the possibility is being considered that the lipopolysaccharide is important for the transmural transport and/or the cytolytic activity of the toxin. If association with lipopolysaccharide is indeed biologically relevant, functional inactivation might be due to conformational alterations in the protein-lipopolysaccharide complexes. Another possibility that merits attention is that the active protein may be fatty acid acylated, and inactivation may be due to removal of nonproteinaceous material from the toxin. A key to the understanding of these problems clearly lies in the function of hlyC, and the nature of the post-translational modification of *E. coli* hemolysin that is required to render the protein cytolytically active.

Pore Formation by E. coli *Hemolysin*

After binding to target membranes, *E. coli* hemolysin resists elution with 1 m*M* ethylenediaminetetraacetic acid and hence is probably inserted into the lipid bilayer. Toxin-attacked cells can be quantitatively protected from osmotic lysis by extracellular dextran 4, but not by sucrose or raffinose. There is rapid efflux of K^+, and influx of Ca^{2+} [80], mannitol and sucrose into dextran-protected cells (fig. 6). These data indicate the presence of transmembrane pores of 15–30 Å diameter generated by *E. coli* hemolysin in the erythrocyte membrane [27].

Sucrose density gradient centrifugation of deoxycholate (DOC)-solubilized target erythrocyte membranes results in the recovery of detergent-solubilized toxin exclusively in monomer from (fig. 7). Electron microscopic examination of toxin-treated membranes have revealed no alterations compared to controls. Hence, pores formed by the toxin have tentatively been proposed to represent toxin monomers inserted into the membranes, whereby the possibility cannot be excluded that toxin oligomers are formed in the membrane that, however, are dissociated by DOC [27]. Although the primary structure of the toxin is known [44], the membrane-inserted regions of the molecule have yet to be identified.

The concept of transmembrane pore formation by insertion of toxin monomers into the bilayer has recently been corroborated and extended

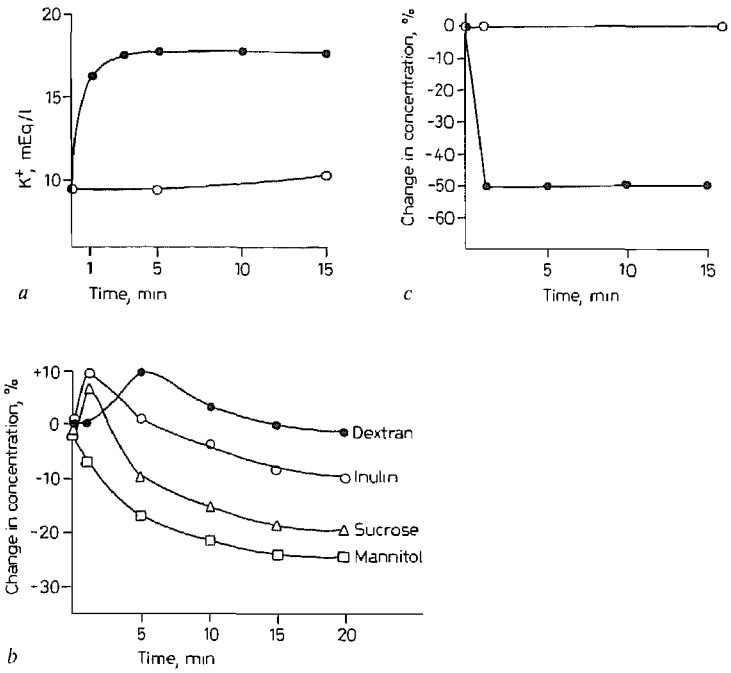

Fig. 6. a Efflux of K^+ from rabbit erythrocytes induced by *E. coli* hemolysin. Erythrocytes were suspended in saline containing 30 m*M* dextran 4 (2×10^9 cells per ml). Toxin treatment induced a rapid efflux of K^+ that was essentially compete within 1 min. Cell lysis was totally inhibited by dextran througout the duration of the experiment. ● = Toxin-treated cells; ○ = control cells. *b* Toxin-induced influx of ^{45}Ca into dextran-protected erythrocytes. A suspension of erythrocytes in dextran-containing buffer was treated with ^{45}Ca. After toxin treatment, samples were removed and radioactivity in the supernatants was measured. Influx of ^{45}Ca was reflected by a decrease in radioactivity, expressed as percent change in ^{45}Ca concentration. Intracellular accumulation of ^{45}Ca was enhanced due to Ca-binding proteins in the cytoplasm. Symbols as in *a*. *c* Influx of radioactive markers into toxin-treated erythrocytes. The experimental design was as in *b*. From Bhakdi et al. [27].

by studies using planar lipid bilayers [Menestrina et al., submitted for publication]. It was found that *E. coli* hemolysin indeed formed functional pores in bilayers of phosphatidylcholine. The pore diameter was calculated to be at least 1.5 nm. In contrast to the α-toxin pore, the *E. coli* hemolysin channel displayed a selectivity of cations over anions. Moreover, channel opening displayed a voltage-dependence similar to that previously described for pore-forming colicins. The channels opened when

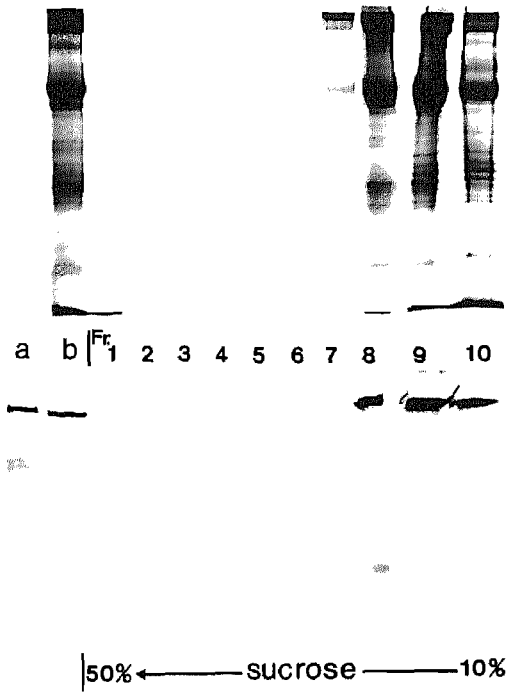

Fig. 7. SDS-PAGE of rabbit erythrocyte membranes lysed with *E. coli* hemolysin (lane b) and of fractions recovered after sucrose density gradient centrifugation of DOC-solubilized membranes (fractions 1–10; direction of sedimentation is from right to left as indicated). Upper panel: gel stained with Coomassie blue; lower panel: immunoblot developed with antibodies to the hemolysin. A sample of *E. coli* hemolysin that was used in the experiment was additionally applied in the SDS-PAGE immunoblot analysis (lane a, lower panel). Note the binding of the 107-kilodalton hemolysin to the erythrocyte membranes and the presence of membrane-derived, detergent-treated toxin exclusively in monomer form in the three top fractions of the gradient. From Bhakdi et al. [27].

the *trans*-side was negative relative to the *cis* (application) side, and closed when the *trans*-voltage became positive ($\geq$ 5 mV) relative to the *cis*-side. Dose-response analyses supported the contention that pores were formed by insertion of toxin monomers, since clear one-hit response curves were obtained. However, these results do not exclude that pore formation ensues through insertion of pre-formed toxin-oligomers. Finally, it was found that pores formed by *E. coli* hemolysin were destroyed by proteases

when these were added to the *cis*, but not to the *trans*-compartment. Hence, this toxin channel differs basically from oligomerizing toxin pores of gram-positive organisms in remaining susceptible to proteolytic destruction even after membrane attachment.

Experiments using unilamellar liposomes have generated further data indicating that *E. coli* hemolysin attaches to bilayers of phosphatidylcholine and effects release of intracellularly trapped markers [Bhakdi et al., unpublished]. Taken together, these data place the pore concept of toxin action on a firm basis and also show that *E. coli* hemolysin, like staphylococcal α-toxin, does not require a specific cell-surface molecule for binding and insertion into a target lipid bilayer.

Secondary Effects Elicited by Pore-Forming Cytolysins

Early studies on staphylococcal α-toxin and sulfhydryl-activated toxins indicated that subcytolytic doses of these proteins were capable of inducing a variety of effects on cells. These observations were made long before the pore concept was advanced, and only recently have attempts been made to obtain explanations on a molecular level for some secondary effects elicited by the toxins. At present, it appears useful to define two potentially important categories of secondary effects: (1) stimulus-response effects evoked by a passive flux of ions and/or small molecules through the pores, and (2) immunological reactions including self-attack processes involving host antibody and complement that are triggered by the insertion of the alien bacterial proteins into the target mammalian membrane. In addition, formation of immune complexes in solution, with concomitant complement activation, may be a further, more indirect mechanism by which bacterial cytolysins exert detrimental effects in the human host.

Stimulus-Response Effects

Since all toxin pores studied to date are either nonselective or cation-selective, they will permit a rapid, passive transmembrane flux of cations including calcium from the extracellular to the intracellular compartment. The transmembrane flux of small molecules, and particularly influx of Ca^{2+}, is bound to profoundly affect cellular physiology. Of the many possible effects ensuing from a toxin-induced rise of intracellular Ca^{2+}, only the stimulation of arachidonate metabolism has been studied in any detail. Treatment of cultured endothelial cells or polymorphonuclear leukocytes

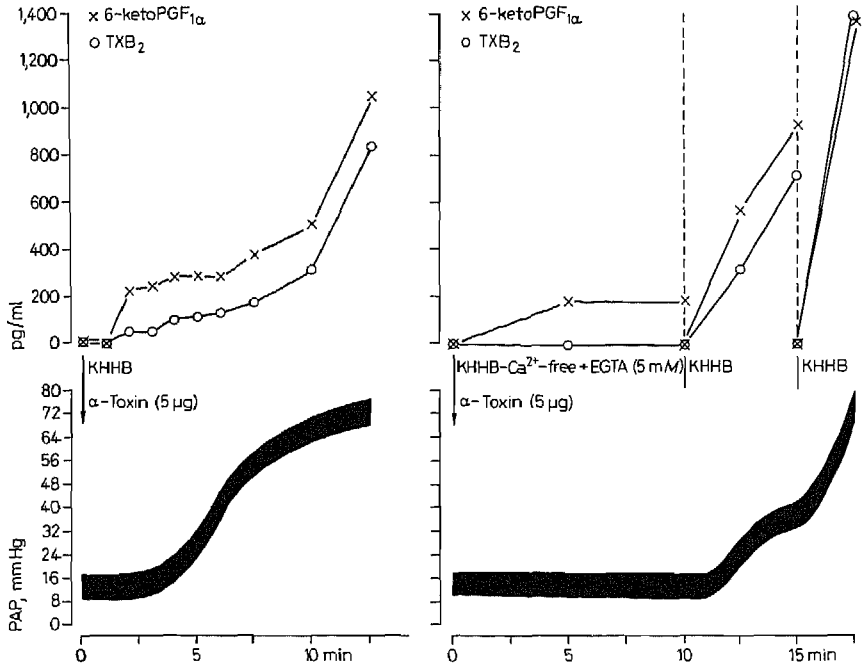

Fig. 8. Provocation of a vascular pressor response in isolated, perfused and ventilated rabbit lung by staphylococcal α-toxin; course of the pulmonary artery pressure (PAP) and the concentrations of thromboxane B_2 (TXB_2) and 6-ketoprostaglandin $F_{1\alpha}$ (6-ketoPGF$_{1\alpha}$) in the recirculating perfusion fluid after application of 5 μg α-toxin in two different lungs in the presence and absence of intravascular calcium. The dashed lines in the right-hand picture indicate the change of perfusion fluid from calcium-free (EGTA-containing) buffer to normal, calcium-containing buffer. Release of arachidonate metabolites and pressor responses were dependent on the presence of calcium in the buffer. From Seeger et al. [115].

with α-toxin has been found to stimulate the synthesis of prostanoids in these cells, this process in turn correlating with Ca-flux into the cells [124].

Another set of experiments was conducted in isolated and perfused rabbit lungs. Addition of the toxin to the recirculating buffer elicited a dramatic increase in prostanoid levels (fig. 8); however, the cells involved in the observed response have yet to be identified. The lung model bears biological relevance because the observed production of prostanoids is causally related to the development of pulmonary hypertension [115] and may thus represent a significant pathophysiological process that contri-

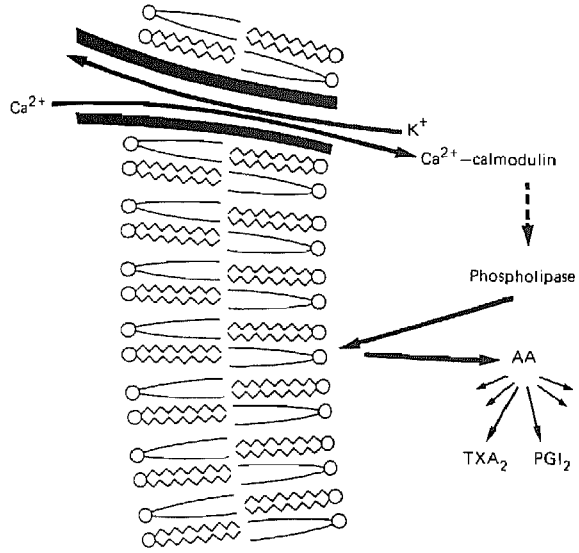

Fig. 9. Proposed mechanism of mode of action of α-toxin. A toxin-created transmembrane pore serves as a nonphysiological calcium channel. Incoming calcium, possibly after binding to calmodulin, activates phospholipases with subsequent cleavage of arachidonic acid and formation of tissue-specific arachidonic acid metabolites. TXA_2 = Thromboxane A_2; PGI_2 = prostacyclin. From Suttorp et al. [124].

butes to the pathogenesis of acute respiratory distress and related syndromes during toxinemia. It is of interest that *E. coli* hemolysin, in addition to eliciting marked pulmonary hypertension in the isolated lung, also induces a rise in vascular permeability and causes pulmonary edema. The mechanisms involved in the latter phenomenon are presently under study [Seeger et al., unpublished data].

Pseudomonas aeruginosa cytolysin, which is probably also a pore-forming exotoxin [86], also stimulates prostanoid generation in cultured endothelial cells [126]. The phenomenon of arachidonic acid cascade stimulation thus appears to be common to most, if not all, pore-forming proteins, and similar observations have also been made with thiol-activated cytolysins [29] and in the complement field [59, 75, 116] (fig. 9). Further search will undoubtedly reveal other secondary effects induced by a passive influx of calcium ions through protein pores.

Immunologic Self-Attack Processes at the Membrane Level

Pore-forming cytolysins belong to the few bacterial proteins that can enter into and become firmly bound to a host cell membrane. All pore-formers studied to date are excellent immunogens, so that antibodies to these proteins are generally present in plasma of healthy adults. Once the proteins have become anchored in the host cell membrane, the antibodies will bind to them and in some cases complement activation will ensue. Many mechanisms regulate complement activation and attack on autologous membranes [e.g. ref. 43, 82], yet they are sometimes unable to halt the cascade. It has been found, for example, that membrane-bound SLO induces fulminant complement activation in an antibody-dependent manner in vitro. This leads to rapid complement consumption, generation of C3a and C5a anaphylatoxins, binding of large quantities of C3b which will act as an opsonin and, finally, generation of C5b-9 lesions in autologous cell membranes (fig. 10) [20]. It is easy to envisage that such processes may support in vivo inflammation, opsonization and phagocytosis of target cells. The unusual stability of SLO oligomers might serve to prolong these detrimental reactions, and phagocytic removal of the afflicted tissue may represent the only possibility of interruption of the vicious cycle. At the same time, deviation of the complement system away from the bacteria may support their invasion and persistence in host tissue. In broader perspective, immunologic self-attack induced by bacterial molecules that enter host membranes may be compared with T cell attack on virus-infected cells that bear alien antigens on their surface. An analogous complement-dependent attack mechanism has recently also been described after spontaneous insertion of bacterial lipoteichoic acid into cell membranes [74].

Formation of Complement-Activating Immune Complexes in Solution

It would probably be wrong to assume that all biological effects of pore-forming bacterial cytolysins are due to their action on cell membranes. Possibly, these bacterial products are also involved in immunopathological processes where they react as soluble components with the immune system. Awareness of this possibility appears warranted especially in view of the fact that the proteins are potent immunogens, and are produced and released in appreciable quantities by the bacteria. Immune complexes formed between SLO and human antibodies, for example, indeed exhibit potent complement-activating properties [20]. At the site of infection, such reactions again would augment inflammation and might

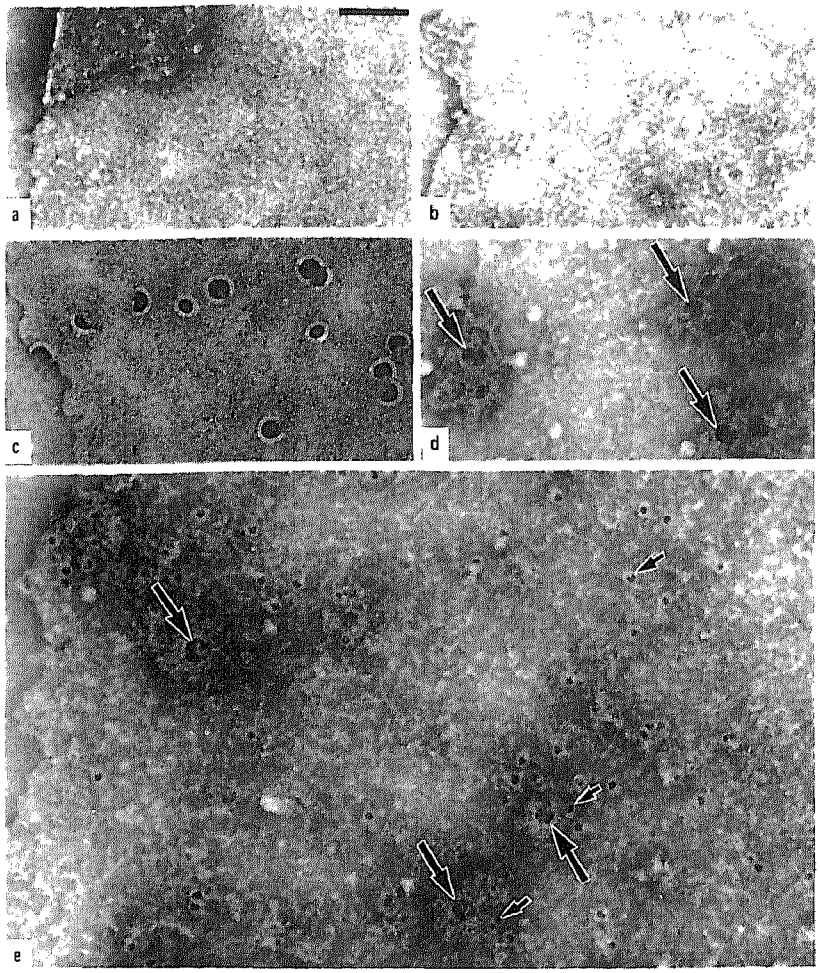

Fig. 10. a Human erythrocyte membrane treated with 5 m*M* dithiothreitol (DTT). Two layers of membrane are superimposed at upper left. *b* DTT-treated membrane after incubation in autologous human serum. Only a slight coarsening of the surface results from this treatment. *c* Membrane treated with SLO; the characteristic SLO lesions are seen. *d* Same membrane preparation as in *c*, post-treated with autologous serum. The toxin structures have become covered by irregular tufts of protein-like material that extend onto the surrounding membrane. *e* as *d*, post-treatment with a high serum dose. Classical complement lesions (small arrows) are scattered over the membrane with a tendency for clustering around the SLO lesions (bold arrows). Bar indicates 100 nm. From Bhakdi and Tranum-Jensen [20].

divert the action of complement away from the bacteria. Deposition of immune complexes in tissues remote from the primary site of infection may possibly contribute to nonsuppurative sequelae, such as are encountered in post-streptococcal disease.

Conclusions and Perspectives

The concept of membrane damage by formation of transmembrane pores through insertion of alien proteins into target bilayers is today well borne out in many biological systems, of which bacterial cytolysins, complement proteins [17, 91] and lymphocyte cytolysins [65] have been studied fairly extensively. Other pore-formers include cytolysins from ameba [87, 149], sea anemone [95], fungi [42] and also the cationic proteins from human eosinophilic leukocytes [150]. The list of pore-formers will certainly grow as general awareness of this mechanism increases. Our objective has been to present an overview of the present knowledge on basic mechanisms that govern the perturbation of membranes by pore-formers. We have concentrated on the three most thoroughly studied bacterial cytolysins since these prototypes present features that will probably be encountered with other pore-formers in the future. The study of these proteins brings together several aspects of protein and membrane biochemistry, cell physiology and immunology. Seen in broader perspective, the phenomenon of a hydrophilic-amphiphilic transition of proteins from a water-soluble to a membrane-bound state appears to be essential for a variety of other important biological phenomena. These include the pH-dependent transition of viral fusion proteins that are required for escape of virus genomes from endosomes [64, 119] and similar pH-dependent transitions of exotoxin fragments (e.g. B fragment of diphtheria toxin) to amphiphilic moieties [81, 112]. Some primarily water-soluble proteins must pass into and sometimes through mitochondrial and chloroplast membranes, and these processes may also involve exposure of lipid-binding areas [111, 114]. Finally, structural similarities may also emerge between membrane-damaging pore-formers and naturally occurring channel proteins that are present in cell membranes [for recent review, see ref. 134]. Further studies on the pore-forming proteins – several of which have been subject to molecular cloning and which are accessible in appreciable quantities and, in very pure form, could contribute to the general understanding of principles that govern the post-translational insertion of proteins

into membranes – are necessary. From a biological viewpoint, it is clear that membrane damage by pore-forming proteins is a very widespread phenomenon that plays an important role both in the context of microbial pathogenesis and as an effector mechanism of mammalian defence systems.

Acknowledgements

Work performed in the authors' laboratories was supported by the Deutsche Forschungsgemeinschaft (Bh 2/2 and 2/1-7) and the Verband der Chemischen Industrie. We thank Drs. F. Hugo, W. Seeger, N. Suttorp, A. Salama, N. Mackman and I.B. Holland for many stimulating discussions, and Margit Pohl, Marion Muhly and Sylvia Krämer for their outstanding technical assistance.

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