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Patron: Stoyanov, Alexander

Journal Title: Medical microbiology and immunology.

Volume: 183 **Issue:**
Month/Year: 1994 **Pages:** 119-144

Article Author:

Article Title: Bhakdi S, Grimminger F, Suttorp N, Walmrath D,; Proteinaceous bacterilal toxins and pathogenesis of sepsis syndrom and sectic shock

Imprint: Berlin, New York, Springer.

ILL Number: 40288041
40288041

Call #: QR1 .M4 V.183/184 (1994/1995)

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Med Microbiol Immunol (1994) 183: 119–144

Medical Microbiology and Immunology

Mini review

Proteinaceous bacterial toxins and pathogenesis of sepsis syndrome and septic shock: the unknown connection

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Received: 13 May 1994

Introduction

Researchers engaged in the study of septic shock pathogenesis generally regard this syndrome to be the consequence of detrimental endotoxin effects in the host organism. This is understandable since causal relationships are firmly established between the action of endotoxin on monocyte/macrophages, granulocytes, thrombocytes and various other inflammatory cells. Foremost in the minds of most investigators is the capacity of lipopolysaccharide (LPS) to potently stimulate production and release of inflammatory mediators such as monokines, in particular tumor necrosis factor and interleukin-1 (IL-1), and eicosanoids, and to activate the complement and coagulation cascades (e.g. [16, 30, 33–35]).

These facts notwithstanding, it is still surprising that proteinaceous microbial toxins have received so little attention as potentially important instigators of systemic inflammation and multi-organ dysfunction. This statement holds for all major cytotoxins save those that are recognized to act as "superantigens" [19]. Vascular leakage and shock evoked by the latter are currently also thought to be due to cytokine overload, analogous to endotoxin shock [19], and will not be dealt with in this review. Our present discussion will focus on much more widespread, yet less-known, bacterial exotoxins that exert direct effects on target cells.

When considering the modes of action of bacterial protein toxins, it is useful to distinguish between toxins that enter the cell, as opposed to those that act primarily at the level of the cell plasma membrane. Examples of the former group are the ADP-ribosylating toxins and the neurotoxins. The molecular structure and mechanisms underlying the action of many of these toxins have been delineated. Although several are of cardinal medical importance, they are not generally associated with septic shock. In contrast, recent evidence has accumulated indicating that membrane-perturbating toxins may directly evoke pathophysiological reactions, leading to vascular damage and shock, and the following discussion will be confined to a discussion of these exotoxins.

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Table 1. Prototypes of pore-forming bacterial exotoxins

Producing organism	Designation	Salient features
Gram-positive bacteria		
Staphylococcus aureus	α-toxin (-hemolysin)	Binds to unidentified specific sites on target cells; forms voltage-independent hexameric pores of 1.0-nm diameter; *identified susceptible human cells:* platelets, monocytes, lymphocytes, endothelial cells
Streptococcus pyogenes A	Streptolysin-O	Prototype of polymerizing toxin that forms large pores (up to 30-nm diameter); probably binds to cholesterol; all mammalian cells examined are susceptible
N. B.: At least 14 other related and probably functionally similar toxins are produced by gram-positive organisms including *Listeria monocytogenes*, *Streptococcus pneumoniae*, and *Bacillus cereus*		
Gram-negative bacteria		
Escherichia coli	*E. coli* hemolysin (Hly A, α-hemolysin)	Receptor problem unclarified, may bind non-specifically to lipid bilayers; forms voltage-dependent, cation-selective pores of 1- to 20-nm diameter possibly through membrane insertion of toxin monomers; potent cytocidal effects on all white blood cells and endothelial cells; triggers G-protein-dependent processes at low concentrations
N.B.: At least 10 other related toxins are produced by gram-negative organisms including *Proteus* sp., *Morganella morganii*, *Pasteurella haemolytica, Actinobacillus pleuropneumoniae,* and *Bordetella pertussis*		
Serratia marcescens	*Serratia* hemolysin	Forms voltage-independent pores of 1-nm diameter
Aeromonas hydrophilia	Aerolysin	Produces oligomeric pores of 1-nm diameter
Other pore-forming toxins are produced by *Vibrio cholerae* and *Vibrio parahaemolyticus*, and *Klebsiella*		

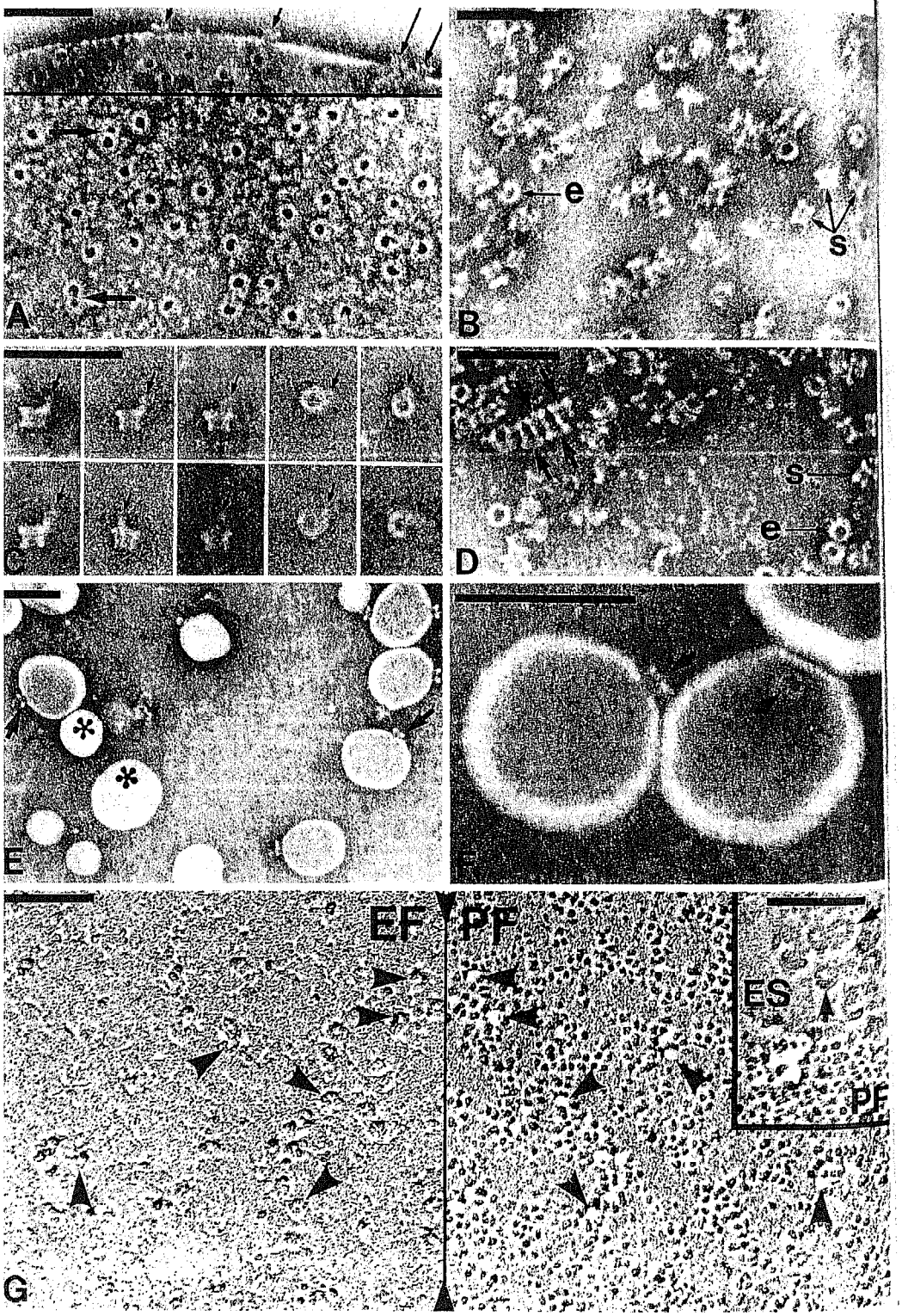
Basically, a protein toxin may attack the plasma membrane either via enzymatic action or via physical perturbation of bilayer integrity. Phospholipase C is the best-known representative of a medically important, membrane-damaging bacterial enzyme. This toxin is elaborated by several bacterial species and has been established as an important pathogenetic factor of *Clostridium perfringens* [32]. Massive release of phospholipase C probably contributes to pathological processes seen in *Cl. perfringens* infections due to its combined effects on blood and tissue cells. Detailed analyses of such phenomena particularly in the context of vascular damage are still wanting, however. The pathogenic significance of other enzymes produced by bacteria, including a broad spectrum of proteases, has not yet been established. The second mechanism of membrane damage by bacterial toxins is via physical perturbation of bilayer integrity evoked, in the main, through the forma-

tion of transmembrane pores [5, 6, 15]. Evidence has accumulated over the past decade that the majority of medically relevant pathogens produce toxins belonging to the category of pore formers. Among the pathogens that are frequently associated with septic shock, *Escherichia coli* and several members of the family *Enterobacteriaceae* and *Staphylococcus aureus* deserve special mention. These and some other producers of pore-forming toxins are listed in Table 1. In the following, we shall first discuss the basic properties of pore-forming proteins and consider the factors that direct their attack to specific cell targets. Thereafter, attention will be drawn to various functional consequences that may follow membrane damage, so that a general concept of how pore-forming toxins may contribute towards the development of vascular injury will evolve.

General features of transmembrane pore formation

All pore-forming toxins are produced and released by bacteria initially as water-soluble proteins, but are able to undergo a unique and possibly always irreversible transition from a hydrophilic to an amphiphilic state upon interaction with a target lipid bilayer. Primary binding to a target membrane may require the presence of specific binder molecules. A well-documented example for this type of interaction is represented by the sulfhydryl-activated cytolysins (e.g., streptolysin-O), which probably initially bind to membrane cholesterol [1, 3, 40]. Often, however, cytolysins will be of the "receptorless" type that binds indiscriminately to lipid bilayers. *E. coli* hemolysin and related toxins are considered representatives of this second group [5, 6]. Staphylococcal α-toxin occupies an intermediate position, since it can interact both with specific binding sites and via nonspecific absorption to lipid bilayers [17, 25]. Only certain cell types express the high-affinity binding sites for α-toxin on their surface. When present at low concentrations, the toxin will bind exclusively so such sites, so that cells lacking the toxin binder will be exempted from attack. At high concentrations, α-toxin will additionally absorb in a non-specific fashion to lipid bilayers, also causing damage to cells that are devoid of the specific binding site. This process is, however, probably not of biological relevance. When present, the high-affinity binding sites are usually only expressed in low numbers; therefore, overall consumption of toxin is low. The attack process is very effective, requiring only a small number of toxin molecules to be deposited on each cell target for membrane permeabilization to take place [7].

Insertion into the lipid bilayer follows as a second and sometimes temporally dissociated step after toxin molecules have bound to the membrane surface. Generally, membrane insertion can take place at 0 °C, albeit at a slower rate [5, 6]. In some instances, pores are probably generated by insertion of protein monomers into the bilayer (e.g., *E. coli* hemolysin) [9]. Alternatively, some pore formers need to aggregate with each other to form oligomers before pores can be generated; this is the case with α-toxin [7] and streptolysin-O [8]. Monomeric pores are too small to be visualized by electron microscopy, whereas several oligomeric pores have been characterized ultrastructurally. In the latter cases, the pores are generally seen as partially or fully circular structures. Oligomeric pores are sometimes heterogeneous in size, due to variations in the number of protomers that constitute the individual channels. *S. aureus* α-toxin oligomers tend to be more homogeneous (Fig. 1). The effective functional diameters of pores span a very large range, from approximately 1.0 nm (α-toxin and *E. coli* hemolysin) to over 35 nm (streptolysin-



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O). In the case of α-toxin, it appears that pore sizes can be heterogeneous, dependent on toxin dose and cell target. In many nucleated cells, low toxin doses create very small lesions that are only freely permeable to monovalent ions [48]. Pores created by *E. coli* hemolysin require a distinct transmembrane potential and display a strong selectivity for cations over anions [31].

Although the primary structure of α-toxin, *E. coli* hemolysin and several sulfhydryl-activated toxins are known, the domains that are membrane embedded have yet to be identified. No details are available on the polypeptide conformation and three-dimensional structure of any protein pore. One surface of the membrane-embedded protein must necessarily by hydrophobic to permit its stable interaction with the lipid domain of the bilayer. The other must be hydrophilic to permit the passage of ions and small hydrophilic molecules across the membrane. It is not known whether the pores always span the bilayer, or whether partial penetration might suffice to generate a functional lesion. The extent to which pores are lined by protein is also unknown. In the case of the α-toxin hexamer and the fully circularized streptolysin-O pores, the channels indeed appear to traverse the interior of protein cylinders. However, incompletely circularized streptolysin-O pores may be lined by an edge of free lipid arising through lateral repulsion of apolar lipid domains from the inserted, hydrophilic faces of the protein [8]. Similarly, it is conceivable that monomerically inserted proteins, such as hemolysin of *E. coli*, do not require circularized protein structures to generate transmembrane channels.

Few studies have addressed the fate of pores after their formation in membranes of nucleated cells. In the case of α-toxin, there is evidence that certain cells are capable of shedding hexamers from their surface, and also of closing a limited number of pores in the membrane (unpublished data). This toxin does not appear to be taken up by endocytosis to any appreciable extent. No study on the deposition of any pore-forming bacterial cytolysin in human tissues has been performed to date.

Cellular reactions

Cell death is the most obvious and inevitable consequence of transmembrane pore formation if a lesion cannot be removed or repaired. Death ensues because the cell is rapidly depleted of ATP, either because of efflux of nucleotides through the pores (Fig. 2) or because the cell is unable to counteract the deleterious effects of ionic

Fig. 1. **A** Negatively stained fragment of rabbit erythrocyte lysed with staphylococcal α-toxin. Numerous 19-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 liposomal membrane and as rings over the membrane (*arrows*). Characteristically, liposomes that escape incorporation of the toxin are impermeable to the stain. **D** Negatively stained erythrocyte membrane lysed by streptolysin-O (SLO) showing numerous curved rods 25- to 100-nm long and approximately 7.5-nm wide with inner radius of curvature of 13–16 nm. Most rods are approximately semicircular, often joined in pairs at their ends. Dense accumulations of stain are seen at the concave side of the 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 in 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. Sodium silicotungstate was used as negative stain in **B**–**F**. Uranyl acetate was used in **A**. From [6]. *Bars* **A**–**F** = 100 nm

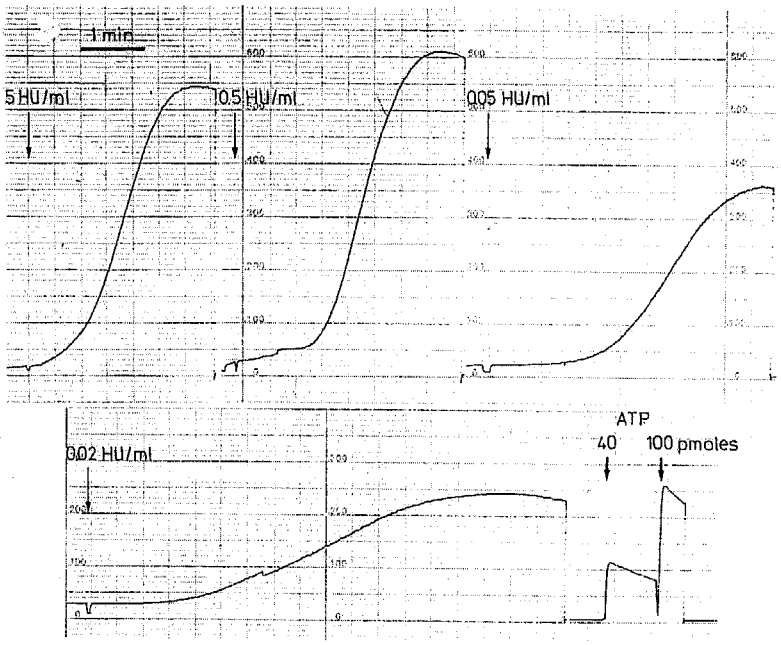


Fig. 2. Release of ATP from polymorphonuclear cells (PMN) (10^6 cells in 400 µl) suspended in PBS induced by *E. coli* hemolysin. ATP was measured continuously using the firefly assay. The final concentrations of toxin applied are given in hemolytic units (HU)/ml. The assays were calibrated with ATP. Note ATP leakage induced by very low concentrations of the toxin (1 HU ~ 0.1 µg/ml). From [13]

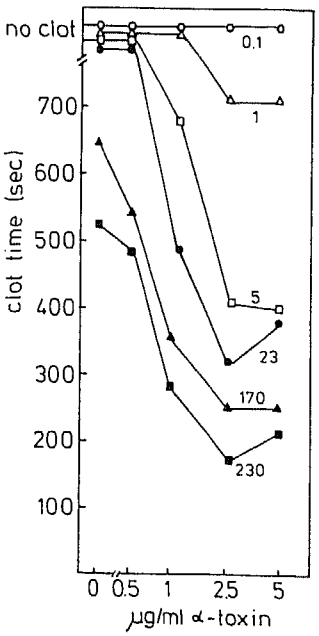


Fig. 3. Reduction in clot times of recalcified human plasma by *S. aureus* α-toxin. Citrated plasma samples containing the depicted numbers of platelets (per nl) were given 12 mM Ca^{2+} and α-toxin at the given final concentrations. Marked reductions in clot times were noted at toxin concentrations of 1 – 2.5 µg/ml, dependent on the presence of platelets in the plasma sample. *No clot* indicates lack of clot formation within 900 s. From [10]

disequilibrium and loss of its "milieu intérieur" that is essential for sustaining metabolic processes. Loss of cellular function can have direct consequences. For example, killing of leukocytes and monocytes by *E. coli* hemolysin cripples the local phagocytic defence system and probably fosters the invasion of other pathogens that colonize the lesions [12, 13]. Damage to endothelial cells by *S. aureus* α-toxin causes massive derangements in lung vasculature, and loss of endothelial cell lining leads to rapid development of pulmonary edema [36, 38]. Renal tubular epithelia are highly susceptible to *E. coli* hemolysin [28] and this may partially explain disturbances in tubular function that commonly develop during renal infections with toxin-producing bacterial strains.

Until recently, awareness that perturbation of the plasma membrane permeability barrier might trigger various cellular reactions was generally lacking. Today, it is apparent that such reactions are inevitable and that they may contribute substantially to the pathogenesis of tissue and organ lesions. Depending on the cell target, a variety of responses may ensue, of which the following have been studied in some detail.

Secretion

Exocytotic liberation of vesicular components has been demonstrated in leukocytes, platelets and neurological cells. Leukocytes succumbing to attack by *E. coli* hemolysin release large amounts of vesicular components [13, 24]. The release of elastase is noteworthy since this enzyme has been discussed as a factor that may contribute towards the development of diffuse vascular leakage and multiple organ injury. Platelets attacked by α-toxin secrete large quantities of granule constituents including platelet-factor 4 and Factor V [2, 10]. Release of the latter leads to assembly of platelet-bound prothrombinase complexes that generate thrombin [2]. *S. aureus* α-toxin, thus, activates human platelets and promotes coagulation (Fig. 3), processes that bear high potential relevance in staphylococcal infections.

Generation of reactive oxygen species

Leukocytes attacked by very low doses of *E. coli* hemolysin produce large amounts of reactive oxygen metabolites. Optimal toxin concentrations (in the subcytocidal range) evoke responses comparable to those observed with formyl-Met-Leu-Phe (fMLP) or phorbol ester doses [4, 24] (Fig. 4). Moreover, neutrophils exposed to low levels of this toxin become hyperresponsive with respect to superoxide production when challenged with conventional stimuli such as phorbol esters [4, 24].

Lipid mediator generation

Subcytolytic concentrations of *E. coli* hemolysin activate the 5-lipoxygenase system in human neutrophils [23, 29]. In the presence of exogenous arachidonic acid, large amounts of leukotrienes and 5-hydroxy-(6E, 8Z, 11Z, 14Z)-eicosatetraenoic acid (5-HETE) are generated (Fig. 5). The 5-lipoxygenase pathway is also activated in alveolar macrophages. Perfusion of isolated, blood-free rabbit lungs with either α-toxin or *E. coli* hemolysin, or with viable hemolytic *E. coli*,

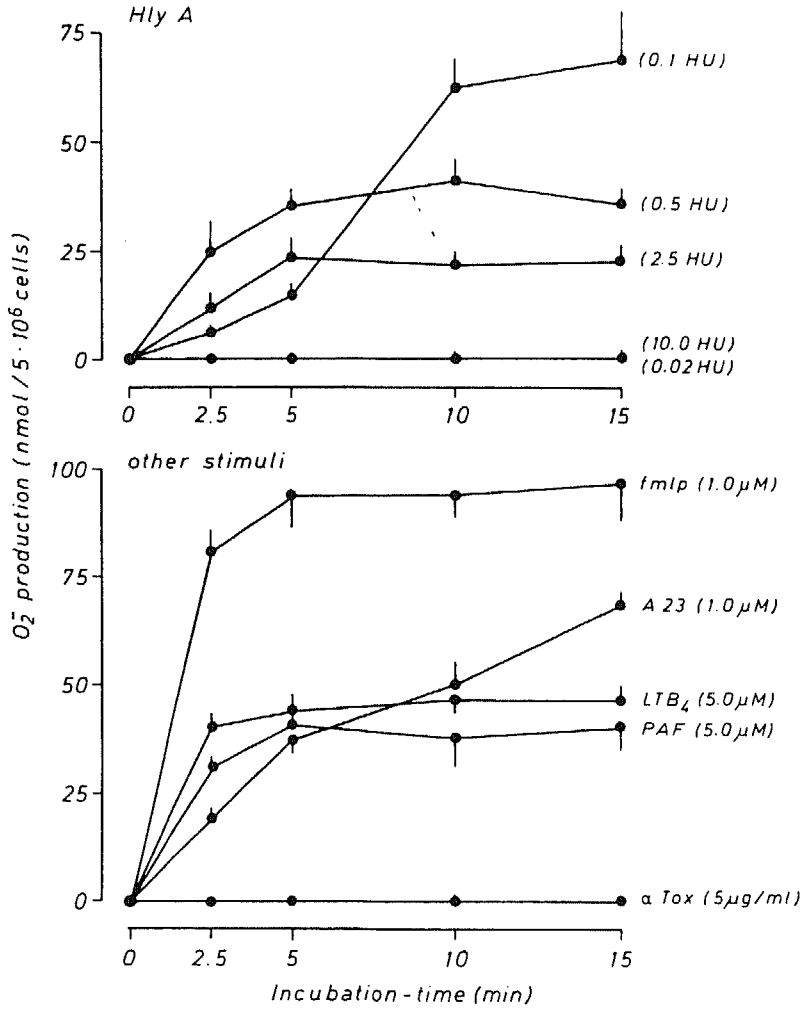
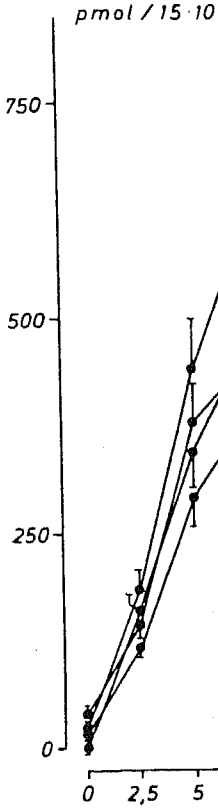


Fig. 4. Time course of neutrophil superoxide production evoked by *E. coli* hemolysin (*Hly A*) in comparison with other stimuli. Cells (10^7) were incubated with different concentrations of Hly A (*top*) or other different stimuli at optimum concentrations (*bottom*) for various time periods. Means ± SEM of five (*top*) and four (*bottom*) independent experiments are given. From [24]

leads to pronounced liberation of both cyclooxygenase and lipoxygenase products of arachidonic acid [18, 21, 22, 36–39]. Low doses of hemolysin and staphylococcal α-toxin evoke release of prostaglandin I_2 and platelet-activating factor (PAF) from cultivated vascular endothelial cells [41, 45]. Human neutrophils challenged with *E. coli* hemolysin also release PAF [24].

Derangement of cytoskeleton organization and function

Staphlyococcal α-toxin and *E. coli* hemolysin induce intercellular gap formation in cultured pulmonary artery endothelial cells, which results in enhanced passage



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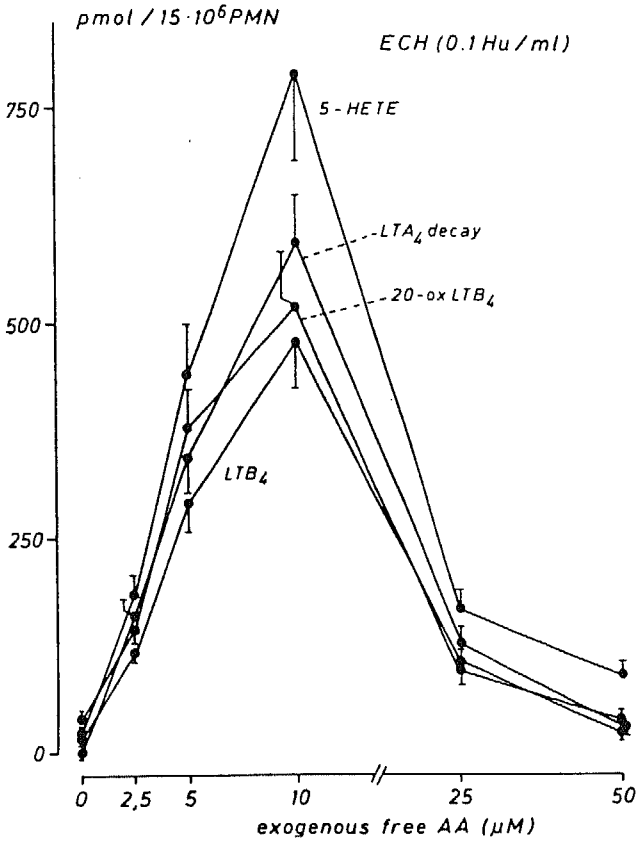


Fig. 5. Dependence on exogenous free arachidonic acid (*AA*) on the 4-series leukotriene and 5-HETE generation in human neutrophils challenged with *E. coli* hemolysin (*ECH*) (0.1 hemolytic unit/ml). Cells (1.5×10^7) were incubated with the hemolysin and incremental doses of AA for 10 min. No 5-series leukotriene or 5-HEPE was detected under these conditions. Means ± SEM of six independent experiments are given. From [23]

of fluid and macromolecules across the monolayer (Fig. 6). Evidence was obtained that this derived from contraction and rounding up of the adherent cells, probably because of rearrangement of the endothelial cytoskeleton [43, 44].

Release of cytokines

Attack on monocytes by *S. aureus* α-toxin and *E. coli* hemolysin causes liberation of large amounts of IL-1β [12, 14]. A prerequisite for this appears to be the prior intracellular accumulation of the IL-1β precursor. Thus, unstimulated monocytes do not release IL-1β in response to toxin attack, but large amounts of cytokine are

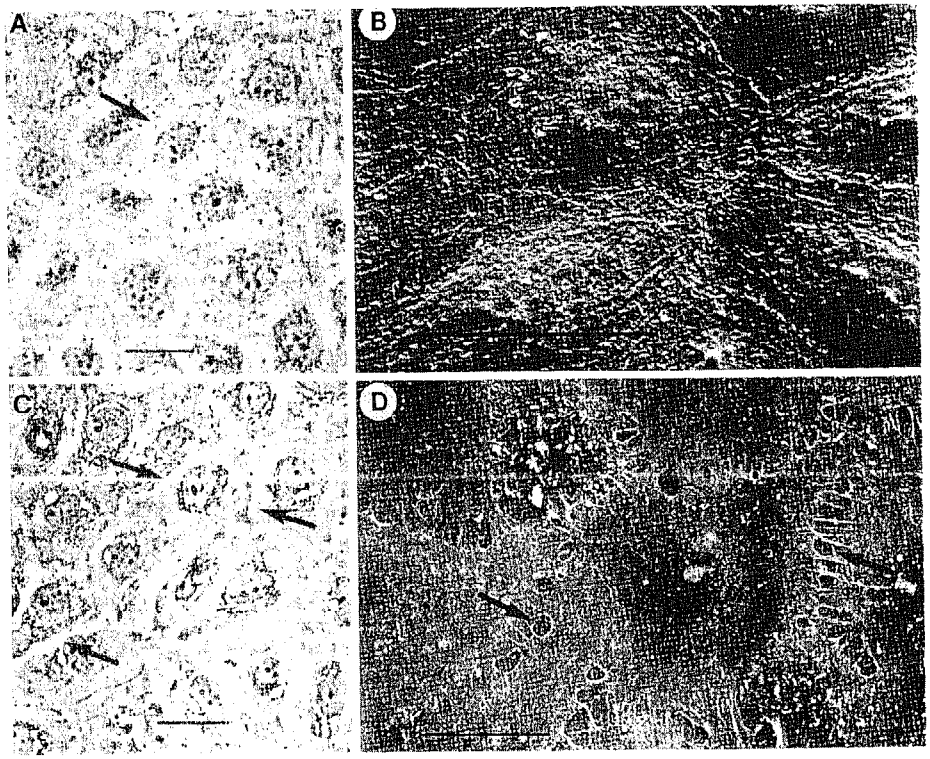
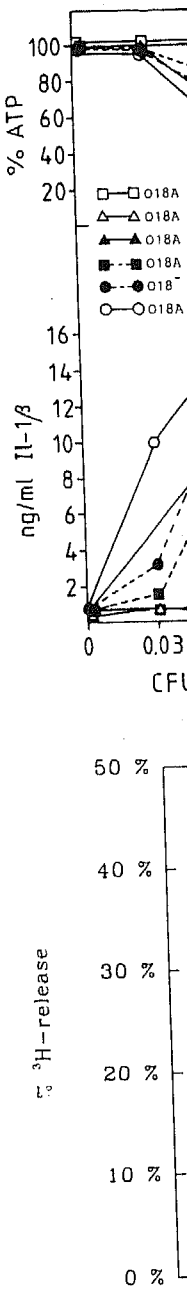


Fig. 6 A–D. Intercellular gap formation in cultured endothelial cells induced by a pore-forming toxin. **A, B** Sealed endothelial monolayer of pig pulmonary artery grown on gelatine-coated cover slips and exposed for 90 min to a hydrostatic pressure of 10 cm H_2O. Examination by phase-contrast (**A**) and scanning electron microscopy (**B**) shows that virtually all interendothelial junctions (intercellular space) are closed. **C, D** Endothelial monolayer exposed for 90 min to 1 µg/ml staphylococcal α-toxin. Note large interendothelial gaps (*arrows*) that are seen by both phase-contrast (**C**) and scanning electron microscopy (**D**). *Bar* = 20 µm. From [43]

liberated from cells that have been in short-term culture or that have been co-stimulated with very low amounts of LPS. Pore-forming toxins may, therefore, synergize with LPS and with other substances that induce IL-1β synthesis. This concept is supported by the finding that incubation of freshly isolated monocytes with toxin-producing *E. coli* at a cellular ratio of only 1 : 1 causes production and release of IL-1β within a few hours (Fig. 7). Such a dramatic and rapid effect was not observed when cells were incubated with genetically related, non-hemolytic *E. coli* [12].

Induction of internucleosomal DNA-degradation

Formation of small α-toxin pores that selectively permit flux of monovalent ions was recently discovered to trigger internucleosomal DNA degradation characteristic of programmed cell death in human T lymphocytes [26] (Fig. 8). This process appears to be at least partially responsible for the lymphocidal action of the toxin.



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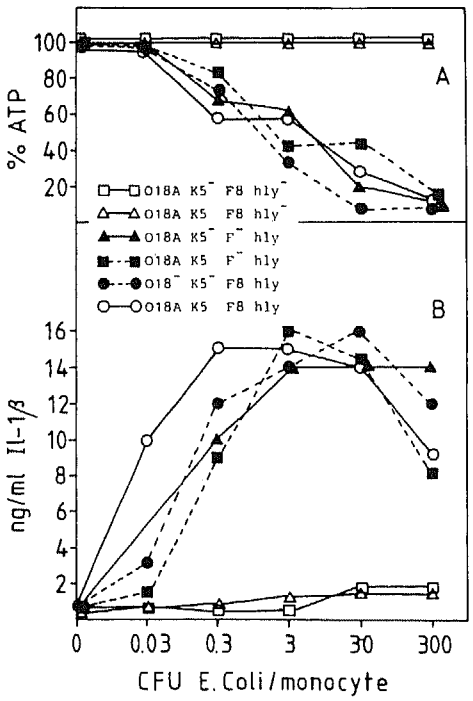


Fig. 7 A, B. Effects of incubation of viable *E. coli* with freshly isolated human monocytes. Six strains were used with the given characteristics and at the given, approximate bacteria to cell ratios. Measurements of cellular ATP (**A**) and of IL-1β (**B**) in the cell supernatants were performed after 70 min incubation at 37 °C. Note that all strains of toxin producers evoked ATP depletion and IL-1β release within the very short incubation period. The 100% ATP content corresponded to ~140 pmol ATP/well. From [14]

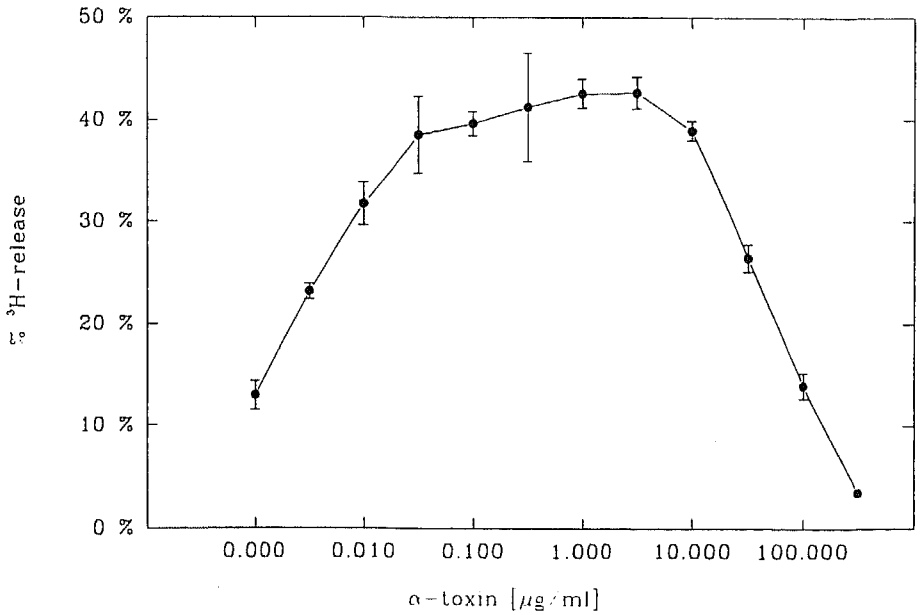


Fig. 8. Dose-dependent induction of DNA fragmentation in T lymphocytes by staphylococcal α-toxin. T cells were activated with phytohemagglutinin and labelled with [^{3}H] thymidine. They were treated with α-toxin at the depicted concentrations and release of [^{3}H] thymidine was measured after 4 h. Note the ball-shaped dose-dependent curve of DNA-degradation evoked by the toxin. From [26]

Release of nitric oxide from endothelial cells

Both *S. aureus* α-toxin and *E. coli* hemolysin increased synthesis of NO by the constitutive NO-synthase in endothelial cells [46]. These results identify a potentially important mechanism by which pore-forming toxins may contribute to the development of hypotension during severe infections and septic shock.

Mechanisms for the induction of cellular reactions

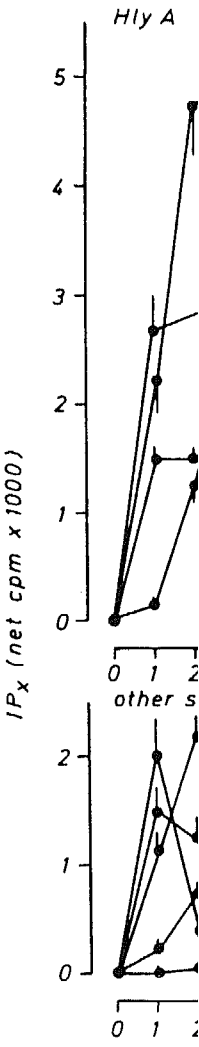
Three concepts have emerged to account for the various cellular reactions observed in different target cells following exposure to pore-forming toxins.

Calcium flux through toxin pores

Generation of prostanoids in perfused rabbit lungs, and prostacyclin generation and cell retraction in cultured endothelial cells in response to staphylococcal α-toxin and *E. coli* hemolysin are strictly dependent on extracellular calcium. Sieving experiments in cultured endothelial cells indicate the generation of small hydrophilic pores that permit passive influx of ^{45}Ca [41]. Transmembrane flux of Ca^{2+} into rabbit granulocytes treated with staphylococcal α-toxin has also been demonstrated [42]. These data suggest that the toxin pores may act as non-physiological calcium gates, thus initiating calcium-dependent cellular processes. These processes include stimulation of phospholipolytic activities in the endothelial cells, calcium-dependent cell retraction phenomena with generation of intercellular gaps, secretory processes, and probably also the stimulation of the constitutive NO-synthase in endothelial cells.

Induction of phosphoinositide hydrolysis and related metabolic responses

A different signal transduction pathway in response to *E. coli* hemolysin challenge has been observed in human granulocytes. The toxin provoked rapid secretion of elastase and myeloperoxidase, generation of superoxide and synthesis of PAF and lyso-PAF. Concomitantly, phosphatidylinositol hydrolysis with sequential appearance of the inositol phosphates IP3, IP2 and IP1, and formation of diacylglycerol occurred [23, 24] (Figs. 9, 10). The metabolic responses displayed distinct bell-shaped dose dependencies, with a maximum at subcytocidal toxin concentrations. These effects were sensitive to pertussis toxin, indicating involvement of G proteins. These data suggest that the marked capacity of low doses of *E. coli* hemolysin to elicit degranulation, respiratory burst and lipid-mediator generation in human neutrophils proceeds via the preformed phosphatidylinositol hydrolysis signal transduction pathway. Further studies will be necessary to elucidate whether this finding is confined to the neutrophils, or whether other cells attacked by *E. coli* hemolysin may similarly employ this signal transduction pathway to mount diverse reactions. The precise mechanism via which certain pore-forming toxins can short-circuit G-dependent regulatory pathways remains to be delineated.



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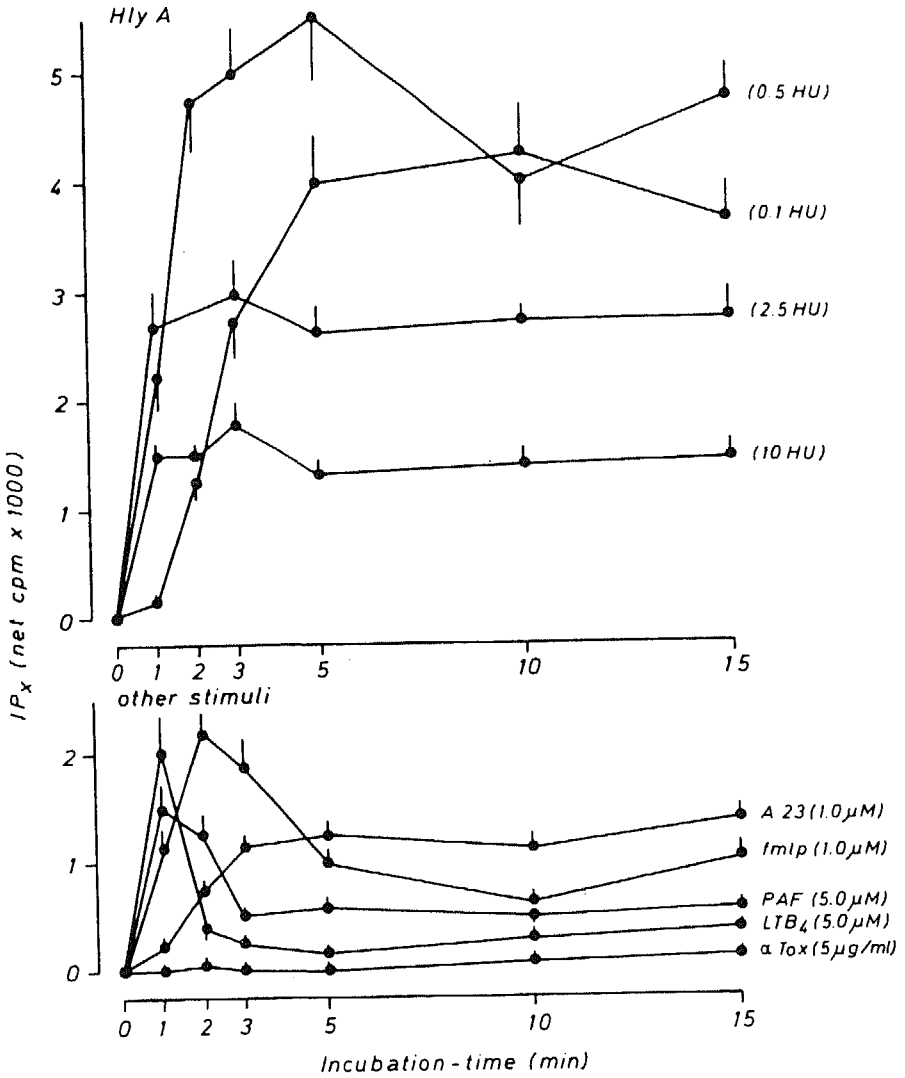


Fig. 9. Time course of neutrophil inositol phosphate generation evoked by *E. coli* hemolysin (Hly A) in comparison with different other stimuli. Human neutrophils (10^7), prelabelled with [^{3}H] inositol, were incubated with different concentrations of Hly A (*top*) or different other stimuli at optimum concentrations (*bottom*) for various time periods. Extracted inositol phosphates were separated by anion-exchange chromatography. IP_3, IP_2, and IP_1 are given as IP_x, corrected for baseline levels in nonchallenged cells (net cpm; baseline range between 540 and 1100 cpm). Means±SEM of five (*top*) and four (*bottom*) independent experiments are given. From [24]

Reactions evoked through selective transmembrane flux of monovalent ions

It appears that generation of very small pores that are selective for monovalent ions may generally trigger a novel spectrum of reactions, such as the induction of internucleosomal DNA degradation and membrane shedding. Furthermore, it appears

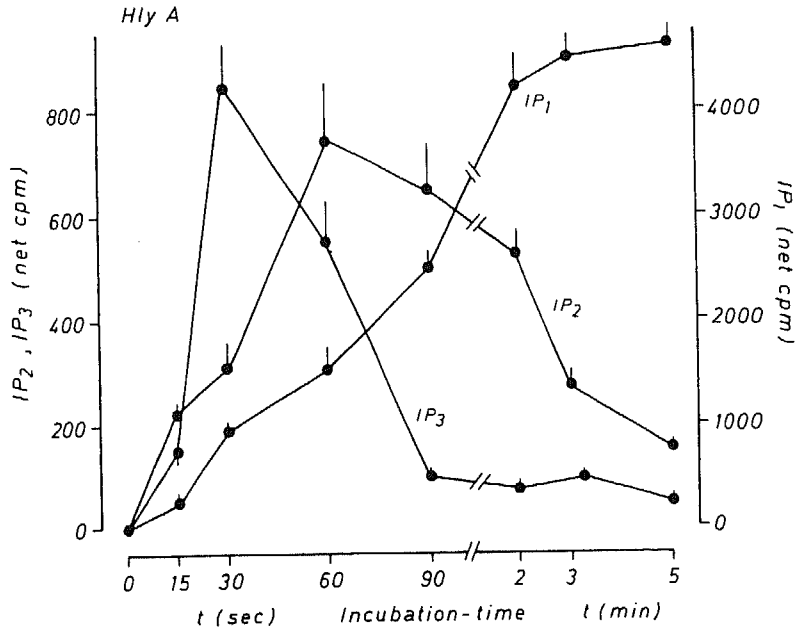
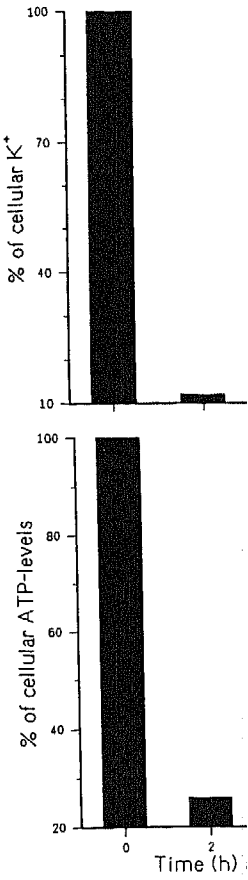


Fig. 10. Sequential appearance of inositol phosphates in neutrophils challenged with *E. coli* hemolysin. Cells (10^7), prelabelled with [^{3}H] inositol, were incubated with 0.1 hemolytic units/ml toxin for various time periods. Extracted inositol phosphates were sequentially eluted from anion-exchange columns and corrected for baseline levels in nonchallenged cells (net cpm; baseline [^{3}H] $IP_2 = 259 \pm 19$ cpm, [^{3}H] $IP_1 = 814 \pm 65$ cpm). Means ± SEM of five independent experiments are given. From [24]

that nucleated cells may often be capable of repairing a limited number of small lesions (Fig. 11). This has been demonstrated by human fibroblasts undergoing attack by α-toxin, where the evidence indicates that pores can be closed in the membrane (unpublished). Future investigations should soon reveal whether other unexpected secondary reactions may be evoked by the creation of very small pores that are temporarily formed in membranes.

Pore-forming toxins evoke acute organ lesions in vitro

Given the broad spectrum of consequences resulting from the attack of pore-forming toxins on nucleated cells, it might be expected that challenge of whole organs with such toxins may provoke acute organ dysfunction. This has, indeed, been amply demonstrated in the isolated rabbit lung model [18, 21, 36–39]. In this system, a rabbit lung is suspended in a chamber, ventilated, and perfused with blood-free buffer. Through the alternate use of two separate perfusion systems, it is possible to perform numerous perfusion phases in the same lung, each with fresh perfusion fluid with or without addition of stimuli. Physical parameters (perfusion pressure, ventilation pressure, and the weight of the isolated lung) are registered continuously. Biologically active mediators such as prostanoids that are released into the perfusion fluid can be directly measured.



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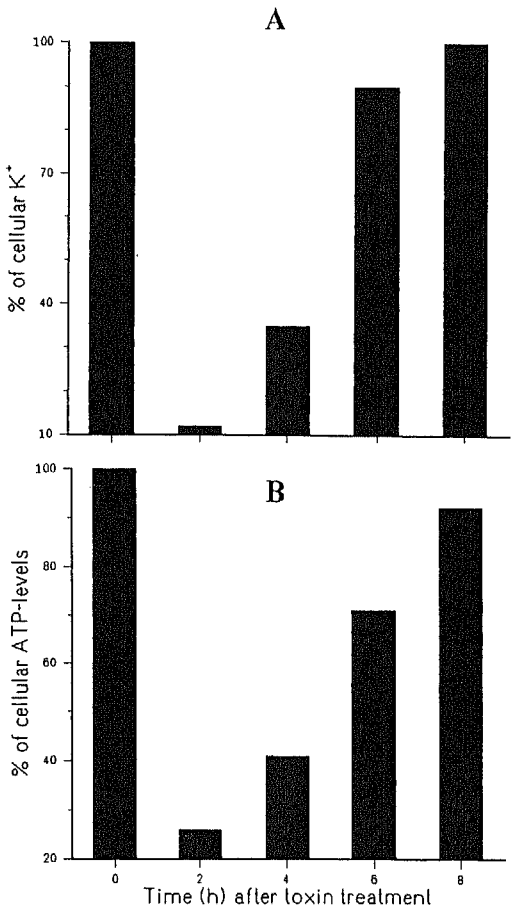

Fig. 11 A, B. Recovery of fibroblasts from attack by staphylococcal α-toxin at pH 7.4. **A** Measurements of cellular K^+. Fibroblasts were treated with 5 µg/ml α-toxin for 60 min, 37 °C in HBSS with 10 mM Hepes, pH 7.4. The medium was then removed and Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, pH 7.4, was added. At the given times, the medium was removed, the cells were washed twice with 150 mM NaCl, and the cells were solubilized with Triton X-100. The K^+ concentrations in the lysates were determined by flame photometry. The K^+ concentrations measured in control cells before α-toxin treatment were taken as 100%. **B** Measurements of cellular ATP. The same protocol was followed as given above. Cells were lysed with Triton X-100 and the ATP concentrations in the lysates were determined using the luciferase chemiluminescence assay. The ATP content of untreated cells was taken as 100%. Data derived from one out of four similar experiments

Addition of α-toxin to the perfusion fluid resulted in an acute vascular pressor response, due to the action of thromboxane B2 that was generated in attacked pulmonary target cells [36]. This process was dependent on extracellular calcium. The dose of α-toxin required to provoke this reaction was only in the order of 25 – 50 ng/ml perfusion fluid. In addition to pulmonary dysfunction evoked by uncontrolled production of thromboxane A2, lungs perfused with α-toxin also displayed direct endothelial cell damage that resulted in massive vascular leakage. Electron microscopy examination of the perfused lungs revealed an increased electron density of microvascular endothelial cell nuclei, followed by detachment of the cells from the basal lamina (Fig. 12). The resulting edema was localized in the blood/gas exchange area. These results raised the possibility that α-toxin might directly contribute to the pathogenesis of acute respiratory failure through its potent cytocidal action on endothelial cells.

When rabbit lungs were similarly perfused with *E. coli* hemolysin, dose-dependent release of thromboxane A2 into the perfusate was again noted and this correlated with pulmonary hypertension [37]. In addition, *E. coli* hemolysin elicited a protracted, dose-dependent increase in the lung capillary filtration coefficient which was independent of the prostanoid-mediated pressor response, and which

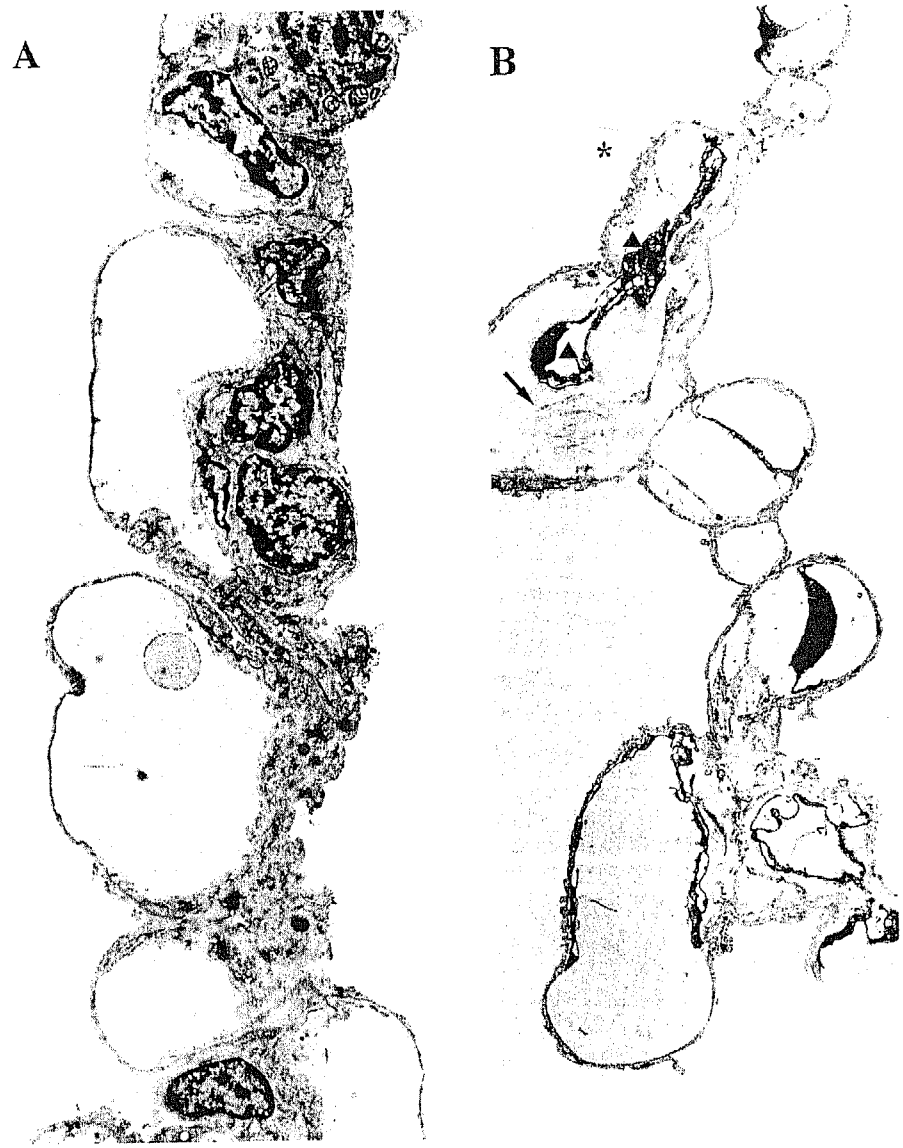

Fig. 12. **A** Electron micrograph of control lung. The lung was perfused according to the standard protocol, without application of staphylococcal α-toxin. The morphological appearance is identical to that of nonperfused rabbit lungs, apart from the far-reaching reduction of intravascular cells. **B** Electron micrograph of lung exposed to 25 ng/ml α-toxin. The interstitial space is markedly enlarged, bundles of collagenous fibers are separated by edema fluid (*arrow*). Nearly all endothelial cells show dark staining of their nuclei and cytoplasma (▲), many of them contain large vesicles, and many of them are detached from their basal lamina. Detachment appears to start in the endothelial nuclear region. Modest undulation of the plasma membranes of epithelial cells type I, with short protrusions bulging into the alveolar space. is visible (*asterisk*). **A, B** ×4200. From [38]

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resulted in severe pulmonary edema. The concentrations of toxins required to evoke these acute alterations were remarkably low, in the order of only 5–20 ng/ml. In contrast to the situation with α-toxin, the increase in lung vascular permeability did not appear to be due to direct cytocidal action of the toxin on the lung endothelium, but appeared to result from unregulated liberation of lipid mediators derived from the lipoxygenase pathway [21]. Overall, these results have demonstrated that both α-toxin and *E. coli* hemolysin emerge as potentially significant contributors to the pathogenesis of acute respiratory failure arising during severe infections.

Similar effects on the lung vasculature were observed when viable, toxin-producing *E. coli* were added to the perfusate [22, 39]. The high sensitivity of the pulmonary vasculature towards hemolysin was observed even in the presence of plasma containing putative hemolysin-inactivating components, such as antibodies and lipoproteins. It is conceivable that direct transfer of the exotoxin from bacteria that are adherent to target cells occurs. In such a hidden microenvironment, plasma inactivators may be virtually ineffective.

Ventilation perfusion mismatch results from toxin-induced vasoconstriction

The vasoconstriction evoked by pore-forming toxins in the isolated lung causes inhomogeneous narrowing of the pulmonary vascular bed. As a consequence, the profile of perfusion distribution is affected, resulting in a severe ventilation perfusion mismatch. Such a mismatch results in severe gas-exchange disturbances even in the absence of lung edema formation and atelectasis (Fig. 13).

Enhancement of biological effects of pore-forming toxins: role of granulocytes and endotoxin

An important and novel aspect relates to the possible synergism between detrimental stimuli, such as endotoxin or reaction products of stimulated cells (e.g., granulocytes), and the action of pore-forming toxins. In the isolated lung, pre-application of human granulocytes which sequested in the microvasculature, caused a marked increase in leukotriene generation when toxin-producing *E. coli* were subsequently introduced into the perfusate [22]. Experiments in which granulocytes and endothelial cells were co-incubated in vitro yielded evidence for a transfer of arachidonic acid from the endothelial cell to the neutrophil, and for transfer of LTA4 from the granulocyte to the endothelial cell when both were subsequently activated [20]. This concept of cooperative leukotriene generation is probably operative when isolated lungs preloaded with neutrophils are subsequently stimulated with very low doses of *E. coli* hemolysin. Interaction of toxin-primed neutrophils with alveolar macrophages and alveolar epithelial cells type 2 also probably causes massive LTB4 generation. This chemotactic agent in turn will be expected to mediate further neutrophil influx into the alveolar compartment, thereby perpetuating inflammatory processes due to its autocrine and paracrine activation of granulocytes and alveolar cells.

Recently, it was also found that priming of pulmonary cells in isolated lungs with endotoxin potentiated vascular abnormalities in response to α-toxin [50] and *E. coli* hemolysin (unpublished). Rabbit lungs were first perfused with endotoxin

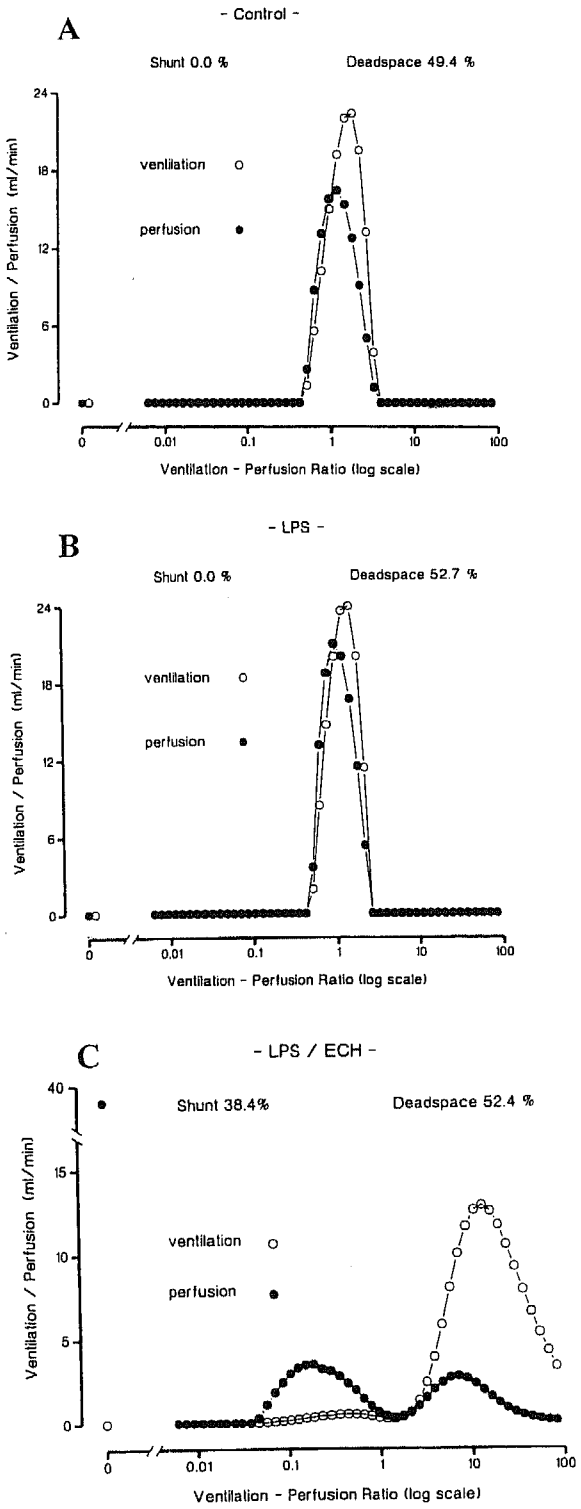

Fig. 13 A, B. Examples of ventilation-perfusion (V_A/Q) distribution in isolated perfused lungs under different experimental conditions. **A** control lung after 180 min of perfusion. **B** Lung perfused for 180 min in presence of 100 ng/ml lipopolysaccharides (LPS). **C** LPS-primed lung stimulated with 0.08 HU/ml of ECH for 10 min. Note the dramatic ventilation-perfusion mismatch ensuing through combined application of LPS and *E. coli* hemolysin

(0.1 – 200 ng/ml) for 60 – 180 min. This treatment caused release of tumor necrosis factor into the medium, but induced no significant alterations of pulmonary hemodynamics or fluid balance. Subsequent application of *E. coli* hemolysin (1 ng/ml) caused massive vasoconstrictive response and lung edema formation. Such low toxin levels were devoid of massive detrimental effects in lungs that had not been primed with endotoxin. The data demonstrated that LPS priming synergizes with *E. coli* hemolysin challenge, and the resulting vascular abnormalities could be relevant to the pathogenesis of organ failure in severe local and systemic infections. Similar synergism may be found to occur between other priming agents (e.g., cell wall components of gram-positive bacteria) and exotoxins.

Lethal effects of α-toxin and *E. coli* hemolysin in experimental animals

The lethal effects of α-toxin in experimental animals has been known for many decades. Only recently, however, are concrete concepts emerging on the causes of lethality. Intravascular application of α-toxin in rabbits and monkeys (100 μg/kg) induced reduction in pO_2 and cystolic blood pressure, and massive reduction in blood platelet counts within 5 min of administration. ECG alterations were noted including prolongation of the QRS complex, depression of the ST segment, extrasystoles, and arrythmias. Repetition of the toxin challenge accentuated these pathological findings, and a third dosis generally led to development of acute hemorrhagic pulmonary edema in monkeys [11]. The fall in blood platelet counts was precipitous and selective compared with other blood cells. This was in accord with the discovery that α-toxin preferentially attacks platelets in human blood [10]. The rapid lethal effect of intravascularly applied α-toxin was ascribed to circulatory collapse due possibly to simultaneous derangement of cardiac function and development of acute pulmonary edema.

Experiments with *E. coli* hemolysin generated surprises. In rabbits, this toxin applied intravascularly at approximately 10 μg/kg was also rapidly lethal [47]. The animals developed fulminant hemorrhagic pulmonary edema and presented with pulmonary failure and circulatory collapse. There was a selective, precipitous drop in blood granulocyte counts, again in accord with the in vitro finding that granulocytes and monocytes represent preferred targets of attack for this toxin. In monkeys, high toxin doses (50 – 100 μg/kg) also provoked a rapid drop in blood granulocyte counts. However, following a short transient drop in blood pressure, these animals recovered and showed no further severe clinical signs of cardiovascular or pulmonary malfunction. Histological examinations revealed accumulation of granulocytes in liver, lung and spleen. Very high leukocyte elastase levels were measured in one animal. These results demonstrated a remarkable tolerance of monkeys towards the selective leukocidal effects of *E. coli* hemolysin. Neither pulmonary sequestration of granulocytes nor massive release of elastase from these cells was in itself sufficient to provoke pulmonary dysfunction in these animals [47]. Thus, the lethal effect in rabbits must be due to additional effects of the toxin, possibly on other cells in the pulmonary vasculature. It is possible that in primates, priming with endotoxin is a prerequisite for *E. coli* hemolysin to induce massive organ damage. This hypothesis can be experimentally tested in the future.

Factors influencing the biological outcome of toxin production in vivo

The in vivo relevance of any cytotoxin can only be understood after it is known which cells are preferentially attacked under physiological conditions, and which secondary reactions occur in the permeabilized cells. Operationally, it may be useful to define two categories of factors, cellular and humoral, that will influence the overall susceptibility of cell towards any given toxin.

Cellular factors

Specific high-affinity binding sites. When present, such sites will direct attack of a toxin to the respective cell target.

Nonspecific, cell-surface characteristics. Nonspecific factors such as the surface organization of charged molecules can probably affect the efficiency and speed at which diffusion of a toxin to a membrane bilayer occurs. For reasons discussed below, slow binding may imply that a toxin will not be able to efficiently attack a cell under physiological conditions.

Intrinsic resistance. Intrinsic resistance towards a toxin is exemplified by human granulocytes and their resistance to *S. aureus* α-toxin (unpublished data). The term implies that a toxin can bind to a cell without inflicting functional membrane damage. The mechanisms underlying such intrinsic resistance have not been delineated.

Repair mechanisms. It is conceivable that nucleated cells can repair a limited number of damaged membrane sites. In isolated cell studies (e.g., attack of leukocytes by *E. coli* hemolysin), repair mechanisms could not be detected even following attack by low toxin doses. However, reversibility of toxin effects was noted in isolated lungs perfused with low doses of this toxin, which might be compatible with repair mechanisms. As mentioned above, recent studies have clearly demonstrated repair of α-toxin lesions in human fibroblasts.

Humoral factors

Pore-forming bacterial toxins are generally potent immunogens, and respective antibodies are, therefore, normally present in healthy individuals. Moreover, many pore formers are bound and inactivated by plasma lipoproteins. When liberated into the host environment, the attack of a given toxin will, thus, be counteracted by several inhibitory humoral factors. The net outcome will be determined by the affinity, binding kinetics, and the concentration of the reaction partners. The speed of binding of a toxin to a target cell may vary considerably. For example, *E. coli* hemolysin binds extremely rapidly to leukocytes, but relatively slowly to erythrocytes (unpublished data). Whenever present, humoral components will, hence, be able to prevent *E. coli* hemolysin from attacking erythrocytes, but their action may often be too slow to similarly protect leukocytes. As a consequence, addition of low amounts of *E. coli* hemolysin to whole human blood results in its selective attack on leukocytes and monocytes [13]. Another example for selective attack of a pore-forming cytolysin is the action of α-toxin on human blood platelets. Upon

addition of this toxin to whole human blood, the cytotoxin binds rapidly to specific sites on blood platelets and leukocytes, less efficiently to lymphocytes, and not at all to erythrocytes. Leukocytes can intrinsically withstand toxin attack, but platelets and monocytes are highly susceptible. As a consequence, selective activation/injury of the latter ensues. In addition to such toxin attack on circulating cells, sessile inflammatory cells (such as macrophages), endothelial cells and parenchymal cells may be highly susceptible to the membrane-perturbating activities of various bacterial agents. An impressive example is the severe injury of capillary endothelial cells in intact rabbit lungs, induced by α-toxin, which occurs concomitantly with activation and release of lipid mediators and nitric oxide from the attached cells.

Pore-forming exotoxins: candidates for immune intervention?

The fundamentally important concept of immune protection against bacterial diseases originated from work on intracellularly acting toxins (tetanus toxins and diphteria toxin). One hundred years later, few microbiologists or clinicians contemplate extending this concept to the realm of membrane-damaging toxins. There are several reasons for this. First, pore-forming toxins never represent the sole pathogenicity factor elicited or expressed by an infectious agent. Second, the pore-forming toxins identified to date are widespread, excellent immunogens, so that all healthy adults already have neutralizing serum antibodies. Is there any rationale for trying to increase or boost these natural antibody titers? Third, the potential biological significance of membrane-damaging toxins has remained unknown or unrecognized by all but a few specialists in the field. Fourth, no data on the beneficial effects of antibodies directed against pore-forming toxins are available. Fifth, the number of potentially important pore-forming toxins is large, and this places obvious constraints on the feasibility of projects aimed at producing specific antibodies for prophylaxis or therapy.

In our opinion, the obstacles impeding development of immune intervention strategies should not be overrated. That a toxic agent does not represent the sole pathogenicity factor of an invading microorganism should not distract from the fact that its neutralization may be very beneficial. Although healthy adults already have neutralizing antibodies, titers are generally low. In fact, such low-titered antibodies are demonstrably incapable of thwarting attack of either α-toxin or *E. coli* hemolysin on the respective, highly susceptible cell targets. Obviously, high-titered antibodies may be successful where low-titered antibodies fail. That general awareness of the existence and potential significance of pore-forming toxins has been grossly lacking should not become an excuse for indulgence.

What, then, are the future prospects for immune intervention, and can they be justified? With regard to the heterogeneity of the toxins in question, this obstacle may not be so great. Pore-forming toxins usually fall within a narrow range of related protein families exhibiting marked immunological cross-reactivity. This applies to the family of sulfhydryl-activated toxins [1, 3, 40], and to the RTX-toxins produced by *E. coli* and other gram-negative bacteria [53]. The development of vaccines probably will not present serious problems. For example, many toxins have been cloned and it is easy to generate inactive, site-directed mutants. In the case of α-toxin, replacement of single amino acid residues results in immunogenic, perfectly non-toxic proteins [27] that elicit high antibody titers in experimental an-

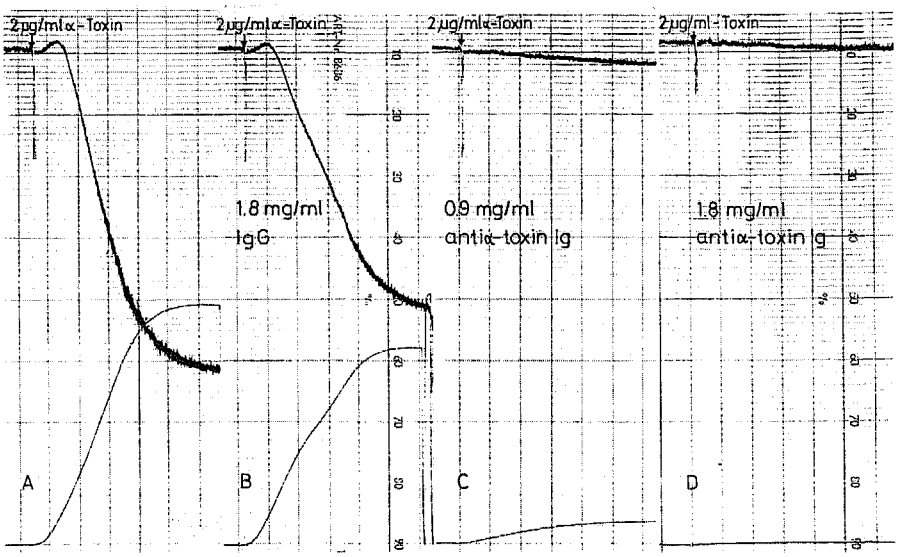

Fig. 14 A–D. Recordings of optical density changes due to platelet aggregation (*upper traces*) and of ATP release (*lower traces*) in platelet-rich plasma induced by 2 µg staphylococcal α-toxin/ml. **A** Control, **B** plasma supplemented with 1.8 mg commercial IVIG/ml; **C, D** plasma supplemented with hyperimmune IVIG. Note effective protection against platelet-activating effects of α-toxin afforded by the high-titered antitoxin antibodies. Chart speed, *one column* corresponds to 1 min. From [11]

imals. High-titered human antibodies against α-toxin have also been produced with the use of a conventional toxoid. These antibodies are capable of neutralizing all detrimental effects of α-toxin in vitro and in vivo in experimental animals [11] (Fig. 14).

A major obstacle to the development and the application of pre-formed antibodies is the fact that an infectious agent and toxin producer must first be identified as the cause of disease before the antibodies can be applied. One area that merits attention is the possibility of vaccinating patients who are to undergo high-risk operations, e.g., with the combination of potentially relevant vaccines. Such a prophylactic regimen would probably be cheap, safe, and may also turn out to be effective. Unfortunately, this possibility continues to be neglected by most infectious disease specialists.

Conclusion and perspectives

In contrast to endotoxins, membrane-damaging bacterial exotoxins have hitherto received little attention as possible contributors to the pathogenesis of septic shock. Despite the fact that they are produced by the majority of important bacterial pathogens, the very existence of these toxins has generally escaped the attention of clinicians. Attack of pore formers on nucleated cells and platelets will evoke complex secondary reactions including exocytosis, stimulation of eicosanoid production, release of reactive oxygen species, and liberation of cytokines. These toxins can, therefore, influence hemostasis and trigger production and release of many

potent mediators whose possible roles in the pathogenesis of septic shock are currently in the forefront of discussion. Indeed, application of purified α-toxin and *E. coli* hemolysin in experimental animals was found to evoke acute symptoms of respiratory and circulatory failure and shock [11, 47]. Furthermore, administration of toxin-producing *S. aureus* or *E. coli* in isolated rabbit lungs produces severe lung vascular injury [22, 39].

Because of their unusual properties, pore-forming toxins are unfortunately very difficult to quantify in biological fluids and tissues. An appreciable fraction will become bound to blood or tissue cells, and another fraction will become bound to antibodies and lipoproteins. Quantitation of the proteins is only feasible if methods can be devised to release them from their bound state. At present, no satisfactory solution to the problem is available. In this regard, the state of the art corresponds to the situation in clinical endotoxin research approximately 10 years ago, when laboratories were struggling with the problem of endotoxin measurements. To further complicate matters, pore-forming toxins need not necessarily be released in measurable quantities into body fluids or tissues to exert their action. Very few molecules are generally required to generate a membrane lesion, and attack on a susceptible cell may occur within a very small, circumscribed area when viable bacteria gain intimate contact with a target. This will probably suffice to evoke the various cytotoxic effects discussed above. For example, seeding toxin-producing *E. coli* onto monocytes at a cellular ratio of only 1 : 1 led to release of IL-1β followed by cell death, in the absence of detactable toxin in the cell supernatants [14]. Similarly, perfusion of isolated, blood-free rabbit lungs with hemolytic *E. coli* also caused massive derangements in the pulmonary vasculature in the absence of detectable circulating toxin [22]. These considerations highlight the obstacles that currently impede acquisition of conclusive evidence that pore-forming toxins do contribute towards the development of sepsis syndromes. The objective of this review has been to confront readers with provocative and suggestive data that might stimulate further research in this neglected area.

Acknowledgements. Our studies have received the continued support of the Deutsche Forschungsgemeinschaft and the Verband der Chemischen Industrie.

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Med Microbiol Immunol (199

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Cell immortalizat
Listeria monocyto

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Received: 20 April 1994

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Introduction

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