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Mini Review

Bacteriophage endolysins: A novel anti-infective to control Gram-positive pathogens

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ABSTRACT

Endolysins (or lysins) are highly evolved enzymes produced by bacteriophage (phage for short) to digest the bacterial cell wall for phage progeny release. In Gram-positive bacteria, small quantities of purified recombinant lysin added externally results in immediate lysis causing log-fold death of the target bacterium. Lysins have been used successfully in a variety of animal models to control pathogenic antibiotic-resistant bacteria found on mucosal surfaces and infected tissues. Their specificity for the pathogen without disturbing the normal flora, the low chance of bacterial resistance, and their ability to kill colonizing pathogens on mucosal surfaces, a capacity previously unavailable, make them ideal anti-infectives in an age of mounting resistance. Here we review the current literature showing the effectiveness of these enzymes in controlling a variety of infections.

Introduction

After replication inside its bacterial host, a bacteriophage (or phage) is faced with a problem, needs to exit the bacterium to disseminate its progeny phage. To solve this, double-stranded DNA phages have evolved a lytic system to weaken the bacterial cell wall resulting in bacterial lysis. Phage endolysins or lysins are highly efficient molecules that have been refined over millions of years for this very purpose. These enzymes target the integrity of the cell wall and are designed to attack one of the 4 major bonds in the peptidoglycan. With few exceptions (Loessner et al., 1997) lysins do not have signal sequences, so they are not translocated through the cytoplasmic membrane to attack their substrate in the peptidoglycan. This movement is controlled by a second phage gene product in the lytic system, the holin (Wang et al., 2000). During phage development in the infected bacterium, lysin accumulates in the cytoplasm in anticipation of phage maturation. At a genetically specified time, holin molecules are inserted in the cytoplasmic membrane-forming patches, ultimately resulting in generalized membrane disruption (Wang et al., 2003), allowing the cytoplasmic lysin to access the peptidoglycan, thereby causing cell lysis and the release of progeny phage (Wang et al., 2000). Compared to large DNA phage, small RNA and DNA phages use a different release strategy. They call upon a phage-encoded proteins to interfere with bacterial host enzymes responsible for peptidoglycan biosynthesis (Young et al., 2000; Bernhardt et al., 2001) resulting in misassembled cell walls and ultimate lysis. Scientists have been aware of the lytic activity of phage for nearly a century, and while whole phage have been used to control infection (Matsuzaki et al., 2005), not until recently have lytic enzymes been exploited for bacterial control in vivo (Loeffler et al., 2003; Nelson et al., 2001; Schuch et al., 2002). One of the main reasons that such an approach is now even being considered is the sharp increase in antibiotic resistance among pathogenic bacteria. Current data indicate that lysins work only with Gram-positive bacteria, since they are able to make direct contact with the cell wall carbohydrates and peptidoglycan when added externally, whereas the outer membrane of Gram-negative bacteria prevents this interaction. This review will outline the remarkable potency these enzymes have in killing bacteria both in vitro and in vivo.

Most human infections (either viral or bacterial) begin at a mucous membrane site (upper and lower respiratory, intestinal, urogenital, and ocular). In addition, the human mucous membranes are the reservoir (and sometimes the only reservoir) for many pathogenic bacteria found in the environment (i.e., pneumococci, staphylococci, streptococci) some of which are resistant to antibiotics. In most instances, it is this mucosal reservoir that is the focus of infection in the population (Coello et al., 1994; de Lencastre et al., 1999; Eiff et al., 2001). Currently, except for polysporin and mupirocin ointments, which are the most widely used topically, there are no anti-infectives that are approved to control colonizing pathogenic bacteria on mucous membranes (Hudson, 1994); we usually first wait for infection to occur before treating. Because of the fear of increasing the resistance problem, antibiotics are not indicated to control the carrier state of disease bacteria. It is agreed, however, that by reducing or eliminating this human reser-

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voir of pathogens in the community and controlled environments (i.e., hospitals and nursing homes), the incidence of disease will be markedly reduced (Eiff et al., 2001; Hudson, 1994). Towards this goal, lysins may be used to prevent infection by safely and specifically destroying disease bacteria on mucous membranes. For example, based on extensive animal results, enzymes specific for *Streptococcus pneumoniae* and *S. pyogenes*, may be used nasally and orally to control these organisms in the community as well as in nursing homes and hospitals to prevent or markedly reduce serious infections caused by these bacteria. This has been accomplished by capitalizing on the efficiency by which phage lysins kill bacteria (Young, 1992). Like antibiotics, which are used by bacteria to control the organisms around them in the environment, phage lysins are the culmination of millions of years of development by the bacteriophage in their association with bacteria. Specific lysins have now been identified and purified that are able to kill specific Gram-positive bacteria seconds after contact (Loeffler et al., 2001; Nelson et al., 2001). For example, nanogram quantities of lysin could reduce 10^7 *S. pyogenes* by >6 logs seconds after enzyme addition. No known biological compounds, except chemical agents, kill bacteria that quickly.

Mechanism of action

Lysin-treated bacteria examined by thin section electron microscopy revealed that lysins exert their lethal effects by forming holes in the cell wall through peptidoglycan digestion. The high internal pressure of bacterial cells (roughly 15–25 atmospheres) is controlled by the highly cross-linked cell wall. Any disruption in the wall's integrity will result in the extrusion of the cytoplasmic membrane and ultimate hypotonic lysis. Catalytically, a single enzyme molecule should be sufficient to cleave an adequate number of bonds to kill an organism, however, it is uncertain at this time whether this theoretical limit is possible. The reason comes from the work of Loessner (Loessner et al., 2002), showing that a listeria phage enzyme had a binding affinity approaching that of an IgG molecule for its cell wall substrate, suggesting that phage enzymes, like cellulases (Jervis et al., 1997), are one-use enzymes, likely requiring several molecules attacking a local region to sufficiently weaken the cell wall.

Endolysin structure

Lysins from DNA phage that infect Gram-positive bacteria are generally between 25 and 40 kDa in size except the PlyC LYSIN for streptococci which is 114 kDa. This enzyme is unique because it is composed of 2 separate gene products, PlyCA and PlyCB. Based on biochemical and biophysical studies, the catalytically active PlyC holoenzyme is composed of 8 PlyCB subunits for each PlyCA (Nelson et al., 2006). A feature of all other Gram-positive phage lysins is their two-domain structure (Diaz et al., 1990; Garcia et al., 1990). With no exceptions, the N-terminal domain contains the catalytic activity of the enzyme. This activity may be either an endo-β-N-acetylglucosaminidase or N-acetylmuramidase (lysozymes), both of which act on the carbohydrate moiety of the bacterial wall, an endopeptidase which acts on the peptide cross-bridge, or an N-acetylmuramoyl-L-alanine amidase (or amidase), which hydrolyzes the amide bond connecting the glycan strand and peptide moieties (Loessner, 2005; Young, 1992). Recently, an enzyme with γ-D-glutaminyl-L-lysine endopeptidase activity has also been reported (Pritchard et al., 2007). In some cases, particularly staphylococcal lysins, 2 and perhaps even 3 different catalytic domains may be linked to a single binding domain (Navarre et al., 1999). The C-terminal cell binding domain (termed the CBD domain) on the other hand binds to a specific substrate (usually carbohydrate) found in the cell wall of the host bacterium (Lopez et al., 1997, 1992; Garcia et al., 1988). Efficient cleavage requires that the binding domain binds to its cell wall substrate, offering some degree of specificity to the enzyme since these substrates are only found in enzyme-sensitive bacteria. The first complete crystal structure for the free and choline-bound states of the Cpl-1 lytic enzyme has recently been published (Hermoso et al., 2003). As suspected, the data suggest that choline recognition by the choline binding domain of Cpl-1 may allow the catalytic domain to be properly oriented for efficient cleavage. An interesting feature of this lysin is its hairpin conformation suggesting that the 2 domains interact with each other prior to the interaction of the binding domain with its substrate in the bacterial cell wall. Other lytic enzymes need to be crystallized to determine if this is a common feature of all lysins.

Comparing the sequences of lytic enzymes of the same enzyme class revealed high sequence homology within the N-terminal catalytic region and very little homology with the C-terminal cell binding region. It seemed counterintuitive that the phage would design a lysin that was uniquely lethal for its host organism, however, as we learned more about how these enzymes function, a possible reason for this specificity became apparent (see below, Bacterial resistance to lysins). However, because of the specificity, enzymes that spilled out after cell lysis had a good chance of killing potential bacterial hosts in the vicinity of the released phage progeny. Because of this, it is believed that the enzymes have evolved to bind to their cell wall binding domains at a high affinity (Loessner et al., 2002) to limit the release of free enzyme.

Because of their domain structure, it seemed plausible that different enzyme domains could be swapped resulting in lysins with different bacterial and catalytic specificities. This was actually accomplished early on by excellent detailed studies of Garcia and colleagues (Garcia et al., 1990; Weiss et al., 1999), in which the catalytic domains of lytic enzymes for *S. pneumoniae* phage could be swapped resulting in a new enzyme having the same binding domain for pneumococci, but able to cleave a different bond in the peptidoglycan. More recently, similar studies were performed for a *Staphylococcus aureus*-specific enzyme (Manoharadas et al., 2009). This capacity allows for enormous potential in creating designer enzymes with high specificity and equally high cleavage potential.

Though relatively uncommon, introns have been associated with certain lysins. For example, 50% of *S. thermophilus* phages have been reported to have their lysin gene interrupted by a self-splicing group I intron (Foley et al., 2000). This also appears to be the case for a *S. aureus* lytic enzyme (Flaherty et al., 2004) and perhaps the C1 lysin for group C streptococci (Nelson et al., 2003). While introns have been previously reported in phage genes, they have rarely been identified in the host genome (Fernandez-Lopez et al., 2005; Tan et al., 2005).

Endolysin efficacy

In most instances, lysins only kill the species (or subspecies) of bacteria from which they were produced. For instance, enzymes produced from streptococcal phage kill certain streptococci, and enzymes produced by pneumococcal phage kill pneumococci (Loeffler et al., 2001; Nelson et al., 2001). Specifically, a lysin from a group C streptococcal phage (PlyC) will kill group C streptococci as well as groups A and E streptococci, the bovine pathogen *S. uberis* and the horse pathogen, *S. equi*, but essentially no effect on streptococci normally found in the oral cavity of humans and other Gram-positive bacteria. Similar results are seen with a pneumococcal-specific lysin, however in this case, the enzyme was also tested against strains of penicillin-resistant pneumococci, and the killing efficiency was the same. Unlike antibiotics, which are usually broad spectrum and kill many different bacteria found in

the human body, some of which are beneficial, lysins may be identified which kill only the disease organism with little to no effect on the normal human bacterial flora. In some cases however, phage enzymes may be identified with broad lytic activity. For example, an enterococcal phage lysin has recently been reported to not only kill enterococci but a number of other Gram-positive pathogens such as *S. pyogenes*, group B streptococci, and *S. aureus*, making it one of the broadest acting lysins identified (Yoong et al., 2004). However, its activity for these other pathogens was lower than for enterococci.

An important lysin with respect to infection control is a lysin directed to *S. aureus* (Clyne et al., 1992; Manoharadas et al., 2009; O'Flaherty et al., 2005; Rashel et al., 2007; Sass and Bierbaum, 2007; Sonstein et al., 1971). However, in most cases these enzymes show low activity or are difficult to produce in large quantities. In one recent publication (Rashel et al., 2007), a staphylococcal enzyme was described that could be easily produced recombinantly and had a significant lethal effect on methicillin-resistant *S. aureus* (MRSA) both in vitro and in a mouse model. In the animal experiments, the authors showed that the enzymes may be used to decolonize staphylococci from the nose of the mice as well as protect the animals from an intraperitoneal challenge with MRSA. However, in the latter experiments, the best protection was observed if the lysin was added up to 30 min after the MRSA. To help overcome the insolubility problem, a chimeric enzyme was produced from 2 different *S. aureus*-specific lysins, however, though soluble, the activity did not show log-fold drop in viability or efficacy in animal models (Manoharadas et al., 2009).

Synergy

When the pneumococcal lysin Cpl-1 was used in combination with certain antibiotics, a synergistic effect was seen. Cpl-1 and gentamicin were found to be increasingly synergistic in killing pneumococci with a decreasing penicillin MIC, while Cpl-1 and penicillin showed synergy against an extremely penicillin-resistant strain (Djurkovic et al., 2005). Synergy was also observed with a staphylococcal-specific enzyme and glycopeptide antibiotics (Rashel et al., 2007). Thus, the right combination of enzyme and antibiotic could help in the control of antibiotic-resistant bacteria as well as reinstate the use of certain antibiotics for which resistance has been established.

Effects of antibodies

The pharmacokinetics of lysins like other foreign proteins delivered systemically to animals is about 20 min (Loeffler et al., 2003). Thus, if lysins are to be used systemically, they will need to be modified to extend their half-life, or they need to be delivered frequently or by IV infusion. An additional concern in the use of lysins is the development of neutralizing antibodies which could reduce the in vivo levels of enzyme during treatment. Unlike antibiotics, which are small molecules that are not generally immunogenic, enzymes are proteins that stimulate an immune response, when delivered mucosally or systemically, which could interfere with the lysin's activity. To address this, rabbit hyperimmune serum raised against the pneumococcal-specific enzyme Cpl-1 was assayed for its effect on lytic activity (Loeffler et al., 2003). It was found that highly immune serum slows, but does not block the lytic activity of Cpl-1. When similar in vitro experiments were performed with antibodies directed to a *Bacillus anthracis*- and a *S. pyogenes*-specific enzyme, similar results were obtained (unpublished data). These results were also verified with a staphylococcal-specific lysin (Rashel et al., 2007).

To test the relevance of this result in vivo, mice that received 3 intravenous doses of the Cpl-1 enzyme had tested positive for IgG against Cpl-1 in 5 of 6 cases with low but measurable titers of about 1:10. These vaccinated and naïve control mice were then challenged intravenously with pneumococci and then treated by the same route with 200 μg Cpl-1 after 10 h. Within a minute, the treatment reduced the pneumococcal titer in the blood of Cpl-1-immunized mice to the same degree as the naïve mice, supporting the in vitro data that antibody to lysins have little to no neutralizing effect. A similar experiment by Rashel et al. (2007) with a staphylococcal enzyme showed the same result and that the animals injected with lysin multiple times exhibited no adverse events.

This unexpected effect may be partially explained if the binding affinity of the enzyme for its substrate in the bacterial cell wall is higher than the antibody's affinity for the enzyme. This is supported by the results of Loessner et al. (2002), showing that the cell wall binding domain of a listeria-specific phage enzyme binds to its wall substrate at the affinity of an IgG molecule (nanomolar affinities). However, while this may explain the inability of the antibody to neutralize the binding domain, it does not explain why antibodies to the catalytic domain do not neutralize. Nevertheless, these results are encouraging since it suggests that such enzymes may be used repeatedly in certain situations to control colonizing bacteria, or in blood to eliminate antibiotic-resistant bacteria in cases of septicemia and bacteremia.

Animal models

Animal models of mucosal colonization were used to test the capacity of lysins to kill organisms on these surfaces; perhaps the most important use for these enzymes. An oral colonization model was developed for *S. pyogenes* (Nelson et al., 2001), a nasal model for pneumococci (Loeffler et al., 2001), and a vaginal model for group B streptococci (Cheng et al., 2005). In all 3 cases, when the animals were colonized with their respective bacteria and treated with a single dose of lysin, specific for the colonizing organism, these organisms were reduced by several logs (and in some cases below the detection limit of the assay) when tested again 2–4 h after lysin treatment. These results lend support to the idea that such enzymes may be used in specific high-risk populations to control the reservoir of pathogenic bacteria and thus control disease.

Sepsis, pneumonia, endocarditis, and meningitis

Similar to other proteins delivered intravenously to animals and humans, lysins have a short half-life (approximately 15–20 min) (Loeffler et al., 2003). However, the action of lysins for bacteria is so rapid, that this may be sufficient time to observe a therapeutic effect (Jado et al., 2003; Loeffler et al., 2003). Mice intravenously infected with type 14 *S. pneumoniae* and treated 1 h later with a single bolus of 2.0 mg of Cpl-1 survived through the 48 h endpoint, whereas the median survival time of buffer-treated mice was only 25 h, and only 20% survival at 48 h. Blood and organ cultures of the euthanized surviving mice showed that only one Cpl-1-treated animal was totally free of infection at 48 h, suggesting that multiple enzyme doses or a constant infusion of enzyme would be required to eliminate the organisms completely in this application. Similar results were obtained when animals were infected and treated intraperitoneally with lysin (Jado et al., 2003; Rashel et al., 2007). Because of lysin's short half-life, it may be necessary to modify the lysins with polyethylene glycol or the Fc region of IgG, to extend the residence time in vivo to several hours (Walsh et al., 2003). In recent studies, phage lysins have also been shown to be successful in the treatment of meningitis by adding the lysin directly to the brain intrathecally (Grandgirard et al., 2008) and endocardi-

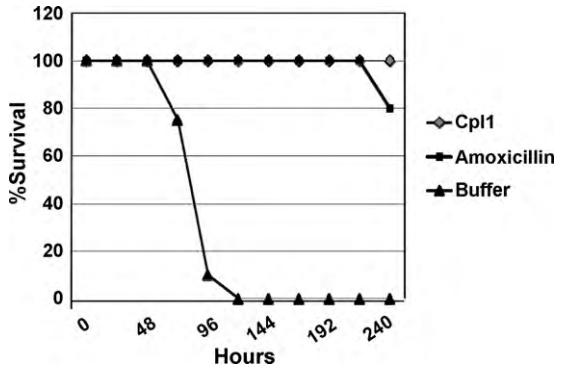

Fig. 1. Survival of mice with pneumococcal pneumonia after treatment with Cpl-1 lysin or amoxicillin. Mice with induced pneumococcal pneumonia were treated IP at 24 h (when focal inflammation was seen) and 12 h thereafter with 1 mg of Cpl-1 lysin or 0.4 mg of amoxicillin. Control animals received buffer. Animals were followed for survival for 240 h (10 days).

tis by delivering the lysin intravenously by constant IV infusion (Entenza et al., 2005). Both these applications would also benefit from modified long-acting lysins.

The ultimate challenge for lysins would be to determine whether they are able to cure an established infection. To approach this, a mouse pneumonia model was developed in which mice were transnasally infected with pneumococci and treated with Cpl-1 or amoxicillin by repeated intraperitoneal injections after infection was established (Witzenrath et al., 2009). From a variety of clinical measurements as well as morphologic changes in the lungs, it was shown that at 24 h mice suffered from severe pneumonia. When treatment was initiated at 24 h and every 12 h thereafter, 100% of the mice treated with Cpl-1 and 90% of mice treated with amoxicillin survived otherwise fatal pneumonia and showed rapid recovery (Fig. 1). Thus, Cpl-1 as well as amoxicillin dramatically reduced pulmonary bacterial counts, and prevented bacteremia.

Using lysins systemically to kill bacteria could result in an increase in cytokine production as a result of bacterial debris being release. In one study to address this issue (Entenza et al., 2005), untreated pneumococcal endocarditis induced the release of interleukin-1α (IL-1α), IL-1β, IL-6, IL-10, gamma interferon, and tumor necrosis factor, but not IL-2, IL-4, or granulocyte-macrophage colony-stimulating factor. Mice treated with a continuous dose of Cpl-1 showed the same pattern of cytokine release but higher than untreated. However, in a mouse model of pneumococcal pneumonia, Witzenrath et al. (2009) showed that the disease caused an increase of pro-inflammatory cytokines and chemokines within 36 h. However, treatment with Cpl-1 was associated with reduced synthesis of IL-1β, IL-6, and the chemokines KC, Mip-1α, and MCP-1, as well as G-CSF in the lung. In addition, decreases were observed in plasma concentrations of IL-6, KC, and Mip-1α, and MCP-1, G-CSF, and IFN-γ concentrations. Pulmonary and systemic IL-10 synthesis was only found in septic animals 60 h after infection and was completely prevented by Cpl-1 treatment. The reason for this difference has not been determined as yet, however, it may depend on the amount of lysin used for the treatments. It is possible that in the former study, in which the animals were treated with a constant IV infusion of lysin that the high concentration of the enzyme resulted in the fragmentation of the bacterial cell wall while in the second study, enzyme was delivered in 12-h intervals resulting in producing holes in the bacterial cell wall without forming wall pieces which are clearly more inflammatory (Kengatharan et al., 1998; Tuomanen et al., 1985).

Secondary infections

Secondary bacterial infections following upper respiratory viral infections such as influenza, are a major cause of morbidity and mortality (Brundage and Shanks, 2007, 2008). The organisms responsible for most of these complications are *S. aureus* and *S. pneumoniae*. Furthermore, otitis media due to *S. pneumoniae* is a leading cause of morbidity and health care expenditures worldwide and also increases after an upper respiratory viral infection (McCullers, 2006). By eliminating or reducing the bacterial burden by these organisms, will significantly reduce or eliminate these secondary infections. However, except for mupirocin and polysporin ointments, for which resistance is being developed, there is no effective way to eliminate these organisms from the upper respiratory mucosa.

In a mouse model of otitis media, using a non-invasive bioluminescent imaging technique, 80% of mice colonized with *S. pneumoniae* naturally developed otitis media upon infection with influenza virus. Treatment of these mice with Cpl-1 lysin before influenza challenge was 100% effective at preventing the development of otitis media (McCullers et al., 2007). Thus, treatment of high-risk individuals with lysin during the influenza season to decolonize them from pneumococci and staphylococci could prove effective in reducing secondary infections by these bacteria.

Bacterial resistance to lysins

Exposure of bacteria grown on agar plates to low concentrations of lysin did not lead to the recovery of resistant strains even after over 40 cycles. Organisms in colonies isolated at the periphery of a clear lytic zone created by a 10-μl drop of dilute lysin on a lawn of bacteria always resulted in enzyme-sensitive bacteria. Enzyme-resistant bacteria could also not be identified after >10 cycles of bacterial exposure to low concentrations of lysin (from 5 to 20 units) in liquid culture (Loeffler et al., 2001; Schuch et al., 2002). These results may be explained by the fact that the cell wall receptor for the pneumococcal lysin is choline (Garcia et al., 1983), a molecule that is essential for pneumococcal viability. While not yet proven, it is possible that during a phage's association with bacteria over the millennia, to avoid becoming trapped inside the host, the binding domain of their lytic enzymes has evolved to target a unique and essential molecule in the cell wall, making resistance to these enzymes a rare event. Since through evolution the phage has performed the "high throughput" analysis to identify the "Achilles heel" of these bacteria, we may take advantage of this by identifying the pathway for the synthesis of the lytic enzyme's cell wall receptor and identify inhibitors for this pathway. This would theoretically result in lead compounds that may be used to identify new anti-microbials that would be difficult to become resistant against.

Conclusion

Lysins are a new reagent to control bacterial pathogens, particularly those found on the human mucosal surface. For the first time, we may be able to specifically kill pathogens on mucous membranes without affecting the surrounding normal flora thus reducing a significant pathogen reservoir in the population. Since this capability has not been previously available, its acceptance may not be immediate. Nevertheless, like vaccines, we should be striving to developing methods to prevent rather than treat infection. Whenever there is a need to kill bacteria, and contact can be made with the organism, lysins may be freely utilized. Such enzymes will be of direct benefit in environments where antibiotic-resistant Gram-positive pathogens are a serious problem, such as hospitals, day care centers, and nursing homes. The lysins isolated thus far are

remarkably thermally stable (up to 60 °C) and are relatively easy to produce in a purified state and in large quantities, making them amenable to these applications. The challenge for the future is to use this basic strategy and improve upon it, as was the case for second and third generation antibiotics. Protein engineering, domain swapping, and gene shuffling all could lead to better lytic enzymes to control bacterial pathogens in a variety of environments. Since it is estimated that there are 10^{31} phage on earth, the potential to identify new lytic enzymes as well as those that kill Gram-negative bacteria is enormous. Perhaps someday phage lytic enzymes will be an essential component in our armamentarium against pathogenic bacteria.

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