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ABSTRACT

Aggregatibacter actinomycetemcomitans is a Gram-negative bacterium that colonizes the human oral cavity and is the causative agent for localized aggressive periodontitis (LAP), an aggressive form of periodontal disease that occurs in adolescents. *A. actinomycetemcomitans* secretes a protein toxin, leukotoxin (LtxA), which helps the bacterium evade the host immune response during infection. LtxA is a membrane-active toxin that specifically targets white blood cells (WBCs). In this review, we discuss recent developments in this field, including the identification and characterization of genes and proteins involved in secretion, regulation of LtxA, biosynthesis, newly described activities of LtxA, and how LtxA may be used as a therapy for the treatment of diseases.

KEY WORDS: leukemia, periodontitis, RTX toxin, LAP.

Aggregatibacter actinomycetemcomitans Leukotoxin: from Threat to Therapy

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a Gram-negative bacterium that inhabits the mouths of one-third or more of the population (Leys *et al.*, 1994; Lamell *et al.*, 2000). In a small number of adolescents, the bacterium can cause a rapidly progressing form of periodontal disease, localized aggressive periodontitis (LAP) (Newman *et al.*, 1976; Zambon, 1985; Slots and Ting, 1999). In contrast to adult or generalized periodontitis, LAP affects the central incisors and first molars. Individuals with LAP have breakdown of the supporting structures of the teeth, and tooth loss occurs when the condition is left untreated. Furthermore, individuals of African-American descent have a 10- to 15-fold greater risk of developing the disease than do Caucasian Americans. Approximately 70,000 adolescents in the U.S. develop the disease *per year* (Löe and Brown, 1991). In addition to causing oral disease, *A. actinomycetemcomitans* is a member of the HACEK group of bacteria, which includes the Gram-negative bacteria that cause infective endocarditis (Das *et al.*, 1997). *A. actinomycetemcomitans* has also been isolated from numerous other diseased sites of the body and is therefore considered a systemic pathogen. Indeed, the link between oral and systemic health is becoming well-established as the molecular and immunological basis for cardiovascular diseases is further studied (van Winkelhoff and Slots, 1999; Slots, 2003; Beck *et al.*, 2005).

To colonize, cause disease, and persist in a host, a bacterial pathogen must maintain an arsenal of virulence factors. *A. actinomycetemcomitans* produces numerous factors that have been well-characterized, including adherence proteins, biofilm polysaccharides, LPS, and toxins (reviewed in Fine *et al.*, 2006). Specifically, 2 protein toxins have been described for *A. actinomycetemcomitans*, cytolethal distending toxin (CDT) (Shenker *et al.*, 1999, 2005) and leukotoxin (LtxA) (Kolodrubetz *et al.*, 1989; Lally *et al.*, 1989b). Both proteins are thought to play a role in immune evasion, but may differ in their target-cell specificity and pattern of expression during disease. This review will focus on the biology of LtxA, with an emphasis on more recent studies.

The ability of *A. actinomycetemcomitans* extracts to cause the death of leukocytes was first described 30 years ago (Baehni *et al.*, 1979, 1981). Soon after this observation, the toxin was isolated from the bacterium (Tsai *et al.*, 1979, 1984). It was shown that leukotoxic activity was due to a protein from *A. actinomycetemcomitans*, leukotoxin. The DNA sequence of the leukotoxin gene (*ltxA*) was revealed by two different groups at about the same time (Kolodrubetz *et al.*, 1989; Lally *et al.*, 1989a; Kraig *et al.*, 1990), and it was found to be similar to other RTX (repeat in toxin) toxin genes. The LtxA protein is ~113 kDa (based on amino acid sequence), shares ~51% amino acid identity with *E. coli* alpha-hemolysin, and is 43% identical to *Mannheimia haemolytica* leukotoxin. The RTX toxin family of proteins includes

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Figure 1. Leukotoxin operon from the 652 strain harboring the full-length promoter region and JP2 strain with a 530-bp deletion at the 3' end of the promoter region.

membrane-active toxins that are found in many Gram-negative pathogens. While there are similarities among the RTX toxins of Gram-negative bacteria, important differences exist, such as host cell specificity and mechanisms by which they interact with and affect host cells.

RTX TOXINS

While none of the RTX toxin three-dimensional structures has yet been determined, different domains of the molecule have been mapped, based on experimental evidence and modeling of proteins with similar sequences. At the N-terminus of the toxin are amphipathic helices, and analysis of the data suggests that these helices interact with the host cell membrane and receptor (Lally *et al.*, 1999). In the middle of the protein are 2 lysine residues that are covalently modified with fatty acid residues (discussed below and reviewed in Stanley *et al.*, 1998). RTX toxins received their designation based on the nonapeptide glycine-rich repeats that occur in the proteins and are responsible for binding calcium ions. Calcium is required for activity of RTX toxins. One calcium ion binds each repeat, and binding causes the formation of a “spring-like” beta-superhelix structure (Baumann *et al.*, 1993). These repeats have recently been shown also to play a role in interaction with the host cell surface (Sanchez-Magraner *et al.*, 2007), and LtxA has been reported to have 12 such repeats (Kraig *et al.*, 1990). At the extreme C-terminus is a ~100-amino-acid domain that is involved in secretion of the toxin by a type I secretion system (discussed below).

THE *ltx* PROMOTER

It was recognized that *A. actinomycetemcomitans* exhibits 2 different leukotoxic phenotypes: minimally leukotoxic (652 strains) and highly leukotoxic (JP2 strains) (Spitznagel *et al.*, 1991). Highly leukotoxic strains produced more LtxA protein and *ltx* mRNA than minimally leukotoxic strains, and an important discovery was the revelation of 2 different types of promoters that control the expression of *ltx* genes (Brogan *et al.*, 1994). DNA sequence analysis of the leukotoxin promoter regions from these 2 types of strains revealed that the highly leukotoxic strain was missing ~500 bp at the 3' end. Thus, the minimally leukotoxic strain harbors a full-length (~1 kb) promoter region, while the highly leukotoxic strain has a truncation (Fig. 1). Leukotoxin promoters are now referred to as either JP2-type or 652-type.

The reason for greater production of LtxA in JP2-like strains is still not completely understood. A logical hypothesis is that the region missing from the JP2-type promoter normally binds a regulatory protein that regulates transcription of the downstream *ltx* genes. In 2003, Mitchell *et al.* identified a factor that binds to the *ltx* promoter region, but the nature of that protein has not yet been identified. Hence, a *ltx* repressor protein has thus far remained elusive. Alternatively, the deleted region may result in a shorter distance between the RNA polymerase binding site and *ltx* structural genes and, consequently, increased levels of transcription.

In 1999, He *et al.* identified, in Japanese individuals, highly leukotoxic strains of *A. actinomycetemcomitans* that did not possess the JP2 promoter. The promoter region in these strains was 1926 bp in length and contained a DNA transposable element (IS1301) that was required for high leukotoxicity. More recently, Schaeffer *et al.* (2008) characterized the mechanism that leads to overproduction of LtxA in these strains. They reported that both the transposase promoter and a protein encoded by an open reading frame just upstream of *ltxC* (OrfA) are required for maximal transcriptional activity of the operon. They proposed that OrfA may act as a regulator protein by binding near the -35 sequence to cause increased transcription. Ongoing studies to characterize the *ltx* promoter region should reveal mechanisms by which *A. actinomycetemcomitans* controls the expression of LtxA in all strains.

SECRETION OF LtxA

For a long time, LtxA was considered to be the only RTX toxin not secreted from bacterial cells, and instead remained associated with the bacterial cell surface. Hence, it was also assumed that a protein required for secretion of other RTX toxins, TolC, was missing in *A. actinomycetemcomitans*. In 2000, it was reported that LtxA was secreted from *A. actinomycetemcomitans* cells (Kachlany *et al.*, 2000). *A. actinomycetemcomitans* actively transports LtxA from the cell, and this secretion is sensitive to numerous factors, including the way the growth medium is prepared, the age of the medium, and the age of the growing culture. All strains that were examined to date secrete LtxA, regardless of their serotype or *ltx* promoter type (Diaz *et al.*, 2006).

In addition to being released into the cell-free supernatant, LtxA has been detected in lipid vesicles that appear to be derived from the outer bacterial membrane (Kato *et al.*, 2002) as well as attached to the cell surfaces of bacteria (Berthold *et al.*, 1992; Johansson *et al.*, 2003; Diaz *et al.*, 2006). Ohta *et al.* (1991) found that LtxA could be released from whole cells by treatment with nucleases, suggesting that nucleic acids may also play a role in binding of LtxA to the bacterial cell surface. The significance of the membrane-bound form of the toxin is unknown, but other bacteria ‘package’ their toxins in vesicles as a mechanism of toxin delivery to host cells (Kesty *et al.*, 2004; Balsalobre *et al.*, 2006).

The genes required for the secretion of *E. coli* HlyA have been well-characterized and serve as a model for related systems

(Schlor *et al.*, 1997; Holland *et al.*, 2005). HlyA is secreted via a type I secretion system that uses an uncleaved C-terminal secretion signal. The type I secretion apparatus in *E. coli* includes 3 different proteins that traverse the inner membrane (HlyB), periplasm (HlyD), and outer membrane (TolC) to form a continuous channel from the cytosol to the extracellular environment. Therefore, proteins secreted via type I secretion do not assume a periplasmic intermediate.

The sequences of the genes, *ltxB* and *ltxD*, were published in 1990, and their function was presumed based on sequence similarity and mutational experiments (Guthmiller *et al.*, 1990a,b, 1995). In addition, Lally *et al.* (1991) showed that LtxB and LtxD are involved in the translocation and insertion of LtxA into the membrane of *E. coli*. However, because LtxA secretion was only recently revealed in *A. actinomycetemcomitans*, little is known about the native secretion process. In an attempt at full characterization of the secretion system for *A. actinomycetemcomitans* LtxA without bias, a random mutagenesis screen was carried out on blood agar plates (Isaza *et al.*, 2008). Non-hemolytic mutants were chosen for study, since these bacteria have a defect in some aspect of LtxA biosynthesis (see below). Through this screen, we identified the secretion proteins LtxB, LtxD, and a third protein, TdeA (for Toxin and Drug Export), which we discovered at about the same time using an independent method (Crosby and Kachlany, 2007).

All 3 proteins—LtxB, LtxD, and TdeA—are required for the secretion of LtxA (Crosby and Kachlany, 2007; Isaza *et al.*, 2008). LtxB is predicted to localize to the inner membrane (IM) and interact with LtxD, which is attached to the periplasmic side of the IM. LtxB has a predicted ATP-binding and hydrolysis site, which would provide energy for the secretion process. Based on the model (Fig. 2), LtxA interacts with LtxB in the cytosol, causing a conformational change in LtxD to allow contact between LtxD and TdeA in the outer membrane. TdeA is a TolC-like protein that likely forms a trimeric pore in the outer membrane. In addition, Gallant *et al.* (2008) recently reported that an inner membrane protein, MorC, is involved in LtxA secretion, but not transcription. MorC affects the structure of the bacterial outer membrane, and loss of MorC resulted in a defect in LtxA secretion. The mechanism by which MorC influences LtxA production is not known; however, MorC may interact directly with other secretion components, or its general effect on the cell membrane could affect assembly of the type I secretion complex.

Like *E. coli* mutants of *tolC* (Wandersman and Delepelaire, 1990; Nishino *et al.*, 2003, 2006; Tamura *et al.*, 2005), *A. actinomycetemcomitans* *tdeA* mutants are significantly more sensitive to antimicrobial agents than are wild-type strains (Crosby and Kachlany, 2007). The reason for this is that TdeA is also part of one or more drug efflux pumps, similar to other TolC proteins. A mechanism used by *A. actinomycetemcomitans* to resist antibiotics and other toxic agents is the active export of these agents after they enter the cell, and drug efflux pumps are responsible for this process. Thus, targeting TdeA would render bacteria not only defective for LtxA secretion, but also highly sensitive to conventional antimicrobial agents, and could be an

Figure 2. Type I secretion system for export of LtxA from *A. actinomycetemcomitans*. The model is based on experimental evidence and data from other systems. Reproduced in modified form from Kuhnert and Christensen (2008) with permission.

effective strategy for targeting *A. actinomycetemcomitans* infections in the future.

REGULATION OF LEUKOTOXICITY

While an *ltx* regulatory protein has not yet been characterized, numerous external factors are known to affect the production or activity of LtxA. These are discussed below.

Carbohydrates

In 2001, Inoue *et al.* reported that the level of fermentable sugars regulates the production of LtxA. They found that growth in a fructose chemostat resulted in low levels of LtxA production and decreased levels of intracellular cAMP. More recently, the genetic system that is responsible for the regulation of *ltxA* production by certain sugars has been identified (Isaza *et al.*, 2008). The connection between sugars and cAMP is through the phosphotransferase system (PTS). The PTS is responsible for the transfer of phosphate to sugars during their active transport into the cell. PTS mutants (*ptsH* and *ptsI*) were attenuated for LtxA production, and cAMP levels in these mutants were lower compared with those in wild-type strains. Interestingly, wild-type production of LtxA could be restored in the PTS mutants by providing exogenous cAMP in the culture medium. In other bacteria, cAMP is able to regulate gene expression by binding to cAMP receptor protein (CRP), which binds to a CRP binding site on the DNA. A CRP binding site has not been identified in the *ltx* operon (Inoue *et al.*, 2001), and so whether *A. actinomycetemcomitans* CRP binds to a different consensus sequence or acts through a novel mechanism is not yet clear.

Fatty Acid Modification

A unique property of RTX toxins is the covalent attachment of fatty acid residues to the protein. Two other RTX toxins, *E. coli* HlyA and *B. pertussis* CyaA, have been shown to be modified with fatty acid residues at internal lysines. Modification is

Figure 3. Hemolytic properties of *A. actinomycetemcomitans* on different growth media. The names on the left indicate different strains of *A. actinomycetemcomitans*, and the letters refer to the media. All media contained 5% sheep blood. Lane **A**, Columbia agar from Accumedia; lane **B**, Columbia agar from BBL-Becton Dickinson; lane **C**, Trypticase soy agar from BBL-Becton Dickinson; lane **D**, AAGM. Reproduced with permission from Balashova *et al.* (2006a).

required for full activity of these toxins, and the fatty acid residues are proposed to play a role in the insertion of toxin into the lipid membrane of the host cell (Stanley *et al.*, 1994, 1998). Recently, it was reported that *A. actinomycetemcomitans* LtxA is also modified at two internal lysine residues; however, the nature of the fatty acids is currently unknown (Balashova *et al.*, 2009). As in *E. coli* and *B. pertussis*, the 'c' gene of the operon (*ltxC*) is responsible for carrying out this modification (Lally *et al.*, 1994; Balashova *et al.*, 2009). An *A. actinomycetemcomitans* *ltxC* mutant was able to secrete LtxA at levels similar to the wild-type strain, suggesting that modification is not required for secretion. As expected, LtxA from the *ltxC* mutant did not kill cells, nor did it cause an elevation in intracellular calcium, an event that occurs early during LtxA-mediated intoxication of a cell.

Iron

A unique feature of LtxA is the high specificity for human and Old World primate WBCs (Taichman *et al.*, 1980, 1987; Simpson *et al.*, 1988). It was noted early on that LtxA can target WBCs, but not RBCs (Tsai *et al.*, 1979, 1984), and in the literature, *A. actinomycetemcomitans* is listed as being non-beta-hemolytic (Bergey and Holt, 1994; Avila-Campos, 1995). However, recently, it was reported that *A. actinomycetemcomitans* exhibits beta-hemolysis on certain types of growth media (Balashova *et al.*, 2006a). Mutants lacking LtxA are non-hemolytic, and purified LtxA has hemolytic properties. Interestingly, the ability of *A. actinomycetemcomitans* colonies to appear beta-hemolytic is dependent on the type of medium used (Fig. 3) and may explain why hemolysis was previously not observed (Balashova *et al.*, 2006a). In contrast to cytolysis of WBCs, hemolysis appears to be non-specific, since human, sheep, and horse RBCs underwent lysis by LtxA, and hemolysis requires a higher dose of LtxA than the killing of WBCs. The nature of the RBC

receptor for LtxA is unknown, but is the focus of ongoing studies in our laboratory.

It is not yet known why LtxA-mediated hemolysis is dependent on medium type, but one explanation is that the growth medium affects the expression or activity of LtxA. Another possibility is that the medium somehow "protects" the RBCs, rendering them resistant to the hemolytic effects of LtxA. The fact that purified LtxA is able to mediate lysis of washed RBCs suggests that the growth medium does not render RBCs sensitive to LtxA.

The discovery that LtxA could cause lysis of RBCs raised new questions about other potential functions of LtxA. *A. actinomycetemcomitans* has been detected in the blood, and the bacterium is not only an oral pathogen, but can also cause numerous systemic diseases, most notably, infective endocarditis (Das *et al.*, 1997). While in the blood and heart tissue, *A. actinomycetemcomitans* could acquire iron by lysis of RBCs to release heme and hemoglobin (Balashova *et al.*, 2006a). Because there is essentially no free iron available in the body, bacteria have evolved several ways of "stealing" iron from host iron-binding proteins. These include the production of siderophores and proteins that can metabolize heme and hemoglobin to release iron. Nagata *et al.* (2002) reported that at least one strain of *A. actinomycetemcomitans* can bind hemoglobin; but, interestingly, JP2 strains, in contrast to 652 strains, do not produce a functional hemoglobin-binding protein (HgpA) and can thus not utilize hemoglobin as an iron source (Hayashida *et al.*, 2002). In addition, *A. actinomycetemcomitans* is unable to obtain iron from human transferrin or lactoferrin (Winston *et al.*, 1993; Hayashida *et al.*, 2002) and does not produce siderophores (Winston *et al.*, 1993; Rhodes *et al.*, 2005). Thus, JP2 strains have an impaired ability to utilize complexed iron from host proteins. We have proposed that perhaps high leukotoxicity (and hence deletion of the promoter region) was strongly selected for in these strains that lacked HgpA, resulting in the JP2 phenotype (Balashova *et al.*, 2006b). Increased lysis of RBCs would result in greater release of heme and hemoglobin and a more favorable chance that some could be used by the bacterium.

If LtxA plays a role in iron acquisition, one possible scenario is that iron could play a regulatory role in the production of LtxA. Indeed, iron regulates secretion, not transcription, of LtxA (Balashova *et al.*, 2006b). In the presence of excess iron, but not other metal ions, LtxA remains intracellular; but under iron-limiting conditions, LtxA is essentially fully secreted. Controlling the secretion step rather than transcription allows the bacterium to respond rapidly to changes in the environment, since transcription and translation of a protein require more time to complete than just secretion. Thus, the data are consistent with the hypothesis that LtxA also plays a role in iron acquisition during infection. The mechanism by which iron regulates secretion is not known, but iron may interact with TdeA to cause a decrease in the pore size to prevent the passage of LtxA out of the cell (Crosby and Kachlany, 2007).

Superoxide Dismutase

After being secreted, LtxA appears to be stabilized by interaction with another *A. actinomycetemcomitans* protein, Cu,Zn superoxide dismutase (SOD). SOD is a bacterial protein that is

required for the detoxification of the superoxide radicals that are generated during a respiratory burst by host cells. SOD interacts with LtxA, and SOD mutants are highly sensitive to superoxide radicals (Balashova *et al.*, 2007). In addition, degradation of LtxA protein occurs more rapidly after exposure to reactive oxygen species (ROS) and reactive nitrogen species (RNS). It has been reported that LtxA and other RTX toxins induce the production of ROS from host cells (Bhakdi and Martin, 1991; Yamaguchi *et al.*, 2001). Hence, during intoxication of host cells, LtxA would likely be exposed to the damaging effects of its own doing. Interaction between LtxA and SOD may result in the stabilization of the toxin during the LtxA-mediated respiratory burst. It is not yet clear where the interaction between LtxA and SOD takes place. It is possible that SOD is secreted at later times, after LtxA has had a chance to act on host cells. Alternatively, SOD may be released from cells that have undergone lysis. SOD is also found associated with the membrane in *A. actinomycetemcomitans* (Fletcher *et al.*, 2001; Balashova *et al.*, 2007), and so it is possible that LtxA interacts with SOD at the cell surface.

LtxA SOD may also have a second function. Interestingly, Cu,Zn SOD from *Haemophilus ducreyi* has been shown to bind heme, and this interaction may not only provide a source of iron for the bacterium, but it may also serve as a mechanism for the detoxification of toxic oxyradicals formed from heme iron and oxygen (Pacello *et al.*, 2001). To date, *A. actinomycetemcomitans* SOD has not been shown to bind heme, although this remains an interesting possibility, given that LtxA can also cause lysis of RBCs. A summary of how different factors regulate leukotoxicity and the proteins required for LtxA production is shown in Fig. 4.

In addition, it has been shown that LtxA is regulated by other environmental factors, such as pH and oxygen. Lower pH values (6.0-7.0) and anaerobic conditions have positive effects on LtxA production and/or stability (Hritz *et al.*, 1996; Ohta *et al.*, 1996; Kolodrubetz *et al.*, 2003), and these have been discussed in greater detail in a previous review (Inoue *et al.*, 2006). Like many other bacterial virulence factors, LtxA is also regulated by quorum-sensing. Quorum-sensing is the mechanism by which bacteria can detect the numbers of other bacteria in their environment by chemical signals. Fong *et al.* (2001) reported that the autoinducer, LuxS, increased production of LtxA. Interestingly, LuxS also increased production of AfuA, a protein involved in iron acquisition (Fong *et al.*, 2001).

INTERACTION WITH HOST CELLS

The primary role of LtxA is in immune evasion. The immune response against *A. actinomycetemcomitans* after it migrates into the periodontal pocket is the infiltration of PMNs to engulf and destroy the bacterium. LtxA is the counter-defense for *A. actinomycetemcomitans*. The goal of a pathogen is not to eliminate its host, but rather to live in symbiosis with it. Hence, *A. actinomycetemcomitans* is an outstanding example of a successful pathogen. By targeting only certain WBCs, the bacterium ensures that the effects of the toxin are limited to only the cells that are most immunologically relevant. In 1997, Lally *et al.* identified leukocyte function antigen-1 (LFA-1) as the

Figure 4. Regulation of LtxA biosynthesis. Activation of the PTS results in the production of cAMP, which has a positive effect on the expression of *ltx* genes. LtxA protein is modified with fatty acid residues by LtxC and then secreted through a channel consisting of LtxB, LtxD, and TdeA. The presence of iron blocks the secretion of LtxA. After being secreted into the environment, LtxA may be stabilized by SOD and protected from the ROS and RNS released by host cells.

cellular receptor for LtxA. LFA-1 is expressed only on cells of hematopoietic origin; hence, LFA-1 serves as an ideal marker for WBCs.

LFA-1 is a heterodimer composed of 2 proteins, CD11a and CD18, which assemble on the surfaces of essentially all human WBCs at some level. While both molecules are required for LtxA to interact with cells, it was found that CD18 confers species specificity on the toxin (Dileepan *et al.*, 2007). Cells expressing bovine CD11a/human CD18 were sensitive to LtxA, but human CD11a/bovine CD18 cells were resistant. Further, the cysteine-rich tandem repeats encompassing integrin-epidermal growth-factor-like domains 2, 3, and 4 of the extracellular region of human CD18 are critical for conferring susceptibility to LtxA (Dileepan *et al.*, 2007). In addition, Kieba *et al.* (2007) showed that a 128-amino-acid domain encompassing the beta-propeller domain of CD11a is important for recognition by LtxA. Thus, LtxA comes into contact with both the CD11a and CD18 domains of LFA-1.

The normal function of LFA-1 is to mediate adhesion to vascular endothelial cells and then migration and extravasation of immune cells during infection (Hogg *et al.*, 2004; Kinashi, 2005). LFA-1 assumes at least two conformations, a resting state and an activated state (also called low-affinity and high-affinity states, respectively). In the resting state, LFA-1 is tucked in toward the cell surface and unavailable for binding to other molecules. However, upon activation of the cell by cytokines (such as during an infection), LFA-1 is extended and becomes available for binding. The natural ligand for LFA-1 is intercellular

Figure 5. Host response to *A. actinomycetemcomitans* infection in the mouth. Interaction between bacterial cells and gingival tissue causes the release of cytokine signals by lymphocytes and other WBCs. Cytokine signaling results in the activation of circulating WBCs in the underlying vasculature. Activation results in the immunological priming of WBCs and a conformational change of LFA-1 from a low-affinity state to a high-affinity, exposed state. High-affinity, activated LFA-1 can bind ICAM-1 expressed by vascular endothelial cells. Interaction between ICAM-1 and LFA-1 results in the migration of WBCs into the infected tissue via the process of diapedesis. In the gingival tissue, WBCs are incapacitated by secreted LtxA binding to activated LFA-1, allowing *A. actinomycetemcomitans* to colonize, persist, and invade deeper into the periodontal pocket.

adhesion molecule-1 (ICAM-1), located on the surfaces of vascular endothelial cells. Activated LFA-1 binds ICAM-1 and then mediates the migration of the cell out of the blood vessel and into infected tissue. Thus, cells that are most immunocompetent are “tagged” with an activated form of LFA-1.

In a recent publication, we examined the relationship between levels of surface LFA-1 and sensitivity to LtxA, and we observed a direct correlation (Kachlany *et al.*, 2009). For example, THP-1 cells had highest levels of CD11a and CD18 on their surfaces, as determined by flow cytometry, and were most sensitive to LtxA. Additionally, LtxA preferentially targets cells with activated LFA-1. Given the natural role of the toxin in immune evasion, this observation makes logical sense, since the WBCs that LtxA encounters will have been activated. A model showing the interplay between *A. actinomycetemcomitans* LtxA and the host immune system in the gingival crevice is shown in Fig. 5. It is not known if the actual levels of LtxA produced by 652 strains *in vivo* are as high as JP2 strains. Examination of gingival crevicular fluid or saliva in persons with LAP may address this issue. Also, bacterial cells can interact directly with neutrophils, and thus, cell-bound LtxA would be important for mediating cell death *via* direct contact (Permanich *et al.*, 2006).

While infiltration of PMNs represents the initial response in the periodontal pocket to *A. actinomycetemcomitans* infection, and thus would be considered the intended biologic target for LtxA, other WBCs are equally susceptible to LtxA *in vitro*

Figure 6. A model for the mechanism of how LtxA interacts with host cells and ultimately causes cellular intoxication by apoptosis. See text for details.

(Taichman *et al.*, 1980, 1987; Kachlany *et al.*, 2009). However, because LtxA would be delivered locally by the bacteria, other WBC types that do not access the diseased site in the body may not be affected by the toxin.

At high doses, LtxA destroys cells by forming pores in the membrane and causing necrosis; at lower (and probably more physiologically representative) doses, LtxA induces apoptosis (Korostoff *et al.*, 1998, 2000). The mechanism by which LtxA interacts with and ultimately kills host cells is poorly understood. Based on the well-accepted model, the initial step involves binding of LtxA to host cells in a non-specific, reversible manner. Fong *et al.* (2006) has shown that the first step to cellular intoxication by LtxA is an increase in intracellular calcium levels, even before interaction with the LFA-1 receptor. The mechanism by which this occurs is unknown. The rise in calcium then leads to activation of calpain, which cleaves talin. LFA-1 is then released from talin protein and clusters into lipid rafts. LtxA interacts with clustered LFA-1, and this interaction may then stimulate an integrin signaling pathway. Morova *et al.* (2008) recently reported that RTX toxins, including LtxA, recognize oligosaccharide subunits on LFA-1; however, LtxA was still partly active, even after the oligosaccharides were removed from WBCs with glycosidases, suggesting that either deglycosylation was not complete or that LtxA may recognize other entities as well. Bound LtxA destroys host cells by apoptosis (Mangan *et al.*, 1991; Korostoff *et al.*, 1998; Lally *et al.*, 1999; Yamaguchi *et al.*, 2001) or activation of caspase 1 through a process that differs from classic apoptosis (Kelk *et al.*, 2003). Others have shown that LtxA affects the mitochondrial membrane potential and causes the release of cytochrome *c* from the mitochondrial intermembrane space and into the cytosol, where it likely activates an apoptosis cascade (Korostoff *et al.*, 2000; Yamaguchi *et al.*, 2001). A proposed model of LtxA action on host cells is shown in Fig. 6. The question marks indicate proteins or pathways that are currently undefined. Structural changes that LtxA necessarily undergoes have been removed for clarity.

RELEVANCE TO DISEASE

Because of the high specificity of LtxA for primate WBCs, it is difficult to test the contribution of this putative virulence factor in well-established mouse and rat models for periodontal

disease (Evans *et al.*, 1992; Baker *et al.*, 2002; Niederman *et al.*, 2002; Lalla *et al.*, 2003; Schreiner *et al.*, 2003; Graves *et al.*, 2008; Lee *et al.*, 2009; Polak *et al.*, 2009). However, analysis of clinical data indicates that LtxA is an important virulence factor for *A. actinomycetemcomitans*. For example, highly leukotoxic strains (JP2 strains) are more often associated with severe disease than minimally leukotoxic strains (652 strains), especially in individuals of African-American descent (Haubek *et al.*, 1997, 2001, 2008; Macheleidt *et al.*, 1999; Haraszthy *et al.*, 2000). In a recent study (Haubek *et al.*, 2008) in Morocco, individuals who harbored the JP2 strains had significantly increased risk of periodontal attachment loss than those with the 652 strains. However, minimally leukotoxic strains are also found in persons with LAP (Kaplan *et al.*, 2002; Fine *et al.*, 2007). Fine *et al.* (2007) studied adolescents in Newark, NJ, and found that the majority of individuals who developed bone loss did not harbor the JP2 strain in their mouths during the study period. Thus, these authors concluded that it is currently not possible to determine if the JP2 strains are more virulent than other minimally leukotoxic strains. It is also important to note that the locations of these studies and ethnicity of the populations differ and could account for clonal variations. However, a rational biological explanation for the observations by Fine *et al.* (2007) is that LtxA is up-regulated to higher levels in the mouth. Thus, strains with the 652 promoter are minimally leukotoxic under laboratory conditions, but produce levels similar to those of JP2 strains in the physiological environment. Studies to investigate the actual levels of LtxA in the periodontal pockets of individuals with disease could test this hypothesis.

POTENTIAL THERAPEUTIC USE OF LtxA

While LtxA has been studied solely because of the threat it poses to the host immune system, we asked if the unique and natural properties of the toxin could be exploited for therapeutic use. Several bacterial toxins have been used clinically, including *Clostridium botulinum* neurotoxin (BOTOX) for the treatment of neuromuscular disorders (Jankovic, 2004) and *Corynebacterium diphtheriae* toxin (ONTAK) for the treatment of T-cell lymphoma (Olsen *et al.*, 2001; Duvic *et al.*, 2002).

Hematologic malignancies include leukemia, lymphoma, and myeloma and are defined as cancer of WBCs. It has been shown that LFA-1 can be up-regulated in many leukemias and lymphomas (Inghirami *et al.*, 1988; Horst *et al.*, 1991; Bechter *et al.*, 1999). Thus, malignant cells may be more sensitive to LtxA-mediated cytotoxicity than normal counterparts. Indeed, healthy PBMCs are relatively resistant to LtxA, while primary

Figure 7. Efficacy of LtxA therapy in a humanized mouse model for leukemia. **(A)** SCID mice were injected with the human leukemia cell line, HL-60, in the peritoneal cavity and then remained untreated (top) or were treated with 3 doses of LtxA (bottom). **(B)** Kaplan-Meier survival plot shows that 7/8 leukemic mice that received LtxA were long-term survivors and remained disease-free, while the untreated leukemic mice all succumbed to disease. Reproduced with permission from Kachlany *et al.* (2009).

PBMCs from leukemia patients are highly sensitive to the toxin (Kachlany *et al.*, 2009). In addition, LtxA is highly effective in a SCID mouse xenograft model for human leukemia (Fig. 7), and the toxin shows targeted activity and safety when administered intravenously to a non-human primate (Kachlany *et al.*, 2009). In the rhesus macaque, a relatively low dose of LtxA (22 $\mu\text{g/kg}$) caused a decrease in WBCs, but no change in hemoglobin values, RBCs, or platelets, or other diagnostic markers of toxicity. Thus, while conventional chemotherapeutic agents show little specificity and high general toxicity, LtxA may represent novel targeted biotherapy for WBC disorders, and LtxA is currently in pre-clinical pharmaceutical development.

While leukemia is unrelated to LAP and oral disease, we believe that understanding the latter could help in the development of LtxA as a therapeutic agent for the former. Just as dispersin B from *A. actinomycetemcomitans* is being investigated for the treatment of wound infections (Izano *et al.*, 2007; Kaplan, 2009), we hope to employ the bacterium for yet another of its natural products. In essence, because of exquisite co-evolution between pathogen and human host, the bacterium has carried out the “research and discovery” phase of the pharmaceutical development process, and we plan to take advantage of the work *A. actinomycetemcomitans* has already done for us.

SUMMARY

Since the discovery of *A. actinomycetemcomitans* leukotoxic activity 30 years ago, much has been learned about the protein, LtxA, which is responsible for this important pathogenic property. Knowledge from other RTX toxins has also shed light on the biology of LtxA; however, important differences exist between LtxA and other RTX toxins, such as regulation of expression and secretion, target cell specificity, host receptor, and structural properties. Thus, simple extrapolation of knowledge from other systems may

not be possible in many cases. Important questions that still remain include the mechanism of LtxA action on host cells, the physiological contribution of LtxA to disease, and how LtxA recognizes and targets RBCs. Furthermore, because of the natural toxicity and specificity of the toxin for certain WBCs, LtxA may show significant therapeutic potential for the treatment of WBC diseases.

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S.C.K. owns stock in a company that has licensed patents from UMDNJ for the clinical use of leukotoxin.

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