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Turnabout is fair play

Use of the bacterial Multivalent Adhesion Molecule 7 as an antimicrobial agent

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Pathogen attachment to host tissues is one of the initial and most crucial events during the establishment of bacterial infections and thus interference with this step could be an efficient strategy to fight bacterial colonization. Our recent work has identified one of the factors involved in initial binding of host cells by a wide range of Gram-negative pathogens, Multivalent Adhesion Molecule (MAM) 7. Interference with MAM7-mediated attachment, for example by pre-incubation of host cells with recombinant MAM7, significantly delays the onset of hallmarks of infection, such as pathogen-mediated cytotoxicity or the development of other adhesive structures such as actin pedestals. Thus, we are trying to develop tools based on MAM7 that can be used to prevent or diminish certain Gram-negative bacterial infections. Herein, we describe the use of bead-coupled MAM7 as an inhibitor of infection with the clinically relevant pathogen *Pseudomonas aeruginosa*.

Keywords: infection, attachment, adhesin, phosphatidic acid, fibronectin, Gram-negative pathogen, anti-adhesion therapy

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Bacterial pathogens are faced with a number of problems when they try to colonize their host—they have to evade immune recognition, invade tissues and modulate cellular signaling events to promote their own survival. A growing number of virulence factors are known to contribute to each of these steps, and more are being discovered continually. However, the initial step giving rise to an infection, the stable attachment of the pathogen to host cells, is often overlooked and as a result a lot less well understood than subsequent events. Many virulence factors are upregulated dependent on host cell contact and are often only expressed at low levels upon the bacteria's first encounter with its host.[1] Often times, their expression only commences after the bacteria receive specific environmental cues they encounter inside the host, such as elevated temperature, higher or lower than usual ion concentrations, or reactive oxygen species generated as a result of the induction of the host's immune response to infection.[2,3] In many cases, the pathogens capacity to exert its full virulence is directly dependent on surface contact and therefore relies to a high degree on its ability to establish a stable interaction with the host cells.[4]

For example, many pathogens express and secrete large autotransporter toxins into the surrounding medium. Delivery of these toxins often requires their localized binding to the host membrane, where they cross into the host cell cytoplasm to exert their cytotoxic effect.[5] Other toxins penetrate the host cell membrane by assembling into a multimeric pore complex, a feature which is highly dependent on local toxin concentration and thus only possible if secreting bacteria and host cells are in sufficient proximity to each other to avoid loss of toxin by diffusive processes.[6,7] Many Gram-negative pathogens possess an arsenal of effector proteins which target host proteins to take over the regulation of host signaling events and manipulate them to the pathogens advantage. For example, effector proteins can inhibit immune responses, induce autophagy and trigger reorganization of a cell's cytoskeleton.[8,9] The delivery of effector proteins into host cells is accomplished by type III, type IV or type VI secretion systems, needle-like complexes which translocate

bacterial proteins directly from the bacterial cytoplasm into the host's cytoplasm.[10,11] Establishment of these translocation machines and correctly timed transfer of proteins relies on direct contact with the host cells.[12]

As such, the initial attachment of pathogens to their eukaryotic host is a logical target of therapeutic interference with bacterial infections. However, developing therapeutic tools interfering with bacterial adhesion remains a major challenge, partly due to the complexity of synthetic receptor or adhesin analogs and variability in receptor-adhesin interactions between different pathogens and tissue types.[13,14] Although many adhesins are known in both Gram-positive and Gram-negative bacteria, their expression is often upregulated as a result of host cell contact and most of them are highly species-specific. In addition, many adhesion factors are large proteins or even multi-subunit protein complexes requiring dedicated cellular machinery for their transport to and assembly at the bacterial outer membrane.[15,16]

Our recent work identified an adhesion factor, termed Multivalent Adhesion Molecule (MAM) 7, which is widely distributed in Gram-negative bacteria, and especially animal pathogens. MAM7 consists of an N-terminal hydrophobic region which is required for outer membrane targeting and anchoring of the protein. This region is followed by a stretch of six to seven mammalian cell entry (mce) domains which are responsible for host cell binding. Mce domains have been described previously—a family of proteins containing one mce domain followed by a domain of unknown function has been identified as a factor promoting cellular attachment and invasion of macrophages by mycobacteria, although the mechanism underlying the invasion process was unknown.[17,18] In Gram-negative bacteria, the mce domain seems to have undergone multiple duplication events to form a distinct family of mce-containing proteins which consist of an N-terminal hydrophobic membrane targeting region, followed by six or seven mce domains.

In contrast to other adhesins, MAM7 is relatively small and constitutively expressed by bacteria, even prior to host cell contact. Upon encountering host cells, it gives the pathogen a distinct advantage, enabling immediate binding to a broad range of host cell types independent of the induction of virulence factors.[19] Outer membrane localization and anchoring of MAM7 does not require any dedicated transport machinery—this enabled us to express the protein at the surface of non-pathogenic *E. coli*. The non-pathogenic *E. coli* laboratory strain BL21 does not possess attachment factors for mammalian host cells, which allowed us to analyze the contribution of MAM7 to host cell attachment independent of other factors promoting pathogen adhesion and dissect the interactions between MAM7 and the host cell surface.

We identified two different host cell receptors for bacterial MAM7—the first receptor is the extracellular matrix protein fibronectin, which has previously been described to serve as a receptor for a variety of bacterial adhesins. In contrast to other adhesins, which exclusively bind fibronectin and have evolved to bind this ligand with exceptionally high affinity,[20-22] MAM7 seems to bind to fibronectin relatively weakly (K_D of 15 μM). This low affinity interaction is complemented by a second receptor, phosphatidic acid, resulting in an overall affinity of MAM7 for host cells that is very high (approx. apparent K_D of 200 nM). The functionality of mce domains as phosphatidic acid binding domain seems to have been conserved across different families—the Arabidopsis chloroblast protein Tgl2, which contains only one mce domain, also binds PA albeit with lower affinity.[23] We hypothesize that the duplication of mce domains in MAM7 leads to an acquisition of an entirely new functionality, the capability to bind to fibronectin. By enabling pathogens to establish interactions with two distinct types of host cell receptors, pathogens have increased their capacity for initial attachment.

Since attachment to fibronectin seems to initialize host cell binding by a wide range of pathogens, we suspected blocking the host cell receptors with MAM7 would potentially inhibit infection by other pathogens. Indeed, we showed that pre-incubation of mammalian cells with non-pathogenic *E. coli* expressing MAM7 could competitively inhibit attachment of a range of Gram-negative pathogens and stop them from exerting cytotoxic effects on host cells (as in the case of *Yersinia pseudotuberculosis, Vibrio cholerae* or *Vibrio parahemolyticus*) and from establishing permanent mechanisms of attachment, such as through formation of actin pedestals (enteropathogenic *E. coli*, EPEC). Our current efforts are directed toward developing improved MAM7-derived tools that may be used as competitive inhibitors of bacterial attachment and thus as agents to attenuate a wide range of Gram-negative bacterial infections.

So far, we have successfully inhibited infection of cultured mammalian cells by a number of important enteropathogenic bacteria. *V. cholerae* is the causative agent of cholera, a severe gastrointestinal disease which is responsible of an estimated 5 million deaths annually and still is one of the leading causes of infant death.[24] *V. parahemolyticus* is a seafood-borne pathogen which usually causes diarrheal disease but can also lead to wound infections and septicemia, particularly in immunocompromised patients.[25] *Y. pseudotuberculosis* is also a food-borne pathogen and mainly causes enteritis or tuberculosis-like symptoms in infected animals.[26,27] EPEC is another major agent of infantile diarrhea and is associated with high mortality rates.[28] For all these pathogens, we observed a 30 to 70% decrease in pathogenicity when host cells were pre-treated with BL21-MAM7.[19]

Our recent efforts to further develop a MAM7-derived agent to attenuate Gram-negative infections have therefore focused on two issues: First, we are seeking to expand the repertoire of pathogens susceptible to MAM7-based inhibition. As discussed in our previous work, we have identified MAM7 homologs in a wide range of Gram-negative pathogens and we currently are testing a selection of them for their susceptibility to MAM7-based inhibition. One of the pathogens we focus on is *Pseudomonas aeruginosa*. *P. aeruginosa* thrives in most environments, including water, soil and on human skin. In immunocompromised patients, it can cause catheter-associated lung and urinary tract infections, but it is also a

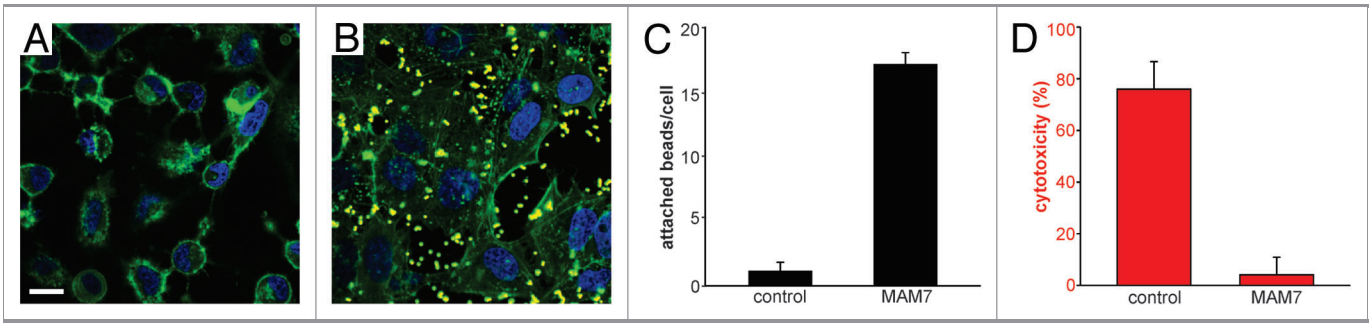

Figure 1. Inhibition of *Pseudomonas aeruginosa*-mediated cytotoxicity using bead-immobilized MAM7. HeLa epithelial cells (80% confluency) were pre-incubated with bead-immobilized GST (A) or GST-MAM7 (B) for 30 min prior to infection with *P. aeruginosa* strain PAO1 at a multiplicity of infection of 20 for four hours. Cells were fixed and stained for DNA (blue) and actin (green). Attached beads, yellow. Scale bar, 20 μm. Attached beads per cell were determined by counting (C, black) and cytotoxicity was determined by measuring LDH released into the culture medium (D, red). Error bars indicate s.e.m. ($n \geqslant 9$). Cloning of expression constructs for GST and GST-MAM7 fusion protein as well as protein purifications have been described elsewhere.[19] Purified proteins were immobilized on 1 μm fluorescent orange latex beads (Sigma) as described by El Shazly et al.[31] For inhibition experiments, a total amount of 7.5 μg protein/10^6 beads/well in PBS were used.

major burden for cystic fibrosis patients and can cause persistent wound infections, for example in burn patients.[29,30] Due to its clinical importance, we studied if *P. aeruginosa*-mediated cytotoxicity could be attenuated by MAM7 in a tissue culture model of infection (**Fig. 1**). The second focus of our current studies in MAM7-based inhibitors is dedicated to finding alternative modes of delivery. While non-pathogenic bacteria expressing surface-bound MAM7 may be a suitable vehicle for gastrointestinal delivery of MAM7 to prevent or combat enteric pathogens, their use on an open wound would most likely exacerbate inflammatory responses and could therefore have adverse effects on wound healing. We are therefore studying alternative modes of delivery for MAM7 to the site of infection. One such approach is to immobilize recombinant MAM7 on the surface of inert polymer beads, which are similar in size to the bacteria we have previously used (1 μm). We tested the efficacy of bead-immobilized MAM7 against *P. aeruginosa* infection of epithelial cells and compared it to control beads displaying GST, which do not bind to host cells (**Fig. 1**). In each case, we counted the number of bound beads per cell (fluorescent beads were used for ease of visualization) and determined the cytotoxic effect of *P. aeruginosa* using lactate dehydrogenase (LDH) release assays. Upon infection, host cells lyse and release LDH into the culture medium, which can be detected colorimetrically and compared with a standard of detergent-lysed cells (100% lysis). GST-beads did not show any significant attachment to host cells and failed to inhibit infection (**Fig. 1A and C**). In contrast, MAM7 beads bound to host cells (17.1 ± 0.9 beads/cell) and, as a consequence, attenuated *P. aeruginosa*-mediated cell killing (cytotoxicity decreased from 76% to 4%).

These studies demonstrate that MAM7-based inhibition may potentially be developed as a tool to attenuate not only enteric pathogens but also hospital-acquired and wound-associated infections, such as those caused by *P. aeruginosa*. The adhesin can be expressed at the surface of non-pathogenic bacteria but may also be delivered by alternative routes, such as immobilized on beads, which may aid in future applications in decreasing risks associated with the introduction of live bacteria into a living organism. In the future, we hope to be able to extend the application of MAM7 to include other clinically relevant Gram-negative pathogens and develop tools for its efficient delivery to the site of potential infection.

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