Pseudomonas aeruginosa Type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos

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Summary

Pseudomonas aeruginosa is an opportunistic human pathogen that can cause serious infection in those with deficient or impaired phagocytes. We have developed the optically transparent and genetically tractable zebrafish embryo as a model for systemic P. aeruginosa infection. Despite lacking adaptive immunity at this developmental stage, zebrafish embryos were highly resistant to P. aeruginosa infection, but as in humans, phagocyte depletion dramatically increased their susceptibility. The virulence of an attenuated P. aeruginosa strain lacking a functional Type III secretion system was restored upon phagocyte depletion, suggesting that this system influences virulence through its effects on phagocytes. Intravital imaging revealed bacterial interactions with multiple blood cell types. Neutrophils and macrophages rapidly phagocytosed and killed P. aeruginosa, suggesting that both cell types play a role in protection against infection. Intravascular aggregation of erythrocytes and other blood cells with resultant circulatory blockage was observed immediately upon infection, which may be relevant to the pathogenesis of thrombotic complications of human P. aeruginosa infections. The real-time visualization capabilities and genetic tractability of the zebrafish infection model should enable elucidation of molecular and cellular details of P. aeruginosa pathogenesis in conditions associated with neutropenia or impaired phagocyte function.

Introduction

Pseudomonas aeruginosa (PA) is a ubiquitous Gram-negative bacterium that can infect a wide variety of plants and animals. It is an important opportunistic pathogen in humans, producing serious infections that can be localized or systemic depending on the clinical setting. Localized infections include keratitis, otitis externa, and most notably, chronic lung infection in patients with cystic fibrosis, in which defective clearance of airway secretions and concomitant impairment of host phagocyte function creates a permissive environmental niche (Lyczak et al., 2000; Knowles and Boucher, 2002; Matsu et al., 2005). In contrast, acute systemic infection occurs principally in neutropenic hosts undergoing chemotherapy and in those with serious burns (Lyczak et al., 2000).

Because PA produces diverse disease, multiple animal models are useful to elucidate the factors and mechanisms of pathogenesis relevant to specific clinical settings. Murine models include corneal infection, burn wound infection, and acute pneumonia and sepsis (Stevens et al., 1994; Tang et al., 1995; Lee et al., 2005; Vance et al., 2005; Zolfaghar et al., 2006). Genetically tractable invertebrate models such as Caenorhabditis elegans (roundworm) and Drosophila melanogaster (fruit fly), as well as the plant model Arabidopsis thaliana (thale cress), have also been developed to study PA virulence.

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D. Embryo survival following infection with GFP-labelled strain PAK. Groups of embryos (n = 15) were inoculated with a low dose (~75 cfu per embryo, small squares) or high dose (~2400 cfu per embryo, large squares) of bacteria at 50–52 hpf and monitored daily for survival over 4 days. Uninfected controls are shown as open circles. Roman numerals (i through v) correspond to photomicrographs (B) of representative embryos. There was an overall significant effect of inoculum size on mean survival during the first 4 dpi (P < 0.0001). Pairwise comparisons showed no significant difference in mean survival for the 200 cfu group relative to the uninfected control group, but significant differences (indicated by *) in mean survival for the 800 and 2400 cfu groups relative to control (adjusted P-values of P = 1.0, P < 0.0001 and P < 0.0001 respectively).

E. Enumeration of bacteria in PAO1-infected embryos. In experiments separate from the survival curves shown above, groups of embryos (n = 45 each) were inoculated with a low dose (~75 cfu per embryo, small squares) or high dose (~2400 cfu per embryo, large squares) of PAO1 at 50–52 hpf, then sorted into subpools for enumeration (n = 25 each) and monitoring of survival (n = 20 each). Error bars indicate standard deviation of cfu per embryo.

Fig. 1. Effect of PA inoculum size on host survival and bacterial growth in zebrafish embryos. Embryo survival and bacterial enumeration experiments were repeated at least three times; representative results are shown.

A. Diagram of a zebrafish embryo at 48 hpf. Injection site (at the axial vein near the urogenital opening) is as indicated. Scale bar, 300 μm.

B. Photomicrographs of embryos infected with GFP-labelled strain PAO1. Representative embryos that had been inoculated with a low dose (i, small square), intermediate dose (ii, medium square) or high dose (iii, large square) of this bacterial strain and imaged at 2 hpi are shown. A representative embryo that received the intermediate dose was highly infected at 1 dpi (v, medium square); it died by 2 dpi. With one exception, embryos receiving the low or intermediate dose that survived to 2 dpi had cleared the infection (iv, small square) and were indistinguishable from each other and the controls. Scale same as for A.

C. Embryo survival following infection with GFP-labelled strain PAO1. Groups of embryos (n = 20 each) were inoculated with a low dose (~200 cfu per embryo, small squares), an intermediate dose (~800 cfu per embryo, medium squares), or a high dose (~2400 cfu per embryo, large squares) of bacteria at 50–52 hpf and monitored daily for survival over 4 days. Uninfected controls are shown as open circles. Roman numerals (i through v) correspond to photomicrographs of representative embryos. There was an overall significant effect of inoculum size on mean survival during the first 4 dpi (P < 0.0001). Pairwise comparisons showed no significant difference in mean survival for the 200 cfu group relative to the uninfected control group, but significant differences (indicated by *) in mean survival for the 800 and 2400 cfu groups relative to control (adjusted P-values of P = 1.0, P < 0.0001 and P < 0.0001 respectively).

Factors and pathogenesis (Darby et al., 1999; Mahajan-Miklos et al., 1999; Rahme et al., 2000; D’Argenio et al., 2001; Fauvarque et al., 2002; Lutter et al., 2008).

While several PA factors are required for virulence across these diverse models (Rahme et al., 2000), others appear to be important only in specific models and clinical contexts. One example is the PA Type III secretion system (T3SS), which translocates exotoxins into host cell cytoplasm and is associated with poor clinical outcomes in acute systemic infection and pneumonia (Roy-Burman et al., 2001; Hauser et al., 2002; Lee et al., 2005). The T3SS is a key virulence factor in Galleria mellonella (greater wax moth), fruit fly, and mouse, but not in roundworms or thale cress (Fauvarque et al., 2002; Miyata et al., 2003; Laskowski et al., 2004; Lee et al., 2005; Vance et al., 2005).

Danio rerio (zebrafish), perhaps best known as a model for investigating the cellular and genetic mechanisms of vertebrate development, is rapidly gaining favour as a model for the study of host–bacterial interactions (Davis et al., 2002; Neely et al., 2002; van der Sar et al., 2003; Pressley et al., 2005; Bates et al., 2006; Rawls et al., 2006; Lin et al., 2007; Clay et al., 2008; Prajsnar et al., 2008). Its genetic tractability and optical transparency early in development make it useful for studying aspects of infectious diseases not accessible in more traditional models. Moreover, while the adult zebrafish has a complex immune system similar to that of humans, with both innate and adaptive arms (Traver et al., 2003), at early developmental stages only innate immunity is operant, allowing for the dissection of innate and adaptive immune responses (Davis et al., 2002; Clay et al., 2007; 2008). Studies of zebrafish development have demonstrated that neutrophils and macrophages are present early in development (Herbomel et al., 1999; Traver et al., 2003; Le Guyader et al., 2008), and have shown the ability of these cells to phagocytose both Gram-negative and Gram-positive bacteria (Neely et al., 2002; van der Sar et al., 2003; Pressley et al., 2005; Lin et al., 2007; Prajsnar et al., 2008).

In this study, we developed the zebrafish embryo as a model for the study of systemic PA infection, and examined the virulence of a PA T3SS mutant in this model. We used differential interference contrast (DIC) and fluorescence microscopy to monitor PA infection in real time in transgenic zebrafish lines with fluorescent macrophages and neutrophils (Mathias et al., 2006; Hall et al., 2007). Finally, we defined the effects of phagocyte depletion on embryos during subsequent infection with wild-type and T3SS-deficient PA strains. Our results show that bacterial T3SS–phagocyte interactions are critical determinants of PA pathogenesis in this model, and suggest that macrophages as well as neutrophils provide protection against systemic PA infection.

Results

Zebrafish embryos are relatively resistant to intravenously injected PA

To determine the effects of introducing PA into the bloodstream, we microinjected a range of doses of two green fluorescent protein (GFP)-expressing laboratory strains (PAO1 and PAK) into the caudal vein of zebrafish embryos (Fig. 1A) at 50–52 h post fertilization (hpf), and assessed infected embryos for survival, and for bacterial
burdens via serial fluorescence microscopy and quantitative plating (Davis et al., 2002; van der Sar et al., 2003; Volkman et al., 2004). The virulence of these PA strains has been demonstrated in other animal models of acute infection (Tang et al., 1995; Lutter et al., 2008). At 2 h post infection (hpi) the embryos displayed bacterial burdens that were proportional to the initial inoculum (Fig. 1B, i–iii). Embryos were resistant to 150–200 colony-forming units (cfu) of either strain; bacteria were invariably cleared within 2 days (Fig. 1B, iv) with no embryo mortality (Fig. 1C and D; the sole 200-cfu-injected embryo that died in Fig. 1C contained no fluorescent bacteria, suggesting that its death was not a direct consequence of PA infection). Dose-dependent mortality was observed with larger inocula (~800–2400 cfu per embryo) of either strain (Fig. 1C and D). While 2400 cfu of PAO1 was uniformly lethal by the first day post infection (dpi) (Fig. 1C), injection with an equivalent amount of heat-killed PAO1 produced no mortality (data not shown), indicating that live PA or heat-labile bacterial products, rather than heat-
stable products such as endotoxin, mediated this effect. Infected embryos that survived the 4 day observation period continued to develop normally thereafter and cleared the infection (data not shown). Thus, zebrafish embryo survival following infection with PA reflected a binary outcome at the individual embryo level (i.e. survival with bacterial clearance or death with rampant bacteriæmia) that was superimposed on the graded dose-dependent mortality observed at the population level.

Embryos infected with 75 cfu of the PAO1 strain cleared 75% of the inoculum within 15 min and continued to clear the remaining bacteria, albeit at a slower rate, during the 8 h observation period (Fig. 1E), confirming the microscopic analysis of fluorescent bacteria (Fig. 1B, i, and data not shown). In contrast, embryos infected with 2400 cfu of PAO1 supported rapid bacterial growth (a 19-fold increase in cfu per embryo over 8 h; Fig. 1E), consistent with their increased mortality (Fig. 1C).

The PAK strain, when inoculated at 2200 cfu per embryo, also expanded rapidly (fourfold increase at 8 h) (Fig. 2A). However, in contrast to PAO1, this strain was cleared rapidly in the first 2 h (78% of the inoculum of ~2200 bacteria) before achieving rapid growth between 4 and 8 hpi (doubling time, ~60 min, compared with ~40 min in log-phase nutrient broth culture), reflecting a 25-fold increase in cfu per embryo over this 4 h interval (Fig. 2A). The basis for the apparent differences in the initial clearance and subsequent growth of these PA strains, which were not compared within the same experiment, is not known.

These experiments show that zebrafish embryos can consistently clear PA doses of up to 200 cfu with minimal mortality, depending solely on innate immunity, whereas doses of > 800 cfu result in rapid proliferation of the inoculum and are frequently fatal. While the initial kinetics of growth varied between the two strains examined, the overall growth of the strains within embryos and the resultant host mortality were remarkably consistent.

The PA T3SS is required for virulence in zebrafish embryos

We next assessed the role of the PA T3SS, a key virulence determinant in acute infection of humans as well as in mammalian infection models (Roy-Burman et al., 2001; Hauser et al., 2002; Laskowski et al., 2004; Lee et al., 2005; Vance et al., 2005), by comparing infection with PA strains that had or lacked the T3SS. The T3SS mutant strain PAKexsA::Ω was cleared rapidly in the first 4 h, identical to the parent PAK strain (Fig. 2A). However, its growth rate thereafter was quite different, increasing only 2.5-fold between 4 and 8 h, compared with the 25-fold increase seen with the parent strain (Fig. 2A). Consistent with these reduced bacterial burdens, 90% of embryos infected with 2400 cfu of PAKexsA::Ω were alive at 4 dpi, compared with survival at 1 dpi of only 30% of embryos infected with 2200 cfu of wild-type PAK (Fig. 2B). In a separate experiment in which embryos were initially infected with ~2600 cfu of PAKexsA::Ω per embryo, the 95% of survivors at 2 dpi had nearly cleared the infection.

Fig. 2. Infection of zebrafish embryos with PAKexsA::Ω, a T3SS mutant. Embryo survival and bacterial enumeration experiments were repeated at least three times; representative results are shown. A. Enumeration of bacteria in PAK- and PAKexsA::Ω-infected embryos at 0.25–8 hpi. Two groups of embryos (n = 45 each) were inoculated with PAK (~2200 cfu per embryo, solid diamonds) or PAKexsA::Ω (~2500 cfu per embryo, open diamonds) at 50–52 hpf, then sorted into subpools for enumeration (n = 25 each) and monitoring of survival (n = 20 each). Note the initial rapid drop in cfu of PAK and PAKexsA::Ω. Error bars indicate standard deviation of cfu per embryo. B. Attenuated virulence of a PAKexsA::Ω T3SS mutant. In a separate experiment, groups of 20 embryos were inoculated with a high dose of PAO1 (wild-type strain; ~2200 cfu per embryo) or PAKexsA::Ω (T3SS mutant; ~2400 cfu per embryo) at 50–52 hpf and monitored for survival over 4 days. There was an overall significant effect of bacterial strain on mean survival during the first 4 dpi (P < 0.0001). Pairwise comparisons showed no significant difference in mean survival for the PAKexsA::Ω group relative to the uninfected control group, but a significant difference in mean survival for the wild-type PAK group relative to control (adjusted P-values of P = 1.0 and P < 0.0001 respectively).

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M. K. Brannon et al.
with residual bacterial burdens of only 19 ± 5 cfu per embryo. These experiments showed that the PA T3SS is a critical virulence determinant in the zebrafish embryo model impacting bacterial burdens and host survival.

Pseudomonas aeruginosa infection causes blood cell aggregation and circulatory blockage

In microscopically monitoring live infected zebrafish, we observed that in contrast to Mycobacterium marinum and Salmonella arizonae (Davis et al., 2002), PA infection resulted in immediate accumulation of bacteria near the site of injection (Fig. 1B, ii). Bacterial aggregates were also observed at sites adjacent to the caudal artery and vein (Fig. 3A and B). Detailed microscopy revealed that these aggregates contained both bacteria and blood cells (Fig. 3B–E). Among the cells present were erythrocytes and occasional infected phagocytes that completely occluded blood flow in the vessel (Fig. 3B and C; Movie S1). Although the duration of this phenomenon was dependent on the dose of injected PA, with aggregation typically resolved within minutes following low doses of PA but minimally if at all following higher doses, cellular aggregates also formed at anatomic sites with fewer bacteria, and appeared to result from adhesions between blood cells and the vascular endothelium (Fig. 3D and Movie S2). This cellular aggregation was observed upon injection of heat-killed wild-type PA as well as live PAKexsA::Ω, but not following mock injections (data not shown).

Both neutrophils and macrophages rapidly phagocytose and kill PA

Our initial results suggested that zebrafish embryos are similar to humans in their capacity to clear systemic PA infection solely through innate immunity. In humans and other mammals, neutrophils are known to provide critical protection against PA infection, although macrophages may also be involved (Tang et al., 1995; Cheung et al., 2000; Lyczak et al., 2000; McClellan et al., 2003). The zebrafish embryo has functional neutrophils and macrophages (Herbomel et al., 1999; Clay et al., 2007; 2008; Le Guyader et al., 2008), and DIC microscopy suggested that some bacteria could be phagocytosed early in infection by macrophages as judged by their distinctive morphological characteristics (Fig. 3C and Movie S1) (Le Guyader et al., 2008). To identify types of phagocytes interacting with PA, we took advantage of two transgenic zebrafish lines that label neutrophils. In the Tg(lyz:EGFP)nz117 line, the lysozyme (lyz) gene promoter drives expression of enhanced GFP in neutrophils and macrophages early in development (Hall et al., 2007), but is expressed almost exclusively in neutrophils starting at 48 hpf (C. Hall, J. Davis, L.

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Ramakrishnan and P. Crosier, unpubl. results). Similarly, in the *Tg(mpx:GFP)uwm1* line, the myeloperoxidase (*mpx*) gene promoter drives neutrophil-specific expression of enhanced GFP (Mathias *et al.*, 2006).

We injected these green fluorescent transgenic strains with red fluorescent protein (RFP)-expressing strains of *PA*. *Tg(lyz:EGFP)*-expressing cells were seen to phagocytose a substantial proportion of the bacteria within 2 h of infection (Fig. 4A and B, and data not shown). Similarly, using embryos of the *Tg(mpx:GFP)uwm1* zebrafish line, we observed colocalization of bacteria with GFP-bright phagocytes (neutrophils) and with non-fluorescent phagocytes (Fig. 4C and Movie S3); the latter were confirmed as macrophages by morphology based on motility, phagocytic capacity, and lack of cytoplasmic granules (Herbomel *et al.*, 1999; Davis *et al.*, 2002). At 2 hpi, most bacteria were intracellular and had been phagocytosed by neutrophils and macrophages (Fig. 4D and E; Movie S3).
Moreover, a substantial number of bacteria had been degraded even within this short time, as indicated by the presence of red debris without clear bacterial morphology within some phagocytes (Fig. 4B, E and F). Both intact PA and degraded remnants were seen within vacuole-shaped compartments in phagocytes, often corresponding to the DIC appearance of bacteria (Fig. 4B, D–F). We did not observe any differences in the phagocytosis of the PAKexsA::Ω T3SS mutant as compared with the PAK or PAO1 wild-type strains within transgenic zebrafish embryos at 2 hpi (data not shown). These data show that neutrophils and macrophages can rapidly phagocytose and destroy PA.

Phagocyte depletion renders zebrafish embryos hypersusceptible to PA infection and restores the virulence of the attenuated T3SS mutant

The differentiation and growth of macrophages and neutrophils in the developing zebrafish embryo are dependent on the myeloid transcription factor gene pu.1 (Rhodes et al., 2005; Clay et al., 2007). To explore the functional relevance of phagocytes to interactions between PA and host cells, we depleted phagocytes from embryos by injection of a modified antisense oligonucleotide (morpholino) directed against pu.1, creating embryos we refer to as pu.1 morphants. The pu.1 morphants succumbed rapidly to infection when injected with ~383 cfu PAK, with 65% surviving as compared with 100% of control embryos at 1 dpi (Fig. 5A); one infected control embryo (5%) died on the second day. In contrast, similar to previous studies (Clay et al., 2007), 95% of uninfected pu.1 morphant embryos (n = 21) survived the 4-day observation period. Control morpholino-treated embryos and untreated embryos were found to respond identically to infection (data not shown), thus only untreated embryos were used as controls in subsequent experiments. These results show that phagocytes play a critical role in protection of zebrafish embryos against PA infection; however, the relative role of macrophages and neutrophils could not be discerned as both cell types are depleted with this morpholino (Clay et al., 2007).

Given the attenuation of the T3SS mutant in wild-type embryos, we next examined whether this bacterial determinant was involved in the phagocyte–pathogen interaction. We found that whereas ~2400 cfu of PAKexsA::Ω had failed to kill wild-type embryos (Fig. 2B), doses of this strain as small as ~384 cfu per pu.1 morphant embryo caused mortality equivalent to that of wild-type PA; only 65% of pu.1 embryos survived at 1 dpi, as compared with 100% of control embryos (Fig. 5B). The finding that phagocyte depletion restored the virulence of a T3SS mutant suggests that the T3SS acts to protect wild-type PA against phagocytes.

Consistent with this decreased survival of PA-infected pu.1 morphants, by 8 hpi bacterial counts in the PAK-infected pu.1 morphants had increased to 1173% of the original inoculum, while control embryos had essentially cleared the infection, with only 2% of the original inoculum remaining (Fig. 5C). Bacterial counts in the PAKexsA::Ω-infected pu.1 morphants also increased over this interval, but to only 280% of the inoculum, one-fourth that of the wild-type strain. The finding that the attenuated T3SS mutant still exhibited a relative in vivo growth defect after phagocyte depletion could be due to increased killing of the mutant bacteria by the small number of residual neutrophils in the pu.1 morphants (Rhodes et al., 2005; Clay et al., 2007), or because phagocytes are not the sole targets of the PA T3SS.

Discussion

This work suggests that the zebrafish embryo is a relevant and tractable model for the study of systemic PA infections in the neutropenic host, allowing for live examination of bacterial interactions with different blood cell types. PA...
rarely causes systemic infection of humans unless they are neutropenic or have had a substantial integumentary breach as in the case of severe burns (Lyczak et al., 2000). Similarly, we have found the zebrafish embryo to be remarkably resistant to PA infection, requiring >2000 cfu of intravenously injected bacteria to produce sustained infection and consistent mortality. This stands in sharp contrast to the low infectious inoculum of <10 cfu of M. marinum or S. arizonae in this model (Davis et al., 2002; van der Sar et al., 2003). Yet PA is relatively pathogenic when compared with non-pathogenic laboratory strains of bacteria such as Escherichia coli K12 or Bacillus subtilis, which are rapidly eradicated by zebrafish embryos (Herbomel et al., 1999). Together, these data indicate that zebrafish embryos possess effective defence mechanisms against systemic PA infection that depend solely on innate immunity. This modest degree of virulence in the zebrafish embryo model is consistent with the reputation of PA as an opportunistic pathogen of humans (Lyczak et al., 2000). Further validation of this model comes from our finding that key host (phagocyte) and bacterial (T3SS) determinants in systemic PA infection of humans (Lyczak et al., 2000; Roy-Burman et al., 2001; Hauser et al., 2002) also play essential roles in zebrafish pathogenesis. Genetic ablation of the bacterial T3SS attenuated bacterial virulence, while phagocyte depletion rendered this host more susceptible to PA infection. Importantly, this work has
directly demonstrated the central role of the bacterial T3SS–phagocyte interaction in determining the outcome of PA infection in vivo, as has been previously suggested through in vitro studies (Coburn and Frank, 1999; Dacheux et al., 2000; Vance et al., 2005). The first effect of Type III secretion in this infection model was observed between 4 and 8 hpi, when the PAK strain (but not a T3SS mutant) began to grow rapidly despite an initial phase of bacterial clearance. Our data suggest that if the initial inoculum exceeds the phagocytic capacity of the host such that sufficient numbers of bacteria survive the initial burst of clearance, induction of T3SS expression in the surviving bacteria results in resistance to phagocytes, bacterial growth and host mortality. This scenario may occur in severe neutropenia when a relatively small inoculum of PA may exceed the capacity of the few phagocytes present and thus initiate an acute systemic infection.

Previous work in a neonatal mouse model of acute pulmonary infection suggested that of the PA strains used in this study, PAK is somewhat more virulent than PAO1 (Tang et al., 1995). In contrast, recent findings in fruit fly models of systemic and intestinal infection suggest that these strains possess similar virulence (Lutter et al., 2008). Our results indicate that both strains are virulent in zebrafish embryos, but do not permit definitive determination of their relative virulence. Both possess three known Type III secreted exotoxins (ExoS, ExoT and ExoY) but lack a fourth exotoxin (ExoU) that confers cytotoxicity (Finck-Barbancon et al., 1997; Ichikawa et al., 2005; Lee et al., 2005; Vance et al., 2005). The biochemical functions and virulence effects of these exotoxins suggest multiple mechanisms for affecting phagocyte function. In addition to their ADP-ribosyltransferase activity, the bifunctional exotoxins ExoS and ExoT are also GTPase-activating proteins that target Rho-like GTPases essential for receptor-mediated phagocytosis; ExoT additionally targets Crkl and Crkll, host kinases involved in cell adhesion and phagocytosis (Caron and Hall, 1998; Goehring et al., 1999; Sun and Barbieri, 2003). The adenylate cyclase activity of ExoY has not been shown to have a significant virulence effect in vivo (Vance et al., 2005); nonetheless, considering that it causes rounding of CHO cells, it could conceivably inhibit phagocyte microbicidal mechanisms (Yahr et al., 1998). Yet, how these effectors act in vivo is not entirely clear, with conflicting data from different animal models and bacterial strains. In combination these exotoxins exert complex synergistic effects on the transcriptional responses of cultured pneumocytes (Ichikawa et al., 2005), but separable and largely nonsynergistic effects on virulence in a mouse model of acute pneumonia (Shaver and Hauser, 2006). Activation of the T3SS and ExoS-dependent inhibition of haemocyte phagocytic function characterizes acute PA infection of fruit fly (Fauvarque et al., 2002; Avet-Rochex et al., 2005); in contrast, inhibition of phagocytosis during in vitro PA infection of a murine macrophage-like cell line is ExoT-dependent (Garrity-Ryan et al., 2000). Moreover, the PAO1 strain, the T3SS of which was not tested in the present study, is known to secrete much less ExoS protein in vitro than does the PAK strain (Frank et al., 1994), potentially explaining observations indicating that the T3SS-mediated virulence of these strains depend on different combinations of effectors (Lee et al., 2005; Vance et al., 2005).

However, PA strains lacking ExoS, ExoU, or even all known Type III effector proteins can kill murine macrophages, with lysis requiring only the presence of a functional Type III translocase (Coburn and Frank, 1999; Dacheux et al., 2000; Vance et al., 2005). Type III effectors are important for systemic spread and survival in blood in a cyclophosphamide-induced leukopenic mouse model of competitive PA infection, but host defences can nonetheless clear PA T3SS mutants from the blood of these mice even after use of a neutrophil-specific monoclonal antibody to induce absolute neutropenia (Vance et al., 2005). In contrast, we observed that depletion of phagocytes in zebrafish embryos markedly hindered the clearance of a PA T3SS mutant. The distinct behaviour of PA T3SS mutants in these models may be attributable to residual macrophages or lymphocytes in the neutropenic mouse. The dramatic dependence of PA on the T3SS to overcome normal phagocyte defences in the absence of adaptive immunity suggests that the zebrafish may be a useful and relevant model to understand the details of T3SS–phagocyte interactions.

A central advantage of the zebrafish embryo model is the ability to monitor infection at a detailed cellular level in real time. Using detailed microscopy of infected embryos, we have made two potentially important observations regarding pathogenesis. First, we confirmed previous observations that both neutrophils and macrophages are capable of phagocytosing and killing of PA (Tang et al., 1995). Indeed, the numbers of each cell type seen to interact with bacteria in this model seemed roughly proportional to their numbers in the caudal haematopoietic tissue, close to the injection site (Murayama et al., 2006). Thus, the relative role of the two cell types in PA phagocytosis and killing may simply be a function of their relative numbers. The greater prominence of neutrophils in acute bactericidal responses in humans may largely reflect their greater abundance in the circulation and more rapid accumulation at sites of acute inflammation (Stossel and Babior, 2003).

Alternatively, macrophages may play a more general role in phagocytosis in zebrafish embryos than in adult mammals. However, this seems unlikely given that embryonic and adult macrophages are similar with respect to production of cytokines, cell surface receptors, and the
inducible isoform of nitric oxide synthetase (Herbomel et al., 1999; Clay et al., 2007; 2008). Also, zebrafish embryonic macrophages have distinct interactions with different pathogens that parallel those of mammalian macrophages. For example, embryonic macrophages challenged with \textit{M. marinum} form epithelioid granulomas, but are most likely to undergo pyroptosis upon phagocytosis of \textit{S. arizonae} (Davis et al., 2002; Fink and Cookson, 2005; Clay et al., 2007). These responses are clearly distinct from the pattern of phagocytosis and digestion that occurs in response to \textit{PA}, and all three pathogen-specific responses are similar to the corresponding macrophage–pathogen interactions observed in adult mammals, suggesting that embryonic and adult macrophages function similarly across a wide range of vertebrate organisms.

Our finding that embryonic zebrafish neutrophils can phagocytose and kill \textit{PA} suggests that they are functionally competent even at this early developmental stage. Recent studies examining interactions of embryonic neutrophils with bacteria have shown that while they are capable of chemotactic attraction to infection sites, they do not phagocytose or kill non-pathogenic \textit{E. coli} as efficiently as macrophages do (Le Guyader et al., 2008), and do not associate with mycobacteria (Clay et al., 2007). However, in zebrafish embryos as in adults, these phagocytic functions are pathogen-specific, because in addition to our observations related to \textit{PA}, others have recently shown that both embryonic neutrophils and macrophages efficiently phagocytose \textit{Staphylococcus aureus} in an acute systemic infection model (Prajsnar et al., 2008). As with \textit{PA}, resistance of zebrafish embryos to \textit{S. aureus} is myeloid cell dependent, because phagocyte depletion with \textit{pu.1} morpholino renders embryos much more susceptible to systemic infection with \textit{S. aureus} wild-type strains and restores the virulence of attenuated mutants of this opportunistic pathogen (Prajsnar et al., 2008).

Our real-time observations of \textit{PA}-infected zebrafish embryos also revealed an erythroid aggregation phenomenon that appears to be transient at lower doses. We have not observed this phenomenon in either \textit{S. arizonae} or \textit{M. marinum} infection, suggesting that it is specific to certain bacteria, including \textit{PA}. The consistent occurrence of this phenomenon intrigued us, because thrombotic complications are associated with \textit{PA} infections in humans (Gupta et al., 1993). While these cellular aggregates are comprised principally of erythrocytes and pro-erythroblasts, we also noted the presence of phagocytes within them. However, these aggregates formed even in \textit{pu.1}-depleted embryos (data not shown), suggesting that phagocytes may be bystander cells trapped within what is fundamentally a \textit{PA}–erythrocyte interaction. Several features of these cellular aggregates are noteworthy. While they are entirely infection-dependent, the number of bacteria present within any given aggregate may be minimal. Second, they occur even in the absence of the T3SS and can be induced by heat-killed bacteria, suggesting that they are not the result of the heat-labile \textit{PA} phospholipase C that has been reported to induce platelet aggregation \textit{in vitro} (Coutinho et al., 1988). Host and bacterial mutational analyses similar to ones that we performed to investigate T3SS–phagocyte interactions in this model could determine what role, if any, these cellular aggregates play in pathogenesis.

Various animal and plant models have been used to study host–pathogen interactions in acute \textit{PA} infection. Interestingly, the T3SS is an important virulence factor of \textit{PA} in hosts that possess professional phagocytes, such as vertebrates and insects, but not in those that lack such cells, such as worms and plants (Fauvarque et al., 2002; Miyata et al., 2003; Laskowski et al., 2004; Lee et al., 2005; Vance et al., 2005; Alper et al., 2007). The fruit fly has been used to examine the role of T3SS in lethal \textit{PA} infection and modulation of haemocyte function (D’Argenio et al., 2001; Fauvarque et al., 2002; Avet-Rochex et al., 2005), and the greater wax moth caterpillar has similarly been used to demonstrate redundancy of ExoT and ExoU and dispensability of ExoY with respect to virulence in this host (Miyata et al., 2003). Advantages of these and other invertebrate models are the feasibility of using large numbers of animals for each test condition, the relative ease of infection, and well-established techniques for genetic manipulation and screening to identify host defence factors. However, these invertebrates have relatively rudimentary innate immune systems that rely heavily on antimicrobial peptide expression as controlled through Toll-like receptor-dependent and -independent signalling cascades (TANJI and Ip, 2005). Given the fundamental nature of such innate immune mediators and the presence of homologues in fish and mammals (Barton and Medzhitov, 2002; van der Sar et al., 2006), invertebrate models can provide important insights into host–pathogen interactions in vertebrates, but the zebrafish embryo model of innate immune interactions with \textit{PA} should provide more accurate simulation of human infection.

In summary, we have developed the zebrafish as a valid and tractable model of acute \textit{PA} infection. This model provides useful tools for exploring the detailed interactions of \textit{PA} with the vertebrate immune system. Experimental tools that are becoming available for zebrafish will further enhance the direct visualization and detailed analysis of innate immune responses to \textit{PA} infection that this model enables. Because the zebrafish is genetically tractable, screens for mutants with aberrant responses to \textit{PA} infection should be feasible. Also, the amenability of zebrafish to small molecule screens should allow for discovery of drugs with anti-pseudomonal activity in the
context of a whole vertebrate animal (Peterson et al., 2000). On the pathogen side, this model should enable investigators to define additional factors that influence PA virulence through the use of PA clinical isolates and mutant libraries. The use of PA strains containing translational fusions of Type III effectors or transcriptional fusions of specific regulatory sequences to fluorescent protein genes (Pederson et al., 2002) should also enable investigators to define cellular targets of Type III secretion and anatomic regions of regulated PA gene expression within the host. Such experimental approaches are expected to provide a richly detailed picture of host–pathogen interactions in vertebrate PA infection.

Experimental procedures

Bacterial strains and culture methods

*Pseudomonas aeruginosa* strains used in this study were PAO1 (Ochsner laboratory strain), kindly provided by E. P. Greenberg, University of Washington, PAK and PAKexsA::T, both kindly provided by D. Frank, Michigan State University (Frank et al., 1994). To confer constitutive expression of GFP or RFP, each strain was transformed with plasmid pMF230, containing the GFPmut2 gene and a carbenicillin-resistance marker, kindly provided by M. Franklin, Montana State University, Bozeman (Nivens et al., 2001), or plasmid pMKB::mCherry, a derivative of pMF230 constructed by replacing the GFPmut2 gene with the mCherry RFP gene, kindly provided by R. Tsien, University of California San Diego (Shu et al., 2006). To obtain log-phase bacteria for injection, single colonies of each strain were inoculated into Luria–Bertani (LB) broth supplemented with 200 mg l–1 carbenicillin, grown overnight at 37°C, subcultured 1:100 in the same medium, and grown at 37°C to an optical density reading at 600 nm of 0.6–0.7. To prepare the final inoculum, 1 ml of cultured bacteria was pelleted by centrifugation at 1500 g for 5 min, resuspended in 0.4 ml of 1× phosphate-buffered saline (PBS), then diluted in additional 1× PBS as needed to achieve the desired bacterial density. To heat-kill bacteria, 20 µl aliquots were incubated in a 50°C water bath for 30 min. Phenol red tracking dye (5% solution) was added to bacterial aliquots (1:20 v/v) prior to injection. To enumerate cfu in the inoculum before, during and after microinjection of each set of embryos, aliquots of the inoculum were spread on LB-agar plates containing 200 mg l–1 carbenicillin and incubated overnight at 37°C.

Maintenance, manipulation and infection of zebrafish embryos

Zebrafish were maintained and handled as described (Davis et al., 2002; Volkman et al., 2004). Animal protocols for this study were compliant with laboratory standards outlined by the University of Washington Institutional Animal Care and Use Committee. Zebrafish embryos used in these experiments included wild-type strain AB, as well as strain AB carrying the myelomonocyte-specific transgenic marker Tg(lyz:EGFP) as a transcriptional fusion (Hall et al., 2007), and strain AB carrying the neutrophil-specific transgenic marker Tg(mpx::GFP) as a transcriptional fusion (Mathias et al., 2006). Embryos were harvested at 3 hpf and incubated overnight in fish water containing 0.01% methylene blue. At 24 hpf, embryos were dechorionated and sorted in fish water containing 0.003% phenylthiourea to prevent melanization. At 50–52 hpf, groups of 15–20 embryos were anaesthetized in 0.1% 3-aminobenzoic acid ethyl ester (tricaine), placed in a depression slide, and microinjected into the axial vein near the urogenital opening. For morpholino experiments, zygotes at the 1–2 cell stage were treated with pu.1 or control morpholino using previously described concentrations and methods (Rhodes et al., 2005; Clay et al., 2007). Morpholino-treated embryos were then infected as described above.

Microscopy of embryos

Microscopy was performed on a Nikon E600 (Tokyo, Japan) equipped with DIC optics, a Nikon D-FL-E fluorescence unit with a 100 watt mercury lamp, and an MFC-1000 2-step controller (Applied Scientific Instrumentation, Eugene, OR). Objectives used included 4× Plan Fluor (0.13 NA), 10× Plan Fluor (0.3 NA), 20× Plan Fluor (0.5 NA), 40× Plan Fluor (0.75 NA), and 60× Water Fluor (1.0 NA). Wide-field fluorescence and DIC images were captured on a Photometrics CoolSnap HQ CCD camera (Roper Scientific, Trenton, NJ) using MetaMorph 7.1 image acquisition software (Molecular Devices Corporation, Sunnyvale, CA).

Image processing

Where indicated, z-stacks were deconvolved using AutoDeblur Gold CWF, Version X1.4.1 (Media Cybernetics, Bethesda, MD), with default settings for blind deconvolution. Dataset analysis and visualization were performed using MetaMorph 7.1 and Imaris x64 6.0 (Bitplane, Zurich, Switzerland). Figure processing and assembly was performed using MetaMorph 7.1 and Adobe Photoshop CS2.

Determination of whole embryo bacterial counts

Infected embryos were randomly assigned to one of two subpools: an enumeration subpool and a survival monitoring subpool. At each time point from 0 to 48 hpi, groups of five embryos were randomly removed from the enumeration subpool, rinsed in 1× PBS, anaesthetized in tricaine, placed in 1.5 ml centrifuge tubes containing 100 µl of 1× PBS with 1% Triton X-100, and homogenized together for 1–2 min with a sterile micropipette. Image processing was performed on a Nikon E600 (Tokyo, Japan), equipped with DIC optics, a Nikon D-FL-E fluorescence unit with a 100 watt mercury lamp, and an MFC-1000 2-step controller (Applied Scientific Instrumentation, Eugene, OR). Objectives used included 4× Plan Fluor (0.13 NA), 10× Plan Fluor (0.3 NA), 20× Plan Fluor (0.5 NA), 40× Plan Fluor (0.75 NA), and 60× Water Fluor (1.0 NA). Wide-field fluorescence and DIC images were captured on a Photometrics CoolSnap HQ CCD camera (Roper Scientific, Trenton, NJ) using MetaMorph 7.1 image acquisition software (Molecular Devices Corporation, Sunnyvale, CA).

Statistical methods

For selected experiments, groups of zebrafish embryos were compared with respect to mean number of days alive, restricted to the first 4 dpi. In instances where subsequent pairwise comparisons between experimental and control groups were performed, the resulting P-values were adjusted for multiple com-
parison testing using the Bonferroni method. For all analyses, embryos that were alive on a given day but no longer alive the following day were assumed to have died at the midpoint between days (e.g. embryos alive on day 2 but no longer alive on day 3 were assigned a value of 2.5 for number of days alive).

Acknowledgements

The authors would like to thank Hilary Clay for first finding the susceptibility of pu.1 morphants to PA, David Tobin, Hannah Volkman, and Heather Wiedenhoft for help and advice with fish handling and microscopy, and Jane Burns for helpful comments on the manuscript. This work was supported by grants from the National Institutes of Health to S.M.M. (R01 AI067653), L.R. (R01 AI054503) and A.H. (R01 GM074827), a Burroughs Wellcome Fund Award to L.R., a National Defense Science and Engineering Graduate Fellowship to J.M.D., and a grant from the New Economy Research Fund (Foundation for Research Science and Technology, New Zealand) to P.S.C.

References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Movie S1. Infected macrophage within the clogged caudal artery, corresponding to Fig. 3C. A PA-infected macrophage (centre) moves within the caudal artery towards an infected cellular aggregate (left) that is clogging the vessel. Scale bar, 20 μm.

Movie S2. Cellular aggregate within the yolk circulation valley, corresponding to Fig. 3D. An aggregation of erythroid cells and occasional macrophages remains in place while blood flow continues adjacent to it. Scale bar, 20 μm.

Movie S3. Uptake of PA by Tg(mpx:GFP)-positive neutrophils shortly after infection, corresponding to Fig. 4C. In this three-dimensional perspective, mpx expression is shown in green. Extracellular bacteria are rendered in white, bacteria inside GFP-bright cells (neutrophils) are pink, and bacteria inside GFP-dim cells (macrophages) are red. Scale grid, 10 μm.

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