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Anthrax toxins

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Abstract *Bacillus anthracis*, the etiological agent of anthrax, secretes three polypeptides that assemble into toxic complexes on the cell surfaces of the host it infects. One of these polypeptides, protective antigen (PA), binds to the integrin-like domains of ubiquitously expressed membrane proteins of mammalian cells. PA is then cleaved by membrane endoproteases of the furin family. Cleaved PA molecules assemble into heptamers, which can then associate with the two other secreted polypeptides: edema factor (EF) and/or lethal factor (LF). The heptamers of PA are relocalized to lipid rafts where they are quickly endocytosed and routed to an acidic compartment. The low pH triggers a conformational change in the heptamers, resulting in the formation of cation-specific channels and the translocation of EF/LF. EF is a calcium- and calmodulin-dependent adenylate cyclase that dramatically raises the intracellular concentration of cyclic adenosine monophosphate (cAMP). LF is a zinc-dependent endoprotease that cleaves the amino terminus of mitogen-activated protein kinase kinases (Meks). Cleaved Meks cannot bind to their substrates and have reduced kinase activity, resulting in alterations of the signaling pathways they govern. The structures of PA, PA heptamer, EF, and LF have been solved and much is now known about the molecular details of the intoxication mechanism. The in vivo action of the toxins, on the other hand, is still poorly understood and hotly debated. A better understanding of the toxins will help in the design of much-needed anti-toxin drugs and the development of new toxin-based medical applications.

Abbreviations *CMG2:* Capillary morphogenesis protein 2 · *DTA:* Diphtheria toxin A chain · *EF:* Edema factor · *EFn:* N-terminal fragment of EF · *ETx:* Edema toxin · *GR:* Glucocorticoid receptors · *GSK3β:* Glycogen synthase kinase 3β · *I domain:* Integrin-like domain · *iNOS:* Inducible nitric oxide synthase · *LF:* Lethal factor · *LFn:* N-terminal fragment of LF · *LTx:* Lethal toxin · *MAPK:* Mitogen-activated protein kinase · *Mek:* MAPK kinases · *PA:* Protective antigen · PA_{20}: 20-kDa N-terminal fragment of PA · PA_{63}: 63-kDa C-terminal fragment of PA · *TEM8:* Tumor endothelial marker 8

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Introduction

Anthrax

Anthrax toxins are virulence factors of *Bacillus anthracis*, a gram-positive rod that causes anthrax. Anthrax is an epizootic disease affecting wild and domesticated herbivores (Mock and Fouet 2001; Turnbull 2002). The disease affects primarily livestock but can occasionally be transmitted to humans who come in contact with infected animals or animal products. It is often reported that anthrax was first described by Virgil as a plague affecting humans and cattle in a district of the eastern Alps (Sternbach 2003). It is also speculated that anthrax was the cause of two of the Egyptians plagues described in the Old Testament, the death of cattle and the appearance of boils. Anthrax played an important role in the history of bacteriology. *B. anthracis* was studied by Koch who used it in 1876 to prove his famous postulates. By growing the bacteria in vitro and challenging animals with the culture he was able to induce disease, disproving the theory of spontaneous generation and introducing the notion of germs. It was Koch too who observed the life cycle of *B. anthracis* for the first time and showed that the bacteria could form highly resistant spores. Anthrax was also studied by Pasteur who, by heat treatments, isolated an attenuated strain that in 1881 proved to be an effective vaccine.

Anthrax occurs when spores of *B. anthracis* gain access to host tissues. In animals this usually occurs by ingestion, whereas in humans, spores usually enter the host via a break in the skin barrier. The latter causes a cutaneous form of the disease with characteristic skin lesions (Friedlander 1999). First, a painless papule develops into a vesicle accompanied by an edema of varying intensity. After a few days the vesicle ruptures, yielding a black eschar. The coal-like appearance of the lesion gave its name to the disease, as anthrax comes from the Greek word for coal. On histology the lesion shows necrosis, lymphocyte infiltration, and edema. With antibiotic treatment the lesion usually heals spontaneously in a few weeks and leaves a limited scar. The main complication of this form of the disease is the systemic spread of the bacteria. The resulting septicemia is often fatal.

Septicemia is immediate when spores gain access to their hosts by inhalation. This causes the inhalation or pulmonary form of the disease, which is highly lethal. After a few days of initial mild symptoms including fever, cough, and malaise, inhalation anthrax takes an abrupt turn: increased respiratory distress, “shock-like” symptoms, coma, and death. A chest X-ray shows a characteristic widening of the mediastinum and pleural effusions. Inhalation anthrax is also often accompanied by acute meningitis.

Pathogenesis and role of the toxins

Regardless of the route of entry, spores are phagocytosed by macrophages where they germinate (Guidi-Rontani 2002). The bacteria, by an unknown mechanism, escape destruction and lyse the macrophages to gain access to host tissue. In its host, *B. anthracis* appears as a large encapsulated, non-motile rod often found in long chains. During infection the bacteria replicates to high titers of up to 10^9 cfu/ml of blood. This exceptional infectivity is due to two main virulence factors coded by genes present on two large plasmids, plasmids pXO1 and pXO2. Plasmid pXO2 carries genes directing the synthesis of a poly-D-glutamic acid capsule that was shown to inhibit phagocytosis of the planktonic bacteria (Makino et al. 1989). A bacteria cured of its pXO2 plasmid was isolated in the 1930s by

Sterne and shown to be avirulent and a good live vaccine (Sterne 1937). Plasmid pXO1 carries genes coding for toxins. The existence of a toxin was reported in the mid 1950s after it was observed that laboratory animals died when they were injected intravenously with clarified plasma from anthrax-infected guinea pigs dying from anthrax (Smith 2002; Smith and Keppie 1954). Subsequent intense research proved that the toxin consists of three polypeptides: protective antigen (PA), edema factor (EF) and lethal factor (LF). It was shown that these three proteins assemble into two distinct toxins with different physiological effects. A mixture of PA and EF forms edema toxin (ETx), which causes edema when injected subcutaneously in laboratory animals. A mixture of PA and LF forms lethal toxin (LTx), which causes death when injected intravenously in laboratory animals.

Several lines of evidence demonstrate that the toxins play a central role in the pathogenesis of anthrax: (1) highly purified preparations of toxins cause death and edema; (2) bacteria cured of their pXO1 plasmid or carrying a plasmid where individual toxin genes have been inactivated are dramatically less virulent (Pezard et al. 1991); (3) an immune response against toxin components and especially PA completely protects against intoxication as well as against anthrax infection; (4) during the course of infection a point is reached where antibiotics can clear bacteremia but are unable to prevent death, most likely due to the remaining circulating toxin that is unaffected by the drugs (Keppie et al. 1955). After the achievements of the 1950s and 1960s, work on anthrax toxin became dormant and was only revived in 1982 when Leppla showed that EF had adenylate cyclase activity (Leppla 1982). In the subsequent decades much work improved our knowledge of the structure, delivery, and cellular activity of the toxins (Ascenzi et al. 2002; Collier and Young 2003; Lacy and Collier 2002). Despite all this work, some molecular details of the intoxication remain unclear. Even more puzzling, the in vivo mechanism of action of the toxins is very poorly understood and subject to debate.

Anthrax as a bioweapon

In animals, once the host is dead and its carcass decays, bacteria come into contact with oxygen and sporulate. Anthrax spores are highly resistant to physical stresses and survive many years in the soil where they can infect their next host. The hardiness of the spores, the ease with which their dispersion can be achieved, and the mortality of the disease all suggest that *B. anthracis* could be used as a biowarfare agent. In 1941 the British government tested the release of anthrax spores on an island near Scotland (Christopher et al. 1997; Manchee et al. 1983). The island remained a hazard until 1986 when tons of seawater and formaldehyde were used to sterilize its soil. Militarization of anthrax was also pursued in the former Soviet Union. In 1979 an epidemic occurred near a Soviet microbiological military facility in Sverdlovsk (Abramova et al. 1993; Meselson et al. 1994). Ninety-six people were reported ill and 64 died. The authorities claimed that an outbreak in cattle was transmitted to humans by consumption of contaminated meat. However, it was later shown that an accidental release of an aerosol from the military facility was the culprit. Lastly, in October 2001 a new outbreak of inhalation anthrax occurred in the United States of America after the intentional release of spores through contaminated letters delivered to politicians and journalists. This attack resulted in five deaths from 11 confirmed cases of inhalation anthrax and 12 confirmed or suspected cases of cutaneous anthrax (Jernigan et al. 2001). Most of the deaths struck postal workers who did not open the tainted letters. This showed that the spores delivered during the attack had been weaponized and thus offered a chilling glimpse of the extent of the possibilities achieved in bioweapon engineering.

One of the challenges posed by an attack involving anthrax spores is the fact that victims who develop inhalation anthrax have symptoms that are difficult to distinguish from influenza-like illnesses. Once symptoms develop, it is often too late to undertake an effective treatment. Antibiotics can eradicate the bacteria, but the huge load of toxins can still ultimately cause death of the victim. In order to fight the toxin the only applicable course of action today is aggressive supportive care. The outbreak in 2001 showed that this approach reduced the mortality rate from nearly 100% to slightly below 50% (Jernigan et al. 2001). However, this approach would prove impossible in the case of a massive release of spores versus the limited occurrence of 2001.

Intoxication mechanism

Anthrax toxin is composed of three polypeptides: PA, EF, and LF. These three proteins are synthesized during the vegetative growth of the bacteria. In vitro expression of the genes coding for the toxin components is induced by addition of bicarbonate and a temperature of 37°C. A specific transcriptional activator, *atxA*, and a repressor, *pagR*, of the toxin genes have been isolated but a full picture of the regulation network has proved to be exquisitely more complex (Koehler 2002; Mock and Mignot 2003). Few specific details are known on the secretion mechanism of PA, EF, and LF. The polypeptides have standard signal sequences and are therefore thought to follow the general secretion pathway and be released in the extracellular milieu independently of one another.

Anthrax toxin belongs to the family of bacterial "AB" toxins. Schematically, these toxins work by the conjugated action of a cell binding B moiety, which allows the binding and translocation of an enzymatically active A moiety in the cytosol of a target cell. More specifically, anthrax toxins belong to the subgroup of binary toxins where the A and B moieties are carried by independent polypeptides. This group comprises adenosine diphosphate (ADP) ribosylating toxins like *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* Sb toxin, *C. difficile* ADP ribosyltransferase, and the vegetative insecticidal proteins from *Bacillus cereus*. However, anthrax toxin is (thus far) unique because it has one central B moiety, PA, which can associate with two enzymatic moieties EF and LF. The mechanism by which the toxins assemble and intoxicate cells is described below (Fig. 1).

Binding of PA to target cells

Mature PA is a 735-residue long, 83-kDa protein. The first step in the intoxication is the binding of PA to its cellular receptor. It was initially observed that a single class of proteinaceous receptors for anthrax toxin was located on the surface of many cell types. Using cross-linking experiments, an 80-kDa cellular protein was found to be associated with PA (Escuyer and Collier 1991). A decade later, a genetic approach was undertaken to find the gene coding for the receptor for the toxin (Bradley et al. 2001). First, Chinese hamster ovary (CHO)-K1 cells were chemically mutagenized and selected for resistance against a mixture of PA and a fusion protein consisting of the N-terminal domain of LF (LFn) and the catalytic domain of diphtheria toxin, DTA. This fusion protein is delivered to the cytosol by PA where DTA blocks protein synthesis. Resistant CHO cell lines were identified and found to lack a receptor for PA. Then, a complementing cDNA was identified by

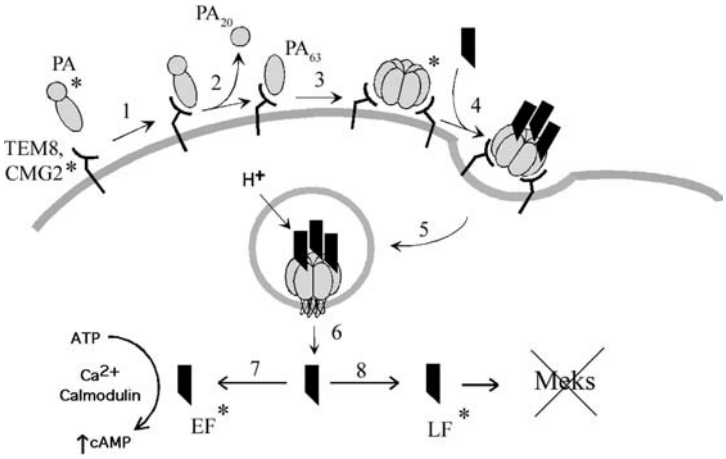

Fig. 1 Model of the intoxication mechanism of anthrax toxins. (*1*) The intoxication starts with the binding of PA to the cellular receptors tumor endothelium marker-8 (TEM8) and/or capillary morphogenesis protein 2 (CMG2). (*2*) Bound PA is cleaved by membrane endoproteases of the furin family, releasing a 20-kDa N-terminal fragment, PA_{20}. (*3*) The remaining 63-kDa C-terminal fragment, PA_{63}, oligomerizes into heptamers. (*4*) The heptamers can then associate with EF and/or LF. Alternatively, PA can be cleaved in the serum of infected hosts, possibly oligomerizing and even forming complexes with EF/LF before binding to cellular receptors (not shown). (*5*) The assembled toxic complexes are endocytosed and routed to an acidic compartment. (*6*) There, the low pH triggers a conformational change resulting in the formation of a cation-specific channel and translocation of EF/LF. (*7*) EF is a calcium- and calmodulin-dependent adenylate cyclase. (*8*) LF is a zinc-dependent endoprotease that cleaves the N-termini of Meks. The *asterisks* indicate the molecules for which a crystal structure has been solved

screening a library. The identified cDNA coded for a membrane protein named tumor endothelial marker-8 (TEM8), previously identified as an up-regulated protein in endothelial cells associated with colorectal cancer. TEM8 appears to be expressed in a broad range of tissues and apparently has three splice variants. The splice variant of TEM8 identified has a short cytoplasmic tail. The extracellular domain of TEM8 contains a von Willebrand factor type A domain, also called integrin-like domain (I-domain). These domains usually mediate protein–protein interactions involved in binding to cell adhesion molecules and extracellular matrix proteins. Consistent with this view, the extracellular domain of TEM8 was found to interact with collagen VI, a form of collagen that like TEM8 is preferentially expressed in tumor endothelium (Nanda et al. 2004). The I-domain of TEM8 is the PA-binding site (Bradley et al. 2001), and it was found that a metal ion-dependent adhesion site motif within the domain was critical for toxin binding. In integrins, this motif coordinates divalent cations like Mg^{2+} or Mn^{2+} that are important for ligand binding. The same type of binding and the implication of a carboxylate group in PA was reported (Bradley et al. 2003).

A second receptor for PA was found encoded by the capillary morphogenesis protein 2 (CMG2) (Scobie et al. 2003). Like TEM8, CMG2 is widely expressed and possesses an I-domain which is highly homologous to that of TEM8. CMG2 binds collagen IV and laminin and thus is also implicated in binding to extracellular matrix proteins. In CMG2 the binding of PA was found to be, like that of TEM8, dependent on the I-domain and its ion binding motif, although the cation specificity was slightly different between CMG2 and TEM8. The crystal structure of the I-domain of CMG2 has recently been solved (Lacy et al. 2004). The structure shows a typical I-domain fold, with a close homology to the

"open" or high affinity conformation of the αM integrin I-domain. Coordination of the metal ion was structurally similar in the two molecules.

Toxin assembly and endocytosis

Once bound to a receptor, PA can be cleaved by furin or a furin-like membrane endoprotease (Gordon et al. 1995). Furin belongs to a class of calcium-dependent serine endoproteases, the prohormone–proprotein convertases. Although furin is prominently found intracellularly, PA cleavage, however, occurs at the cell surface. On polarized cells, receptor and furin are both localized on the basolateral surface (Beauregard et al. 1999). The structure of furin with a substrate-derived inhibitor has recently been solved (Henrich et al. 2003). This structure helps to elucidate the stringent specificity of this protease towards the sequence R-X-K/R-R. In PA, this sequence is found in the N-terminus, and cleavage results in the release of a N-terminal 20-kDa fragment (PA_{20}). This fragment is not thought to play a further role in the intoxication. The remaining 63-kDa fragment (PA_{63}) oligomerizes quickly into a heptamer (Milne et al. 1994), a step that PA_{20} sterically prevented. Cleavage of PA at the normal furin cleavage site can also be achieved in solution using moderate amounts of trypsin (Novak et al. 1992). After cleavage in solution, however, PA_{63} will not oligomerize spontaneously as is the case on cells. Oligomerization in solution requires the addition of ligand or separation of PA_{63} from PA_{20} by running cleaved PA on an anion exchange chromatography column (Miller et al. 1999). Similarly, cleavage of PA has been reported to occur in vivo in the serum of animals (Brossier et al. 2000; Ezzell and Abshire 1992). In that case it is unclear, however, if PA_{63} is oligomerizing in the serum, and if so, whether this occurs with or without the presence of EF, LF, or both.

Once assembled into a heptamer, PA_{63} can associate with EF and LF. This yields the fully assembled toxic complex. It was found that the PA-binding domains of LF or EF were in the ~250 residue-long N-terminal domains termed LFn or EFn, respectively (Arora and Leppla 1993). It was demonstrated that the stoichiometry of the complex at saturating concentrations of enzymatic moieties (either EF or LF) is three ligand molecules per heptamer (Mogridge et al. 2002a). Moreover, PA_{63} mutants unable to oligomerize could not bind LFn, whereas a ternary complex of a PA_{63} dimer associated with one molecule of LFn could be purified (Mogridge et al. 2002b). These data suggest that there are seven binding sites for EF/LF binding on the heptamer located across the boundary between monomers. The unexpected stoichiometry is explained by the fact that binding to one site of the heptamer sterically blocks the adjacent ones, therefore only allowing a maximum of three molecules bound per heptamer (see below and Cunningham et al. 2002). It is still unclear, however, if both EF and LF molecules can bind to one heptamer of PA_{63}.

Regardless of whether it associates with EF and/or LF, it was shown that proteolytic cleavage of PA triggers and is absolutely necessary for the internalization of PA (Beauregard et al. 2000). This led to the hypothesis that the oligomerization process triggered a cell event leading to endocytosis. Consistent with this hypothesis it was observed that only PA_{63} oligomers could be endocytosed (Liu and Leppla 2003).

Prior to cleavage, PA bound to its receptor is found in detergent-soluble parts of the plasma membrane, but after cleavage it is rapidly relocalized to detergent-insoluble parts, also termed lipid rafts (Abrami et al. 2003). It was found that the clustering of anthrax toxin receptors, either via PA_{63} oligomerization or using an antibody, causes its relocalization into lipid rafts. As monomers, receptor molecules are taken up slowly, but upon clustering

the endocytosis is rapid, caveolae-independent, and clathrin-dependent. These molecules are therefore ideal receptors for PA. By associating with a receptor that is slowly endocytosed as a monomer, PA can stay at the cell surface until it is able to oligomerize. Once oligomerization is achieved, then it can rapidly proceed with the intoxication. Indeed, even a PA mutant that cannot be cleaved by furin can be internalized when clustering is triggered using an antibody. The mechanism for relocalization and triggering of endocytosis are unclear. The cytoplasmic tail of TEM8 was found not to be involved in this process, as even an anchored version of the extracellular domain of TEM8 was functional in an intoxication assay (Liu and Leppla 2003).

After endocytosis it was demonstrated by several approaches that the toxic complex is delivered to an acidic compartment. Treatment of cells with lysosomotropic amines can block LTx intoxication (Friedlander 1986). Similarly, treatment with chloroquine, which prevents endosome acidification, prevented ETx intoxication (Gordon et al. 1988). Lastly, treatment with bafilomycin, which inhibits the vacuolar ATPase proton pump and therefore acidification, blocks LTx action (Menard et al. 1996).

Translocation of EF/LF

It was observed that PA_{63} heptamers could form pores in planar lipid bilayers when shifted to a low pH (Blaustein et al. 1989).These channels were found to be cation selective. Indeed, a K^+ efflux could be measured from liposomes incubated with PA_{63} heptamers at low pH (Koehler and Collier 1991). Later, the ability to form channels was also observed on cells by measuring an efflux of $^{86}Rb^+$ when cells are incubated in the presence of PA_{63} heptamers at low pH (Milne and Collier 1993). In the latter experiments, the optimal pH for release was found to be approximately pH 5 and negligible above pH 6. However, in planar lipid bilayers, channel formation could be measured around pH 7. The reason for this discrepancy is unclear. Collectively these experiments suggest that once PA_{63} heptamers reach the endosome they form a channel.

Channel formation in the endosome is associated with translocation of EF/LF, but the mechanism is unclear. It was shown that LFn could direct the translocation of heterologous polypeptides fused to its N or C termini when cells were incubated with a mixture of PA and the chimera (Arora and Leppla 1993; Milne et al. 1995). Thus, LFn seems to encompass all the information necessary for translocation, and the remaining part of the polypeptide is passively transported along. Moreover, since both N-terminal and C-terminal fusions are equally translocated, there does not seem to be directionality in the translocation process.

By incubating cells on ice with trypsin-activated PA and radiolabelled LFn, complexes of LFn bound to PA_{63} heptamers can be formed but, because of the low temperature, remain on the cell surface. When those complexes are then exposed to low pH, it was shown that the radiolabelled ligand becomes inaccessible to a protease added extracellularly, and thus has been translocated into the cytosol (Wesche et al. 1998). Translocation is therefore observed in conditions triggering pore formation, whether in endosomes or on the cell surface. All known PA mutants specifically impaired in translocation are also deficient in pore formation (Mourez et al. 2003; Sellman et al. 2001b). These data led to the hypothesis that translocation occurs through the channel of the pores formed by PA_{63} heptamers. Strikingly, it was observed that pore formation on cells was blocked by addition of LF or LFn (Zhao et al. 1995). The size of the channel formed by PA_{63} heptamers was assessed

in planar lipid bilayers and shown to be around 12 Å (Blaustein and Finkelstein 1990). This implies that the ligands cannot be transported in a folded state. The translocation of fusions between LFn and dihydrofolate reductase (DHFR) or between LFn and DTA was studied in the cell surface assay described above. It was shown that translocation was blocked in the presence of the ligands of the heterologous parts of the DHFR and DTA chimeras, namely methotrexate and adenine, respectively (Wesche et al. 1998). This suggests that, indeed, stabilization of folding prevents translocation and that low pH must trigger some partial or complete unfolding of the ligands bound to the heptamers. Translocation of unfolded enzymatic domains via a cell-binding domain forming a pore has also been observed in other AB toxins like diphtheria toxin (Oh et al. 1999) and *C. botulinum* neurotoxin (Koriazova and Montal 2003). However, the nature of the "channel" in those cases is thought to be quite different from that of the heptamer of PA_{63}.

Since enzymatic moieties are translocated unfolded, refolding must occur in the cytosol where chaperones might be involved. In a recent report the role of such a cytosolic chaperone, Hsp90, in the toxicity of C2 toxin was tested (Haug et al. 2003). It was found that Hsp90 is necessary for translocation and activity of C2. It was also necessary for the activity of iota toxin but not for anthrax lethal toxin. The reason for the discrepancy is unclear but could be due to the use of a different cell type in the LTx assay or to true differences. It is interesting to note that diphtheria toxin, another AB toxin with ADP ribosyltransferase activity, was also shown to require Hsp90 (Ratts et al. 2003). Therefore, one possibility might be that Hsp90 is specifically involved in the translocation/refolding of ADP ribosyltransferase toxins.

There are, however, numerous questions challenging such a model for the translocation step. Since the lumen of the heptamer can only accommodate an unfolded polypeptide, or at the most a helix, one has to assume that only one polypeptide can be translocated at a time. The fact that up to three ligand molecules can be bound per heptamer raises the question as to how these three molecules can be sequentially translocated. It is easier to envision the translocation of multiple molecules in a model where the ligands are translocated on the sides of the heptamer instead of through its lumen. Consistent with this hypothesis, it was observed that LF and EF at low pH are able to destabilize and/or insert into lipid bilayers by themselves (Kochi et al. 1994a; Wang et al. 1996; Wang et al. 1997). This suggests that they might participate in their own translocation. Another experiment yielded an intriguing result about the translocation ability of PA: a polycationic tail fused to DTA could promote its translocation in the cytosol of cultured cells in a PA-dependent manner (Blanke et al. 1996). The efficiency of translocation was less than that of the LFn–DTA fusions, and the translocation was not inhibited by the presence of LFn. One model to explain this peculiar result would be that the polycationic tail allows DTA to interact with the anionic phospholipids of the membrane and get translocated at the interface with PA_{63} heptamers when pH becomes acidic. Alternatively, the cationic tail could target DTA to the lumen of the heptamers.

Enzymatic activity

After translocation, the enzymatic moieties have access to the cytoplasm, where they can exert their activity. Because the edema response induced by ETx was reminiscent of that induced by cholera toxin, it was hypothesized and then shown that EF, like cholera toxin, can increase the intracellular cyclic adenosine monophosphate (cAMP) concentration

(Leppla 1982). In fact, it was shown that EF is itself an adenylate cyclase strictly dependent on the presence of calcium and calmodulin. *Bordetella pertussis* also produces a highly homologous adenylate cyclase toxin. EF has a specific activity 1,000-fold higher than that of mammalian calmodulin-activated adenylate cyclases, but because bacteria lack calmodulin, this high activity is restricted to the cytosol of eukaryotic cells.

The enzymatic activity of LF remained a mystery for a long time. The first clue came when a zinc-binding motif, HEXXH, present in all zinc-dependent endoproteases was recognized in LF (Klimpel et al. 1994; Kochi et al. 1994b). The only substrates of LF identified to date are mitogen-activated protein kinase (MAPK) kinases (Meks) (Duesbery et al. 1998; Vitale et al. 1998). LF cleaves all known Meks (Mek 1 to Mek 7) except Mek 5 (Duesbery et al. 1998; Pellizzari et al. 1999; Vitale et al. 2000; Vitale et al. 1998). The cleavage site is located in the N terminus of Meks in a proline-rich region. As a consequence of the cleavage, Meks are unable to dock with their substrate MAPK. Furthermore, it was recently shown that this cleavage also reduces the intrinsic kinase activity of Meks (Chopra et al. 2003). The N-terminal region of Meks is not the only region recognized by LF, since point mutations in a conserved C-terminal region could prevent proteolysis without affecting kinase activity (Chopra et al. 2003).

Structural studies

The structure of PA was solved both as a monomer and as a trypsin-activated, purified soluble heptamer of PA_{63} (Petosa et al. 1997). The soluble heptamer structure is thought to be identical to that of the heptamer assembled on the cell surface prior to endocytosis. Hence it was termed "prepore" to differentiate it from the structure of the membrane-inserted "pore" at low pH. There is little structural difference between the two forms of PA_{63} in the monomer and the prepore. This confirms the idea that the only role of PA_{20} is to sterically prevent oligomerization of PA_{63}. PA has four domains (Fig. 2). Mutational studies have helped define the function of each of the domains which are described below. Recently, a global cysteine scanning study of PA_{63} has helped to give a global view of the structure–function relationships in PA, summarizing most of these studies (Fig. 3, Mourez et al. 2003).

Domain 4 (residues 595–735) starts with a hairpin and helix connecting this otherwise independent domain to the rest of the molecule. The rest of the fold of domain 4 is that of a β-sandwich with an immunoglobulin fold. Mutations in domain 4 affect the binding of PA to target cells (Brossier et al. 1999; Rosovitz et al. 2003; Varughese et al. 1999) and monoclonal antibodies binding to this domain affect PA binding (Little et al. 1996). This proves that domain 4 is the receptor-binding domain. To date the receptor and receptor-binding domain have not been implicated in any intoxication mechanism other than binding on cells (Bradley et al. 2001) and, possibly, triggering endocytosis (Abrami et al. 2003). It would be interesting to assess if the receptor and domain 4 are involved additionally in pore formation and translocation.

Domain 3 (residues 488–595) has a ferredoxin-like fold. In a random mutagenesis approach undertaken to elucidate the function of this domain, inactive mutants were found to be unable to form heptamers (Mogridge et al. 2001). The primary role of domain 3 therefore seems to be the oligomerization of PA_{63}. In this hypothesis, residues of domain 3 (centered on aspartate 512) on one face of PA_{63} would make one oligomerization interface. The complementary interface would be formed by a patch of residues from do-

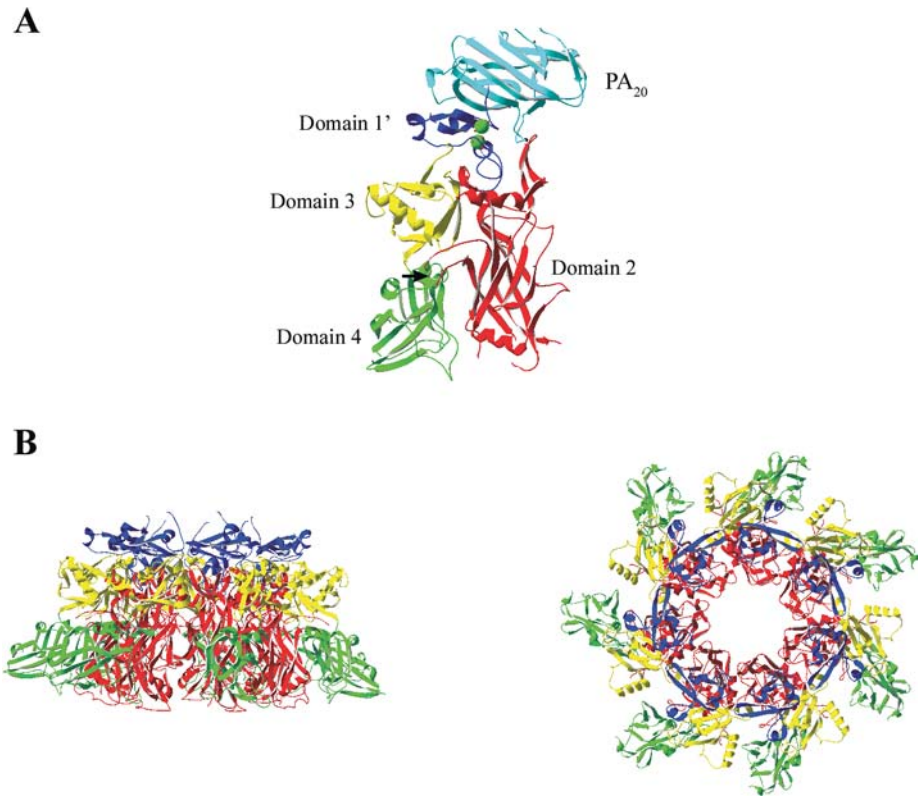

Fig. 2A, B Crystal structure of PA. Ribbon representation of the crystal structure of (**A**) PA and (**B**) the PA_{63}-soluble heptamer believed to represent the structure of the prepore (the membrane-bound heptamer before its encounter with low pH) (Petosa et al. 1997). The four domains are represented with different colors: domain 1 consists of PA_{20} in *cyan* and domain 1′ in *blue*; domain 2 in *red*; domain 3 in *yellow* and domain 4 in *green*. **A** The two calcium ions bound in domain 1′ are represented as *green spheres* and the *arrow* indicates the flexible loop of PA_{63} that inserts into the membrane at low pH. **B** Side view (*left*) of the prepore (the lipid bilayer would be at the bottom of this view) and top view (*right*) of the prepore (the lipid bilayer would be at the back of this view). The structure images in this figure and Figs. 3 and 4 were generated using Swiss-PDBviewer 3.7 (http://www.expasy.org/spdbv/)

mains 1 and 2 (centered on lysine 199 and arginines 468 and 470) on the other face of PA_{63}. Indeed, mutations of the latter residues also prevent oligomerization (Mogridge et al. 2002b). Furthermore, by mixing PA mutants where one or the other oligomerization faces have been mutated, stable dimers can assemble through the only intact interface and be purified (Mogridge et al. 2002b).

Domain 2 (residues 249 to 488) has a β-barrel core structure and makes up most of the lumen in the prepore structure. Domain 2 was shown to possess a chymotrypsin-sensitive region critical in the translocation process (Novak et al. 1992). The deletion of two phenylalanines in this region was also found to prevent translocation (Singh et al. 1994). This region, corresponding to the loop joining the $2\beta2$ and $2\beta3$ strands, is disordered and unresolved in the PA structures (Petosa et al. 1997), suggesting that it is highly flexible. By analogy with the structure of the pore-forming toxin α-hemolysin of *Staphylococcus aureus*, which is also a heptamer that forms channels in membranes (Song et al. 1996), it

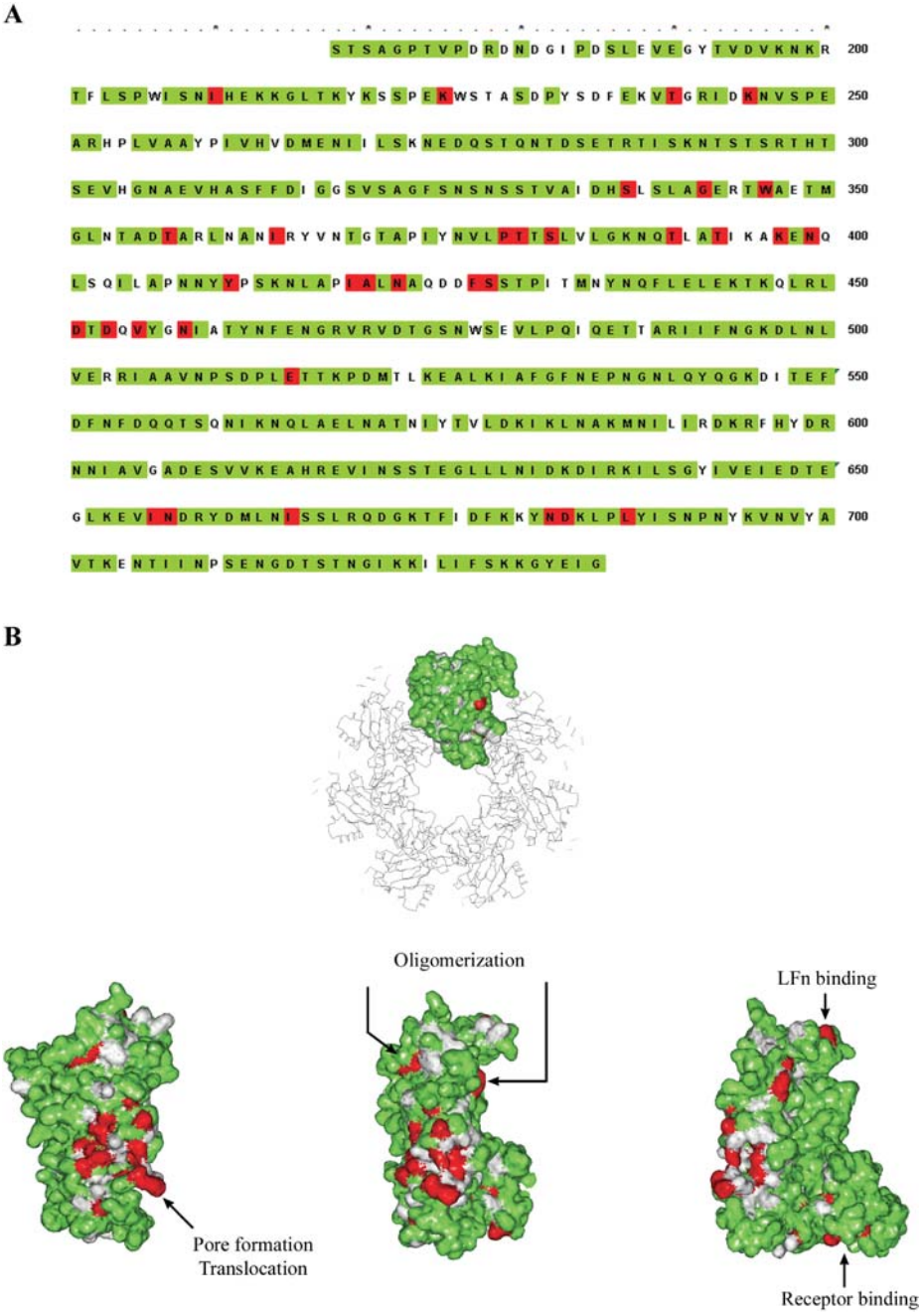

Fig. 3A, B Global structure–function study of PA_{63}. Summary of the results of the cysteine scanning experiment performed on PA_{63} (Mourez et al. 2003). **A** Sequence of PA_{63}. **B** Molecular surface of a PA_{63} monomer isolated from the heptamer, top view (*top*) of the heptamer with the highlighted PA_{63} monomer; and three views of the monomer: a left-side view (*bottom left*), a center view (*bottom middle*) and a right side view (*bottom right*), as viewed from inside the lumen of the heptamer. Both in **A** and **B**, the residues for which changes to a cysteine resulted in an unstable, active, or inactive mutant are colored *white*, *green*, and *red*, respectively. The functions affected by mutations of the red residues are indicated in **B**

was hypothesized that this flexible loop might insert into membranes at low pH (Petosa et al. 1997). The loops of all the monomers would then form a β-barrel channel in the membrane. The structure of the pore form of the heptamer remains elusive, but various approaches were developed to test this hypothesis. By replacing each residue of the flexible loop with cysteine the accessibility of the mutants at low pH can be tested with a thiol-reactive membrane impermeant reagent (Benson et al. 1998). The reagent used adds a charge, restricting the conductance of the channel that can be measured in planar lipid bilayers. This showed that the loop inserts into the membrane and structures itself as two β-strands of alternating hydrophilic and hydrophobic residues. This is the typical structure of the strands of a β-barrel.

The insertion of the flexible loops would require a major rearrangement in the rest of domain 2 and presumably the unfolding of a Greek-key motif consisting of four β-strands (Petosa et al. 1997). To test this hypothesis the cysteine mutagenesis and accessibility study was extended beyond the flexible loop (Nassi et al. 2002). This showed that the barrel structure extends beyond the flexible loop and confirms the existence of a major rearrangement in the rest of domain 2 upon insertion. The structural rearrangement taking place upon pore formation is likely responsible for the sodium dodecyl sulfate (SDS) resistance of the pore, whereas the prepore is SDS sensitive (Miller et al. 1999; Milne et al. 1994).

The identification of the residues that provoke this conformational change upon a drop in pH, the "pH trigger," remains elusive. It was postulated that histidine residues, which have a protonation state likely to change between pH 7 and pH 5, might be involved (Petosa et al. 1997). In domain 2, histidines are located at the top of the flexible loop or in a loop with a pH-sensitive mobility. No confirmation that these residues are indeed part of the pH trigger has been obtained.

In the cysteine-scanning study (Mourez et al. 2003), most of the mutations that abolished the activity of PA were found in domain 2 and clustered in the region forming the lumen of the prepore. This region seems therefore peculiarly important. Most of these mutants were unable to make pores and translocate LFn (M. Mourez and R.J. Collier, unpublished data; Mourez et al. 2003). Mutants in some charged residues in the lumen of the prepore mutations were shown to have similar effects (Sellman et al. 2001b). Some of these mutations were found to have a dominant-negative effect, with likely as little as one mutant molecule per heptamer being enough to block the activity of the whole heteroheptamer (Sellman et al. 2001a). In the cysteine scanning study, dominant-negative mutants were found only in domain 2 and clustered in the 2β6 strand, 2β7 strand, and the 2β10–2β11 loop (Mourez et al. 2003). All these residues are located in the lumen and are solvent accessible in the prepore structure. This suggests that the lumen, in addition to the flexible loop, is critically important in pore formation/translocation. The dominance of these mutations suggests that the pore formation process involves a structural rearrangement where some residues of the lumen of the prepore find themselves making contacts in the pore structure or in a transition state. This again suggests that the conformational change taking place upon lowering the pH is extensive.

The N-terminal domain, domain 1 (residues 1–249), contains the furin cleavage site at position 167. The domain has therefore two subdomains, PA_{20} (residues 1–167) and domain 1′ (residues 167–249). Domain 1′ has two calcium ions bound through a modified EF-hand motif. These calcium ions are tightly bound and most likely play a structural role (Gao-Sheridan et al. 2003; Gupta et al. 2003). Consistent with these results, most of the unstable cysteine mutants mapped to the calcium binding sites (Mourez et al. 2003).

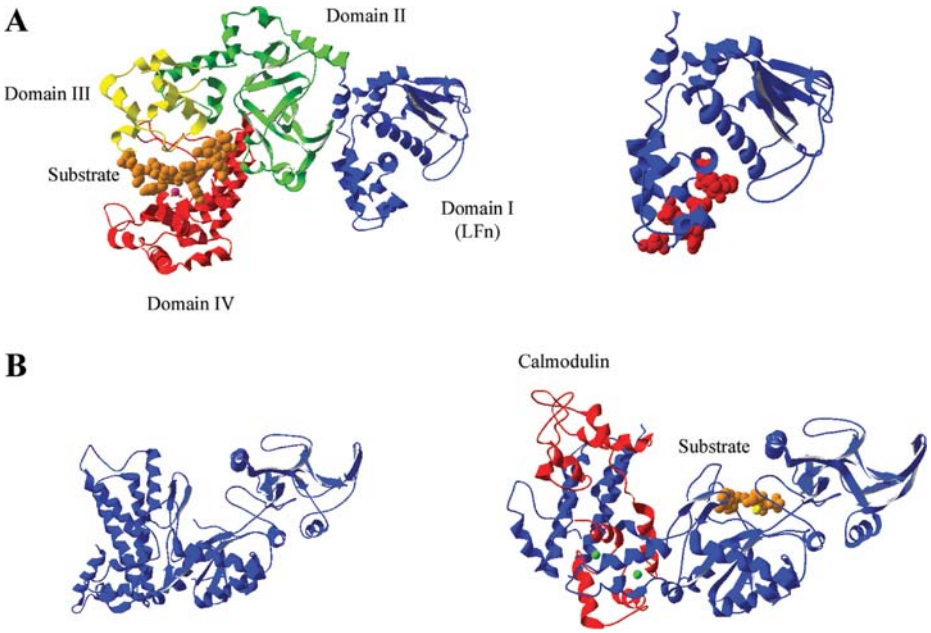

Fig. 4A, B Crystal structure of LF and the catalytic domain of EF. **A** Ribbon representation of the crystal structure of LF bound to an optimized peptide substrate (Turk et al. 2004): whole molecule (*left*) and close up view of the structure of LFn in the same orientation (*right*). The four domains are represented by different colors: domain I in *blue*, domain II in *green*, domain III in *yellow*, and domain IV in *red*. The zinc atom is represented as a *pink sphere* and the substrate as *orange spheres*. The residues that, when changed to an alanine, cause the resulting mutants to be unable to bind to PA (Lacy et al. 2002) are represented by *red spheres*. **B** Ribbon representation of the crystal structure of the catalytic domain of EF alone (*left*, Drum et al. 2002) or bound to calmodulin and a high-affinity inhibitor (*right*, Shen et al. 2004). EF is represented in *blue* and calmodulin in *red*. The calcium atoms bound to calmodulin are represented as *green spheres*, the divalent cation bound to EF as a *yellow sphere* and the inhibitor, adefovir diphosphate, as *orange spheres*

In addition to this structural role, domain 1′ is also important in PA_{63} oligomerization (Mogridge et al. 2002b) and is the site of interaction of PA with its ligand (Chauhan and Bhatnagar 2002; Cunningham et al. 2002). The release of PA_{20} uncovers a surface that was buried before. Critical residues in the binding of LFn were found by mutating this surface (Cunningham et al. 2002). By combining oligomerization mutants in order to make PA_{63} dimers together with the mutations in domain 1′ it was possible to define the residues involved in the unique ligand binding site of a PA_{63} dimer (Cunningham et al. 2002). This showed that the EF/LF binding sites are located across the monomer–monomer interface and are close to one another. Binding on one site will therefore likely sterically prevent binding on the adjacent ones, and gives a rationale for the odd stoichiometry of three ligand molecules bound per heptamer (Mogridge et al. 2002b).

The structure of LF was solved in the presence of a Mek 2-derived peptide substrate (Pannifer et al. 2001) as well as with an optimized substrate or inhibitors (Panchal et al. 2004; Turk et al. 2004). LF has four domains (Fig. 4). The N-terminal domain, domain I or LFn, corresponds to residues 1–263. The first 27 residues were not resolved in the structure, but truncation of up to residue 36 has no impact on binding or translocation (Lacy et al. 2002); therefore, this region is not believed to have major functional importance. LFn is composed of a bundle of 12 α-helices and 6 β-strands making two sheets on

one face of the molecule. Conserved residues between EFn and LFn could be mapped on the surface of LFn (Lacy et al. 2002). The conserved residues clustered to a patch of LFn that by mutagenesis studies was shown to be the PA binding site (Lacy et al. 2002). Domain II (residues 263–550, excluding residues 300–386) is the only part of LF having structural similarities with other proteins. It resembles the fold of the ADP ribosyltransferase of the vegetative insecticidal toxin of *B. cereus*. Domain III (residues 3000–386) is an α-helical bundle made up of four imperfect sequence repeats from a feature of domain II. It is inserted in domain II, and there is evidence that it has functional significance (Arora and Leppla 1993), possibly helping in substrate recognition. The C-terminal domain, domain IV (residues 551–777), has the zinc protease site. The active site is similar to that of the thermolysin proteases family with the HEXXH motif located in an α-helix neighboring a four-stranded β-sheet. The substrate binding site is a long cleft formed by domain II and parts of domains III and IV (Tonello et al. 2003; Turk et al. 2004). In the original structure the peptide substrate was not bound in a productive conformation, but a later structure shows a peptide substrate bound in a correct orientation (Tonello et al. 2003; Turk et al. 2004). Peculiarly, the folds of domains IV and I (LFn) are highly similar, except that catalytic residues (and especially the HEFGH motif) have been mutated in the LFn structure (to a YEIFGK sequence). This modularity based on duplication and mutations suggests an interesting evolution scenario (Lacy and Collier 2002).

The crystal structure of the catalytic C-terminal portion of EF (residues 291–800, which excludes EFn) was solved alone, in the presence of calmodulin, and in the presence of calmodulin and a noncyclizable analog of ATP (Fig. 4 and Drum et al. 2002). Since EFn and LFn have sequence homologies and are functionally identical, it is believed that they are structurally related, and therefore a complete picture of EF could be modeled. Contrary to expectations, there are no significant homologies with mammalian adenylate cyclases. The structures show the conformational changes arising upon binding of calmodulin. This is the first insight into how calmodulin modulates a biologically active substrate. An α-helical domain undergoes a 15-Å translation and 30° rotation leading to the activation of the catalytic site. The catalytic site has a single metal ion coordinating the substrate and positioning it for the catalytic action of histidine 351. This contrasts with the mechanism of mammalian adenylate cyclases, which do not possess catalytic histidines and use a second metal ion instead. Calmodulin itself is bound in an extended conformation that is also different from previous structures obtained with peptides derived from mammalian adenylate cyclases. These differences might explain the enhanced activity of EF compared to mammalian counterparts.

The structure of an assembled toxic complex has yet to be obtained. However, the structure of LF, the structure of the prepore, and the identification of the binding sites on those structures opens the possibility to compute molecular models of an assembled toxic complex. These models can then be tested by measuring molecular distances, for instance using fluorescence resonance energy transfer. One such study was recently published (Croney et al. 2003) and it is expected that more of these studies will yield a complete and possibly dynamic picture of the interactions between PA_{63} heptamers and their bound substrates.

In vivo effects of anthrax toxins

Targeting of macrophages by LTx and death by inflammatory shock

After many unsuccessful attempts at finding a cell line with a measurable phenotype when treated with LTx, it was noted that some murine macrophage-like cell lines were lysing after 2 h in contact with LTx (Friedlander 1986). It was also noted that the sensitivity of these macrophages was correlated to that of the mice strains from where the cell line originated (Hanna et al. 1993). This led to the hypothesis that macrophages play a central role in anthrax pathogenesis. Reports that sublytic doses of exposure to LTx triggered release of pro-inflammatory cytokines by sensitive macrophages offered a possible explanation for their role during infection (Hanna et al. 1993): low doses of LTx would induce in the macrophages a cytokine build up which would ultimately be released upon lysis and induce a shock. This is consistent with the observation of "shock-like" death observed in animals challenged intravenously by LTx (Smith et al. 1955). Consequently, the "macrophage hypothesis" has been cited in many reviews and focused the attention of research groups on macrophages and on cytokine expression modulation by LTx. However, conflicting reports soon emerged.

What is the relevance of macrophage lysis by LTx?

When the cellular targets of LF, Meks, became known, it also became apparent that there was no difference in their cleavage between "sensitive" and "resistant" macrophages (Salles et al. 2003; Watters et al. 2001), raising the possibility that macrophage lysis is due to unspecific mechanisms rather than the direct action of the toxin. Moreover, the susceptibilities of animals to LTx and that of their macrophages is not perfectly correlated as (1) strains of inbred mice that harbor "resistant" and "sensitive" macrophages are both sensitive to LTx with only relatively minor differences; and (2) some resistant macrophages come from species, such as rats or humans, that are sensitive to LTx and anthrax (Cui et al. 2004; Kim et al. 2003; Popov et al. 2002a).

Three linked loci, Ltxs1–3, have been implicated in the difference of resistance of macrophages from resistant (C57BL/6, DBA/2J) versus sensitive (C3H/HeJ, BALB/c) strains of inbred mice (McAllister et al. 2003; Watters et al. 2001). The only gene positively identified from these loci is *kif1C*, encoded in Ltxs1 (Watters et al. 2001). Kif1c is a ubiquitously expressed kinesin-like motor protein likely involved in the intracellular transport of some molecular cargo. A human homolog of Kif1C has been shown to be involved in the retrograde transport from Golgi to the endoplasmic reticulum, but the nature of the cargo is unknown. Consistent with a role of molecular shuttling in LTx sensitivity, brefeldin A treatment increased sensitivity of "resistant" macrophages to LTx and correlated with Kif1c relocalization (Watters et al. 2001). However, no direct link between Kif1c and LF activity can be made at this time, and therefore the role of Kif1c in the lysis phenomenon strengthens the idea that lysis is only a peripheral event during LTx action.

Recent reports have shown that destruction of macrophages might not be restricted to the sensitive cell lines. LTx was shown to induce the apoptosis of macrophages when these cells are activated by lipopolysaccharide (LPS) (Park et al. 2002). Another study also found the hallmarks of apoptosis in LTx-treated macrophages (Popov et al. 2002b). Such an event might occur in vivo as caspase inhibitors were reported to have protective

effects during infection of mice with *B. anthracis* (Popov et al. 2004). Another group reported that macrophage lysis by LTx could be triggered in "resistant" macrophages by addition of peptidoglycan or poly-D-glutamic acid capsule (Kim et al. 2003). The authors propose that the gain of sensitivity is due to tumor necrosis factor (TNF)-α release by these components and an autocrine effect. However, in this study lysis was found to be consistent with necrosis but not with apoptosis. Regardless of this discrepancy with the previous reports, collectively these studies revive the hypothesis that macrophage destruction is a relevant phenomenon in anthrax.

In order to understand the extent of the impact of LTx treatment on macrophages and how lysis/apoptosis/necrosis could be triggered, one group undertook a transcriptional profiling experiment using DNA array (Tucker et al. 2003). This approach showed the differential regulation upon exposure to toxin of a number of genes controlled by glycogen synthase kinase 3 β (GSK3β). This signaling pathway is not well studied in macrophages but is better understood in the context of embryonic development, especially that of the zebrafish. Thus, among other experiments designed to confirm LTx action on the GSK3β pathway, the authors challenged zebrafish embryos with LTx. Developmental abnormalities appeared which correlated well with what would be expected from impairment of GSK3β activity. More work is needed in order to ascertain the identities and roles of the genes regulated by GSK3β that could be implicated in LTx action on macrophages or other host cell types. It is interesting to note that among the genes identified in this study were genes coding for kinesins.

The picture of LTx action on macrophages is even more complex than previously thought. A recent report showed that sensitive macrophages can be "desensitized" to lytic challenge with LTx by prior incubation with sublytic amounts of toxin (Salles et al. 2003). This "toxin-induced resistance" might represent an example of the adaptability of macrophages during infections. On the molecular level the authors showed that despite proteolysis of Mek 1 and Mek 2, the sublytic challenge induced a recovery of downstream targets of these kinases by an unknown compensatory mechanism. The authors speculate that this compensation might account for the observed resistance. Indeed, the authors showed that the kinetic of Mek cleavage was much faster in sensitive cell lines compared to resistant ones. Thus, resistance to LTx might be due to a slower kinetic of Mek cleavage, giving more time to allow a compensatory mechanism to establish itself. Interestingly, this is consistent with a careful study of the kinetics of LF cleavage performed with a model substrate (Tonello et al. 2003). This study suggests that because of differences in the structure of their cleavage sites, Meks are likely to be cleaved non-uniformly, implying that LF will have a considerably variable activity in different cell types and that even in one cell type the activity is likely to differ, depending on environmental signals. These phenomena might contribute to the discrepancies between the studies performed with LTx and macrophages.

Does LTx induce or suppress inflammatory responses?

Contrary to the previous report described above (Hanna et al. 1993), in recent years many groups reported that LTx did not induce pro-inflammatory cytokines either in cells or in animals (Erwin et al. 2001; Moayeri et al. 2003; Moayeri and Leppla 2004; Pellizzari et al. 1999; Popov et al. 2002a). In fact, the induction of such cytokines and the release of nitric oxide in response to classical stimuli like LPS or interferon (IFN)-γ were inhibited

(Pellizzari et al. 1999). Furthermore, if the hypothesis of the release of pro-inflammatory cytokines were to be true, inhibiting or preventing the inflammatory response during LTx challenge should decrease the effects of the toxin. However, an irreversible inhibitor of the inducible nitric oxide synthase (iNOS), which decreases the inflammation response, increased LTx sensitivity of mice (McAllister et al. 2003); and strains of "knock-out" mice for the receptor of IL-1 or iNOS were shown to be more susceptible to *B. anthracis* (Kalns et al. 2002). Inhibition of pro-inflammatory cytokine release in human peripheral blood mononuclear cells stimulated by *B. anthracis* cell wall was also reported (Popov et al. 2002a). Recently, it was shown that LTx had an inhibitory effect on IFN-regulatory factor 3 activation by LPS (Dang et al. 2004). Since this regulatory factor is essential in type I interferon expression during LPS activation, this could explain the inhibitory effects of LTx on cytokine expression.

It was also shown that LTx could inhibit glucocorticoid receptor (GR) function in transfected cells and in BALB/c mice (Webster et al. 2003). Glucocorticoids play an important role in the regulation of the inflammatory response. The perturbation of the inflammatory response by the inhibition of GR could prevent the host from clearing an infection with *B. anthracis*. Little is known, however, about how LTx can affect the GR, other than the fact that a catalytically active LF is required. Moreover, this study raises the possibility that GR are not the only nuclear receptors affected by LTx. Indeed, the same study showed an effect on progesterone receptor and estrogen receptor α, but none on mineralocorticoid receptor and estrogen receptor β. The relevance of these observations is unclear.

Lastly, it should be noted that it is difficult to assess the role of the toxin on cytokine release in an infectious setting from the studies with purified LTx. Two reports by the same group show, on the one hand, LTx-mediated inhibition of pro-inflammatory cytokine release in vitro (Popov et al. 2002a), and, on the other hand, significant cytokine release in vivo in mice infected with *B. anthracis*, with some differences between LTx sensitive and resistant mice strains (Popov et al. 2004). The authors suggest that the cytokines are released by cells undergoing LTx-induced apoptosis, especially in the liver. The controversy over whether or not LTx induces cytokine release persists. It is unfortunately very important to solve this question, as cytokine-oriented therapies are being advocated to treat anthrax.

Are there other cellular targets of LTx?

Toxin production is induced very early after germination, while bacteria are still in the macrophages. Two studies have addressed the role of toxins in those early events of the infection by looking at germination of spores in macrophage cell lines. These studies yielded conflicting results. In one study, after germination the bacteria were found to escape the macrophages phagosomes, replicate in the cytosol, and get released from the macrophages, all events being independent of toxin production (Dixon et al. 2000). In another study, the newly germinated bacteria did not escape phagosomes, did not replicate, and escaped macrophages only thanks to toxin production (Guidi-Rontani et al. 2001). Few new data have helped resolve this contradiction, and it is therefore still unclear if the toxins play a role in the escape from macrophages. Another report showed that when using bestatin, an aminopeptidase inhibitor with some activity against LTx, spore-infected human mononuclear cells showed higher sporicidal, bactericidal, or both activities (Popov

et al. 2002a). This supports a role for LTx in early events of infection like macrophage escape.

It was recently shown that LTx has a profound impact on the function of dendritic cells by disrupting their interactions with T cells (Agrawal et al. 2003). Such interactions are a prerequisite for activation of the T cells and subsequent induction of an inflammatory response, production of antibodies by B cells, and differentiation of B and T cells into memory cells. These are main events in the establishment of acquired immunity. The authors propose that by its effect on dendritic cells, LTx could prevent the host from containing an infection by *B. anthracis*. Although this hypothesis is tempting, it remains to be tested. It is, for instance, unclear if the host has time to mount an effective response involving dendritic cells during an infection with *B. anthracis*.

It is puzzling that only immune cells seem to be affected by LTx. Since virtually all cell types have receptors for the toxins and Meks are so central in cellular physiology, it is likely that LTx can have consequences on other cell types. As a first example of such a possibility, an in vitro experiment showed that LTx could have a cytotoxic action on endothelial cells (Kirby 2004). This is highly significant because of the numerous observations that LTx action in animals is consistent with a vasculature collapse (see below). This study showed that LTx induced caspase-dependent apoptosis of different kinds of endothelial cells. In this cell type, LTx cleaved Meks and inhibited downstream pathways that the author suggests are responsible for the cytotoxicity observed.

Synergy with ETx and role of ETx

Strikingly, little work has been done on the mechanism of action and the role of ETx in an infection compared to all the reports with LTx. This is an unfortunate oversight. By inactivating the genes coding for EF or LF in an attenuated strain of *B. anthracis* still lethal to mice, it is clear that both genes are required for full virulence in a mouse model of infection (Pezard et al. 1991). This suggests that ETx and LTx act synergistically during infection. It is interesting to note that ETx was shown to down-regulate the production of TNF-α and IL-6 by monocytes stimulated with LPS (Hoover et al. 1994). These effects are consistent with the suppression of the inflammatory response of macrophages reported in studies with LTx, and could explain why there is a synergy between the two toxins.

Little is known about the mechanism of edema formation by ETx in vivo. It is usually assumed that ETx acts similarly to cholera toxin, causing an increase in cAMP in cells and an efflux of water. However, the nature of the cells affected by ETx during an infection is undetermined. A mouse adrenocortical cell line and a human polarized epithelial cell line have been shown to undergo macroscopic changes upon treatment with ETx (Beauregard et al. 1999; Soelaiman et al. 2003).

How do toxins cause “shock-like” death in animals?

The consensus that emerged from the original work on LTx is that the toxin caused death by inducing shock-like symptoms and vascular leakage (Beall and Dalldorf 1966; Smith and Stoner 1967). Before that consensus was reached, other theories had been put forth, such as blockage of the capillaries (Dalldorf and Beall 1967) or an effect on the central nervous system (Bonventre et al. 1967; Remmele et al. 1968; Vick et al. 1968). In one ex-

periment, primates challenged intrathecally with LTx died within 10 min of the injection, from respiratory blockade (Vick et al. 1968). Unfortunately, these experiments were never reproduced later and in our hands intracerebral challenge of Fisher 344 rats with ten times the minimum intravenous lethal dose of LTx did not cause any symptoms (M. Mourez, unpublished data). This could be due to inherent differences between animal models. Death of animals by shock is therefore the currently accepted hypothesis.

Several different events, however, can cause a shock. Because of the previously reported lysis of macrophages and induction of pro-inflammatory cytokine release, it was argued that in anthrax the shock could be induced by cytokine release (Hanna et al. 1993). However, the recent animal studies cast doubts on this hypothesis. Most notably, a careful pathological study was undertaken by Leppla and co-workers to look at the effect of LTx on "resistant" and "sensitive" strains of inbred mice (Moayeri et al. 2003). This study did not find any evidence of inflammation related pathology or any systemic release of pro-inflammatory cytokines. Depletion of circulatory mononuclear cells that could be attributed to LTx-mediated lysis was observed in sensitive mice strains and not in resistant ones but was not paralleled in fixed tissue macrophages and was not correlated with an alteration of the pathological findings. Ultimately, this study determined that animals died of pleural fluid accumulation and liver failure probably due to hypoxia. Both effects could be due to vascular permeability alterations. The hypoxic response induced by LTx is highly significant, since a severe hypoxia has often been reported in cases of anthrax. It is then interesting to note that the strain of mice more resistant to LTx is also more resistant to hypoxia. No vascular collapse, coagulopathy, or endothelial cell damage could be seen. Lastly, this study showed that LTx treatment induced a high neutrophilia and induction of KC chemokine expression, a potent neutrophil recruiter. Both mediators seem to be expressed by hepatocytes and therefore the neutrophils and hepatocytes might represent specific targets of LTx. The precise mechanism and order of the hypoxia and vascular collapse still have to be determined.

The effect of a continuous infusion of LTx in rats was also recently published by the same group and, again, no inflammation or cytokine release could be observed (Cui et al. 2004). In this model, however, no hypoxia was observed. Instead, hypotension and acidosis was noted. This is in contrast to the previous belief that the response of rats to LTx is a pulmonary change causing edema and cardiovascular collapse (Beall and Dalldorf 1966). In the uniquely sensitive Fisher 344 rat model, a pulmonary edema is evident as quickly as 60 min postchallenge with LTx (Ezzell et al. 1984).

As discussed above, LTx was recently shown to have an inhibitory effect on glucocorticoid receptors (Webster et al. 2003). The inhibition of GR might exacerbate the hypoxic shock. Interestingly, the Fisher 344 rat strain is known to have an aberrant glucocorticoid response that in some situations can cause death of the animals. Since the same strain of rats is exquisitely sensitive to LTx, it raises the possibility that GR inhibition by LTx is responsible for this sensitivity. Combining this result with the study of LTx effects in rats (Cui et al. 2004), one hypothesis could be that through inhibition of GR, LTx might affect vascular integrity or vasoconstrictor functions.

Conclusion: what are the roles of the toxins in an infection?

The effects and roles of the toxins in vivo are far from clear. With so many possibilities being discussed, it seems especially important at this stage to put the question back in the context of a *B. anthracis* infection. With that perspective it can be expected that the toxins possibly exert three kinds of effects: (1) As the spores germinate in the macrophages the bacteria start producing toxins; thus it is conceivable that the toxins have an intracellular effect that could ultimately result in the release of bacteria from macrophages. Once bacteria are released they continue to secrete the toxins, which can then have a (2) local as well as a (3) systemic effect.

With only two studies available yielding opposing results, the ability of the toxins to promote release of the bacteria from phagocytes is an interesting hypothesis, but it still awaits confirmation. The local effect of the toxins, on the other hand, seems to be reaching a consensus. It is likely that LTx and ETx act synergistically locally to promote bacterial infection by perturbing the host immune response. This is probably achieved through multiple actions including cytokine release inhibition and macrophage destruction. Edema is another local response mediated apparently solely by ETx. It is assumed that the adenylate cyclase activity on some cells can cause an electrolyte imbalance through a cAMP concentration increase, similarly to cholera toxin. Lastly, death is a systemic effect mediated apparently only by LTx. This "effect" remains mysterious, and this raises the question: Is it an aberration or a relevant pathological mechanism? One challenging observation, for instance, is that in vivo LTx sensitivity can be inversely correlated to the sensitivity to anthrax infection, i.e., mice more sensitive to LTx are more resistant to bacterial challenge (Welkos et al. 1986). This casts doubts about the real significance of death caused by LTx. Lastly, the prevailing hypothesis put forward to explain death has been the induction of shock by cytokine release. It seems likely that this hypothesis will come under more scrutiny and might even be replaced by another in which LTx causes shock through hypoxia and vascular collapse. These effects could, for example, be mediated by toxicity to endothelial cells or by an effect on glucocorticoids.

Fighting anthrax toxin

In most cases, cutaneous anthrax can be treated easily with antibiotics. In the case of inhalation anthrax, however, as detailed above, antibiotic treatments are not enough, presumably because the toxins are unchecked. The recent use of anthrax in an attack has therefore triggered an intense search for drugs that act against the toxin.

Even before the toxins were discovered, passive immunization was demonstrated to protect rabbits using sheep hyperimmune serum (Gladstone 1946). The source of antigen was in the serum of infected animals, and therefore it was stated that *B. anthracis* secreted a "protective antigen." It was later realized that this antigen is part of anthrax toxin. An attenuated strain of *B. anthracis* was discovered by Sterne and found to be a very effective vaccine in animals (Sterne 1937). This Sterne strain is not encapsulated but produces toxin and it was found that the main immune reaction was directed against the toxin components, and mainly PA. Consequently, a cell-free vaccine was prepared from supernatants of cultures of a Sterne strain adsorbed to aluminum hydroxide gel. This vaccine is the only licensed commercial vaccine used in humans (Friedlander et al. 2002). The immune response is believed to help clear the toxin from the serum of infected patients and is there-

fore, in a sense, an anti-toxin therapy. The acellular anthrax vaccine adsorbed is very effective, but the immunization schedule is cumbersome: it has to be administered at 0, 2, 4 weeks and 6, 12 and 18 months with yearly boosters.

Many new formulations (recombinant PA and/or LF, adjuvant) and delivery vectors (naked DNA, live bacteria, viruses) have been tested to try and improve the safety and immunization schedule (Friedlander et al. 2002). For instance, it was shown that additional antigens besides the toxins are needed for optimal immunization, like antigens from spores or capsules that could enhance phagocytosis (Brossier et al. 2002). Consequently, promising experimental vaccines, including capsule and PA antigens have been designed (Rhie et al. 2003; Schneerson et al. 2003).

A prophylactic approach might not be economically viable, however, or even accepted by the public. Therapeutics able to neutralize the toxin after the exposure to *B. anthracis* spores are therefore needed. There are a number of steps in the intoxication process that can be targeted and various types of inhibitory molecules that can be used to block them.

Inhibitors of receptor binding

Experiments showed that a polyclonal anti-PA antibody could save some guinea pigs from a lethal anthrax challenge (Little et al. 1997). However, neither anti-PA monoclonal antibodies nor anti-EF or anti-LF polyclonal antibodies had any effect (Little et al. 1997). Similar experiments showed that polyclonal anti-PA antibodies were effective in treatment of challenged guinea pigs (Kobiler et al. 2002) and in the treatment of challenged mice in conjunction with antibiotics (Karginov et al. 2004). Efforts are now being made to obtain antibodies that could be used in humans. For instance, recombinant single-chain antibodies have recently been obtained that showed inhibitory activities against LTx in vitro and in vivo (Maynard et al. 2002). In another approach, human antibodies fragments from phage-displayed libraries generated from immunized donors were screened against PA. Selected clones displayed inhibitory activity in vivo (Wild et al. 2003). There is some evidence that the protective epitopes are located in domain 4 of PA, and therefore it is likely that all these active antibodies prevent PA binding on cells (Flick-Smith et al. 2002; Rosovitz et al. 2003).

The discovery of receptors for anthrax toxin opened up the possibility to directly target these molecules. An inhibitor could be designed to bind on the metal ion-binding motif and compete with PA for binding. Alternatively, a soluble mimic of the receptor could function as a decoy preventing binding of PA on cells. Since the functions of TEM8 and CMG2 are not known, those approaches have potential toxicity risks. Still, soluble parts of TEM8 were shown to have potent inhibitory effects in cell cultures and in rats challenged with LTx (Bradley et al. 2001; Scobie et al. 2003).

Inhibitors of furin activation

Small molecules could be designed to block the furin-like proteases and prevent PA activation. Furin is a protease involved in many biological processes, and therefore inhibiting its activity might prove to have serious toxic consequences. Despite these concerns, a small peptide of hexa-D-arginine proved to be a stable inhibitor of furin and was shown to be able to inhibit anthrax toxin in vivo and in cell cultures (Sarac et al. 2004). The struc-

ture of furin (Henrich et al. 2003) will probably help in the design of specific small molecule inhibitors of this protease, which might prove effective.

Inhibitors of assembly

Some molecules could prevent the binding of EF/LF to PA_{63} heptamers. Such an inhibitor was developed based on peptides selected from phage-displayed libraries (Mourez et al. 2001). The peptides were selected in order to specifically bind to the surface involved in the interaction of PA_{63} heptamers with EF/LF. The authors assumed that each peptide could bind to one of seven sites on a heptamer. If true, then by grafting multiple copies of the peptides on a flexible polymeric backbone, a molecule could be designed that would bind PA_{63} heptamers at multiple sites simultaneously. This strategy allowed for the design of polyvalent molecules with dramatically improved efficacy compared to the peptide alone. The molecules were able to prevent the toxicity of LTx administered to rats. Peptidic inhibitors are, however, notoriously prone to degradation and the backbone used was polyacrylamide. Further improvements are therefore required before these molecules can be made into viable therapeutics.

Inhibitor of translocation

As stated above, some PA mutants unable to promote translocation were shown to be dominant negative and block in vitro the action of LTx (Mourez et al. 2003; Sellman et al. 2001a; Yan and Collier 2003). These mutants have also proved to be very effective in vivo. Compared to the inhibitors described above, the PA mutants have the advantage of being "optimized" for in vivo stability and targeting. In addition, PA is already administered to humans in a vaccine formulation. One disadvantage, however, is the fact that PA mutants will bind to the ubiquitous receptors and therefore are likely to be taken out of the systemic circulation quickly.

Inhibitors of enzymatic activities

With the solving of the structures of EF and LF and the understanding of their catalytic activities, the door has been opened for an intense search of small molecule inhibitors. There are already a huge number of commercial drugs active against proteases that could have an effect against LF. Since the discovery of the enzymatic activity of LF many groups have designed high-throughput screens and used the crystal structure of LF to look for small molecule inhibitors (Cummings et al. 2002; Panchal et al. 2004; Tonello et al. 2002). As a result, hydroxamate derivatives of substrate peptides have been designed and have in vitro inhibitory constants in the nanomolar range (Tonello et al. 2002). Non-peptidic inhibitors have also been obtained with an in vitro inhibitory constant in the micromolar range (Panchal et al. 2004; Turk et al. 2004).

Using the crystal structure of EF it became possible to screen a database of small molecule compounds that could specifically inhibit its enzymatic activity. This allowed the identification of a number of lead compounds with inhibitory constants around 20 μM (Soelaiman et al. 2003). Recently, the same group showed that adefovir dipivoxil, a drug already approved in the treatment of chronic infection with hepatitis B virus, was active

against EF (Shen et al. 2004). The inhibition constant was 27 nM, lower than the previous leads, and crystal structure of the inhibitor in complex with EF and calmodulin revealed a strong interaction of the active metabolite of the drug with EF. This tight contact explained a 10,000-fold higher affinity of EF for the drug over its natural substrate, ATP. Interestingly, these compounds are also effective against *B. pertussis* adenylate cyclase.

Unknown mechanism of action

A number of other drugs have been shown to have protective effects either in cell cultures or in animals: inhibitors of proteasome (Tang and Leppla 1999); intracellular calcium agonists (Bhatnagar et al. 1989; Shin et al. 2000); antioxidants like *N*-acetyl-L-cysteine and mepacrine (Hanna et al. 1994); phospholipase C and protein kinase C inhibitors (Bhatnagar et al. 1999); protein synthesis inhibitors (Bhatnagar and Friedlander 1994); and inhibitors of an unknown protein phosphatase by calyculin A (Kau et al. 2002) all showed some protecting effects against LTx challenge. These effects are difficult to interpret and/or transpose in a clinical setting.

Using anthrax toxin

Some investigators have decided to capitalize on the ability of anthrax toxins to be translocated in vivo in the cytoplasm of almost any cell type to design innovative therapeutics.

Epitope delivery system

As stated above, LFn was found to be required and sufficient for binding to PA_{63} and for delivery of recombinant polypeptides fused to its N or C terminus in the cytosol of target cells in the presence of PA. Although this system does not allow the delivery of all polypeptides (Wesche et al. 1998), it was shown to allow the in vivo delivery of a cytotoxic T lymphocyte epitope from an intracellular pathogen in mice (Ballard et al. 1996). This delivery stimulated a cytotoxic T lymphocyte response in the absence of any other co-stimulant, which could protect mice from a challenge with the pathogen. This showed that an engineered anthrax toxin could be used as a basis to design cellular vaccines effective in vivo. A cytotoxic immune response against human immunodeficiency virus (HIV) could also be achieved using this system (Lu et al. 2000).

Antitumor effect

Some tumors have elevated levels of active MAPK including breast carcinoma and glioblastoma. The idea that LTx could be used as an antitumor therapeutic was recognized when it was shown that LF inhibited MAPK signaling (Duesbery et al. 2001). LTx antitumor activity was tested in vitro on transformed cells and in vivo on transformed cells implanted in athymic nude mice. In both cases LTx was able to inhibit transformation and growth (Duesbery et al. 2001). The use of unmodified LTx raised safety concerns and subsequent attempts were made to specifically target tumor cells. To that end, engineered tox-

ins that can only be activated on tumor cells were designed. Recombinant PA molecules were constructed where the furin cleavage site was replaced with those of membrane proteases that are dramatically overexpressed on tumor cells (Liu et al. 2001, 2003). A mixture of the recombinant PA molecules with a fusion of LFn to the enzymatic part of *Pseudomonas exotoxin* A displayed a specific and dramatic cytotoxicity toward a range of transplanted tumors.

Further improvements can still be achieved by preventing recombinant PA binding to normal cells and redirecting it specifically towards tumor cells. In addition, instead of fusing an unspecific toxic moiety to LFn, one could envision grafting an enzymatic moiety with some specific activity against tumor cells. The high specificity against tumor cells that would be achieved by redirecting the binding, activation, and enzymatic activities of a single chimeric toxin toward those cells would make for a safe and efficient therapeutic.

Concluding remarks

The recent years have brought numerous advances in the anthrax toxin field: the structures of LF and EF have been solved; two receptors have been identified; and critical information has been gathered on the molecular details of the toxin's binding, assembly, endocytosis, pore formation, and the enzymatic activities of EF and LF. This progress has made possible the design of potent toxin inhibitors and the use of toxin-based therapies in cancer treatment and vaccines.

Much remains to be learned. The structure of the pore formed by PA_{63} heptamers upon exposure to low pH remains elusive. The same is true about the conformational changes taking place during the transition from prepore to pore. Now that they have been identified, the role of the toxin receptors in these and other events of the intoxication mechanism can also be tested. The fundamental question of how EF and LF are translocated is also unanswered. We know that EF and LF require some unfolding and that PA heptamers are making pores in the membrane. We do not know whether EF and/or LF use the channel as a conduit for their translocation or if they can cross the lipid bilayer around the channel. We also do not know if cellular proteins help in the translocation process or in the refolding of EF and LF. These questions remain a major challenge.

It is strikingly ironic that in vivo studies have been central to the identification of the toxins and yet it is the in vivo effects of the toxins that we understand the least. Edema and death caused by the toxins have been observed for decades; however, the mechanisms that have been suggested to cause these physiological effects are subject to debate. New hypotheses are required. We need to know which cells the toxin targets during an infection and what the effects of the toxin's ability to increase cAMP concentration and cleave Meks are on these cells. We need to keep in mind that effects other than death and edema could be more relevant to the infectious process. And, lastly, we must not forget that EF and LF act synergistically when assessing the roles and action of the toxins. The need for anti-toxin drugs to counter the use of *B. anthracis* spores as weapons and the potential applications of toxin-based new therapeutics has made this research even more essential.

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