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The intestinal environment of surgical injury transforms *Pseudomonas aeruginosa* into a discrete hypervirulent morphotype capable of causing lethal peritonitis

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Abstract

BACKGROUND—Secondary peritonitis continues to carry a high mortality rate despite aggressive use of imaging, drainage and antibiotics. Although host factors and microbial burden contribute to the outcome of peritonitis, here we propose a role bacterial virulence as a determinant of outcome from peritonitis. Bacterial virulence is an inducible trait that is activated in response to specific local “cues” that we have previously shown to be present in the mouse gut exposed to surgical stress and injury.

METHODS—*Pseudomonas aeruginosa* was harvested following its intestinal inoculation into the cecum of mice subjected to surgical injury (30% hepatectomy) or sham surgery (controls). Harvested strains were then injected into the peritoneum of non-injured (naïve) mice and mortality determined.

RESULTS—*P. aeruginosa* harvested from the intestines of surgically injured mice caused 100% mortality whereas strains harvested from control mice caused no mortality. Among recovered strains a distinct *P. aeruginosa* morphotype (wrinkled shape) was demonstrated to cause lethal peritonitis compared to smooth shaped strains which were non-lethal. Wrinkled strains were associated with a tendency to elicit a more pro inflammatory response in mice compared to smooth shaped strains.

CONCLUSIONS—Surgical injury transforms the morphotype of intestinal *P. aeruginosa* to express a hypervirulent response in the peritoneum of mice. Enhanced virulence of intestinal pathogens in response to surgical injury may play an important role in predicting the outcome of peritonitis.

INTRODUCTION

Recent large cohort studies comparing infection rates among surgical and non-surgical patients demonstrate that not only are infections more frequent among surgical patients, but

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they are also more lethal¹². Even when controlling for co-morbid conditions, surgical patients experience more frequent and severe infections despite being healthier than their comparative non-surgical cohorts². This conclusion remains statistically valid even when surgical site infections are eliminated from the comparison and suggests that surgical injury itself might shift the dynamics of the host pathogen interaction resulting in a greater degree of infectious related morbidity and mortality. Although current paradigms of the causes of surgical infection posit that infection develops merely as a result of the permissive effects of a dysfunctional immune system unable to contain or clear an invading pathogen, there is now increasing evidence that the ability of microbial organisms to dynamically sense host stress signals and respond with enhanced virulence governs the occurrence, course, and outcome of infection³⁻⁸. While this view does not dismiss the important role of the immune system, it raises the possibility that surgical patients may harbor more virulent strains of pathogenic bacteria because of the selective pressures imposed by the surgical injury itself and the ability of bacteria to sense host stress change their phenotype or genotype. Once bacteria are exposed to the key activating environmental “cues” released during host injury, they can process this information and respond in a context dependent manner. As most host injury is short lived (i.e. elective surgery, rapid return of bowel function and normal oral intake), a return to homeostasis is the norm and usually ends in a type of molecular détente between the host and its newly acquired pathogenic flora. Yet for patients colonized by certain pathogens whose virulence is highly inducible, prolonged and severe surgical injury can lead to the release of host factors and environmental cues that trigger these otherwise dormant colonizers to become invasive and lethal pathogens. We have modeled such events with the human opportunistic pathogen *Pseudomonas aeruginosa* and determined that it is *in vivo* transformed to express enhanced virulence in response to host tissue factors released during surgical injury such as interferon gamma⁵, opioids⁹, and end-products of ischemia¹⁰ (i.e. adenosine) via direct interactions with its quorum sensing system. Yet whether *P. aeruginosa* can express a phenotype to a degree sufficient to cause mortality in a non-injured and presumably immunocompetent host, has not yet been demonstrated. Therefore the aims of the present study were to determine if exposure of intestinal *P. aeruginosa* to surgical injury (30% hepatectomy) results in its transformation to a more pro-inflammatory and lethal phenotype when it is cross transferred into the peritoneal compartment of separate groups of untreated (naïve) mice.

MATERIAL AND METHODS

Bacterial Strains and Inoculum Preparation

P. aeruginosa wild-type strain MPAO1 was obtained from the *P. aeruginosa* mutant library¹¹ and was routinely grown overnight before all experiments in tryptic soy broth (TSB), a phosphate-rich media containing 45 mmol/L phosphate. A single colony was selected and suspended in liquid TSB media overnight (12-14 hours). 200 µL of the bacterial suspension was centrifuged at 5,000 RPM for 5 minutes, the excess TSB removed, and the remaining pellet suspended in sterile 10% glycerol to a final optical density at 600 nm of 0.20, corresponding to 10⁷ CFU/ml. This suspension was immediately placed on ice; bacteria were used within 2 hours of preparation.

Mouse model of surgical injury and cross transfer of intestinal *Pseudomonas aeruginosa* into the peritoneum of sham operated mice

All experiments were approved by the Animal Care and use Committee at the University of Chicago (IACUC Protocol 72090). Specific pathogen free C57B/L6 mice weighing 18 to 22g and 6-8 weeks old were used for all experiments. Mice were routinely fed tap water and Harland Teklad feed under 12 hour light/dark cycles and were allowed to acclimate for at least 48 hours prior to surgery. To test *in-vivo* virulence transformation of *P. aeruginosa*,

mice were sterilely draped and underwent either sham laparotomy or 30% hepatectomy immediately followed by inoculation of 2×10^6 CFU of *P. aeruginosa* into the cecum via injection into the terminal ileum (Intestinal inoculation). This model was developed in our lab and has been repeatedly reported to result in sepsis and high mortality (60-100%) in mice following hepatectomy and no mortality and healthy appearing mice in sham operated mice at 48 hours. Partial (30%) hepatectomy is associated with *in vivo* virulence activation of *P. aeruginosa* in the cecum of mice but not in the cecum of sham operated mice. After 24 hours, cecal contents were collected from both groups of mice and plated on *Pseudomonas* isolation agar (PIA) to isolate *P. aeruginosa* from the stool of each group. After overnight culture, 2×10^6 CFU of pure *P. aeruginosa* were suspended in 10% glycerol and admixed with sterile control (untreated) mouse feces and a small fixed weight pellet cross transferred and implanted in the right lower quadrant of separate groups of mice following either sham laparotomy or 30% hepatectomy. This lead to cross transfer of intestinal *P. aeruginosa* from sham operated (Sham) and hepatectomy (Hep) mice into the peritoneum of sham operated and hepatectomy mice generating four treatment groups (Figure 1 and Table I).

Morphotype Identification

P. aeruginosa was retrieved from cecal stool contents from mice in the intestinal inoculation phase 24 hours after the initial procedure. Mice were sacrificed and cecal stool contents were collected under sterile conditions. Samples were diluted with sterile normal saline and plated onto PIA. After overnight culture, plates were observed under light microscopy to identify the morphotype patterns of individual colonies. Two predominant morphotypes were identified and defined as either “smooth” or “round.” These morphotypes were then sub-cultured overnight and subsequently suspended in 10% glycerol to an OD of 0.20 for mixture with sterile stool and used in intraperitoneal cross transfer studies.

Pyocyanin Assay

P. aeruginosa was retrieved from cecal stool contents from mice in the intestinal inoculation at 24 hours after the initial procedure. Mice were sacrificed and cecal stool contents were collected under sterile conditions. Samples were diluted with sterile normal saline and plated onto PIA for pyocyanin formation as previously described⁶. Briefly, after overnight culture on PIA, bacterial cultures were spun down by centrifugation and 1 mL of supernatant was extracted using 500 μ L of chloroform, then re-extracted with 150 μ L of 0.2 mol/L HCl. Absorbance was measured at OD 520 nm. Appropriate controls were analyzed including bacterial suspensions containing high phosphate (15 mmol/L), low phosphate (0.1 mmol/L), as well as phosphate solutions containing no bacteria. All experiments were performed in triplicate.

Cytokine Assay

Assessments of cytokine profiles were performed using a commercially available assay by Millipore (Milliplex MAP Mouse Cytokine/Chemokine–Premixed 12 Plex). Simultaneous measurement of 12 cytokines/chemokines (IL-1 beta, IL-6, IL-12(p40), IL-12(p70), IL-15, TNF- α , IL-10, IL-13, IL-17, IFN- γ , IL-9, IP-10) was performed. All assays were performed according to the manufacturer’s protocols. Samples were collected from the blood and peritoneal washings of mice following intraperitoneal cross transfer at 24 hours post-procedure, and diluted 1:1 in the assay buffer provided with the kit, and at least two replicate wells were plated per sample. Cytokine concentrations were determined using BeadView software (Millipore).

Photon camera imaging of *P. aeruginosa* in the peritoneum of mice

In order to demonstrate that the *P. aeruginosa* fecal pellet admixture distributed within the peritoneum to cause peritonitis as others have described¹², we performed photon camera imaging of bioluminescent bacteria following 6 and 12 hours following intraperitoneal injection. *P. aeruginosa* distributed from the fecal pellet to the generalized peritoneal cavity within 12 hours.

Statistical Analysis

For survival curves standard Kaplan Meir statistical analysis was applied. Virulence factor analysis was compared using student's t test. Non-parametric Kruskal-Wallis sum rank ANOVA was used to compare the results of the cytokine data. Standard statistical software was used for all analyses (STSS Chicago, Illinois).

RESULTS

P. aeruginosa isolated from the intestine (cecum) of mice subjected to 30% hepatectomy produces significantly increased pyocyanin production compared to isolates from sham laparotomy mice

When stool from mice subjected to 30% hepatectomy was cultured on selective media for *P. aeruginosa*, it appeared grossly green compared to stool cultures from sham operated mice. Pyocyanin assay revealed that *P. aeruginosa* cultured from mice subjected to 30% hepatectomy demonstrated a statistically significant increase in pyocyanin production compared to that from sham operated mice ($p < 0.01$) (Figure 2A).

Intestinal *P. aeruginosa* isolates from mice following intestinal inoculation and 30% hepatectomy, in contrast to isolates from sham operated mice, cause high mortality rates following intraperitoneal cross transfer

Cross transfer of intestinal *P. aeruginosa* from sham operated mice to the peritoneum of sham operated mice (Sham_{intestine}→Sham_{peritoneum}) group I resulted in 100% survival (6/6) and healthy appearing mice at 24 hours. Cross transfer of intestinal *P. aeruginosa* from sham operated mice to the peritoneum of 30% hepatectomy (Hep) mice (Sham_{intestine}→Hep_{peritoneum}, group II), demonstrated a lower survival rate (83%; 5/6) at 24 hours that was not statistically significant ($P=NS$) with all surviving mice appearing healthy. However cross transfer of *P. aeruginosa* from the cecum of 30% hepatectomy mice to the peritoneum of sham operated mice (Hep_{intestine}→Sham_{peritoneum}), group III, resulted in rapid appearing sepsis and 100% mortality (8/8 $p < 0.05$). Similarly cross transfer of intestinal *P. aeruginosa* from 30% hepatectomy mice to the peritoneum of 30% hepatectomy mice (Hep_{intestine}→Hep_{peritoneum}, group IV) also resulted in rapid appearing sepsis and 100% mortality ($N=6$, $p < 0.05$). All mice were sacrificed at 24 hours and autopsied for the presence of peritonitis (Figure 2B).

Intraperitoneal *P. aeruginosa* within a sterile fecal pellet distributes within the general peritoneum within 12 hours

In mice that developed severe peritonitis and death, photon camera imaging of the abdominal cavity of bioluminescent strains confirmed that the fecal pellet containing *P. aeruginosa* diffusely distributed in the general peritoneal cavity within 12 hours following their intraperitoneal injection as has been reported by others¹² (Figure 3).

Intestinal *P. aeruginosa* isolates from mice subjected to 30% hepatectomy are transformed to distinct wrinkled surface morphotypes

When stock wild type strains of *P. aeruginosa* PAO1 are observed under light microscopy they demonstrate a round/smooth surface morphotype on PIA. Similarly strains harvested from the intestine of mice undergoing sham laparotomy following the intestinal inoculation phase displayed a similar round appearance. In contrast, isolates taken from mice undergoing 30% hepatectomy demonstrated a high proportion of the wrinkled morphotype (as depicted in Figure 4A), while proportionally demonstrating a smaller number of the smooth surface morphotype.

Wrinkled surface *P. aeruginosa* isolates cause high mortality rates in mice following intraperitoneal inoculation compared to no mortality with smooth surface isolates

To evaluate the relationship between morphotype and virulence, wrinkled or round/smooth isolates of *P. aeruginosa* were directly harvested from the culture plates generated during the intestinal inoculation experiments. Selected wrinkled or round/smooth isolates were then re-introduced into naïve mice via intraperitoneal inoculation without additional surgical stress. Interestingly, mice who received inoculations of smooth morphotype isolates taken from both sham and hepatectomy mice appeared healthy and had 100% survival at 24 hours. However, wrinkled morphotype isolates taken from hepatectomy mice cause 100% mortality by 24 hours, suggesting that a wrinkled surface morphotype confers a virulent and lethal phenotype (Figure 4B) (n=8/group, p<0.01).

Differential effect of *P. aeruginosa* isolates on serum and local peritoneal cytokine production

Cytokines expression across groups (I-IV) showed a trend toward activation of the serum cytokines IL-1 β and INF- λ when isolates from hepatectomy mice were intraperitoneally transferred into separate groups of mice although this was not statistically significant. Wrinkled isolates compared to round isolates also demonstrated a trend toward a pro-inflammatory phenotype, although again, this was not statistically significant (see Figure 5).

DISCUSSION

Data from the present study underscore the importance of phenotype transformation or phase variation that develops as microbes adapt and respond to novel environments. Here we show for the first time, that the local intestinal microenvironment during surgical injury can shape the phenotype of colonizing pathogens like *P. aeruginosa* to play a causal role in mortality. That this effect is due to a particular property of the pathogen and not to impaired immune clearance per se is suggested by the use of cross transfer experiments of intestinal *P. aeruginosa*. These experiments allow for the independent assessment of microbial phenotype while maintaining the host immune response constant using mice not previously exposed (i.e naïve) to *P. aeruginosa* inoculation. We have previously observed and reported this round/smooth versus wrinkled or rough surface phenotype in *P. aeruginosa*¹³. However the ability of these morphotypes to differentially express a lethal potential was not assessed. Further work to determine the precise mutation, epigenetic, or phenotype switching mechanisms that are responsible for the expression of this lethal phenotype will be necessary. Yet this will be methodologically challenging given that the smooth and round phenotypes are not stable upon subculture and revert back and forth (data not shown). This finding makes pure DNA and RNA extraction from a single phenotype challenging. Phase variation or phenotype switching is a well described phenomenon among heterogeneous bacterial populations¹⁴. Phase variation can be defined as the random switching of phenotype that appears to occur at frequencies much higher than reported spontaneous mutation rates. Phase variation is a major mechanism by which bacteria express virulence and population

heterogeneity¹⁵¹⁶. Phase variation might be accomplished by riboswitches, small molecules or mRNA that can bind to target molecules and affect ENREF 15 gene expression without necessarily affecting mutation or gene structure. This could be yet another mechanism by which bacteria sense unique host environments and respond in a contextually appropriate manner¹⁷⁻¹⁹ ENREF 15 ENREF 15 ENREF 15. That riboswitches turn on and off particular phenotypes has been proposed by others and could also explain the variation in mortality among the isolates observed in the present study²⁰. The round/smooth versus wrinkled phenotype switch has been observed with other strains of *Pseudomonas* and appears to be highly dependent on adaptation to a fluctuating environment²¹. Sequence analysis of the various isolates in present model is being pursued and will clarify the various mechanisms that shift *P. aeruginosa* from an indolent colonizer to lethal pathogen when present in the gut of a surgically stressed host.

The variation in immune response between intestinal *P. aeruginosa* isolated from mice following hepatectomy versus sham laparotomy is intriguing but will require further study. Although many cytokines were measured, the data were very noisy and not statistically significant. Accurate cytokine measurements in these types of studies are heavily dependent on timing, degree of mouse illness, hydration status, normalization to a background protein etc. For these reasons we did not pursue additional studies in this regard as we felt they would only show that when mice appeared healthy, were feeding well, and were well hydrated, their cytokines profiles would remain at baseline and when they appeared frankly septic, they would be altered. Similarly results from experiments in which wrinkled isolates were compared to round/smooth isolates for their serum and peritoneal cytokine response following intraperitoneal injection demonstrated a trend toward a pro-inflammatory phenotype. However again this was not statistically significant. We also did not pursue these studies further because depending on the strain and character of quorum sensing molecule expression, *P. aeruginosa* can either suppress or stimulate cytokine production⁴ ENREF 4. In addition depending on the particular environmental context (i.e. *in vivo* cell growth dynamics, local physico-chemical cues (i.e. pH, redox, presence of immune cells etc)), a uniform cytokine pattern may not emerge between groups under the conditions of the present study. Nonetheless, studies are in progress to examine the effect of wrinkled versus round/smooth variants on the immune response using *in vitro* assays where *P. aeruginosa* phenotypes can be stabilized (i.e. they do not revert back and forth) and the local environment controlled. This will require that strains be fully sequenced and media conditions rigorously controlled.

Finally, results from the present study could explain, in part, the poor prognosis of patients who develop secondary or tertiary peritonitis²². It is generally recognized that when hospitalized patients develop intestinal perforations or require multiple laparotomies to treat peritonitis, mortality rates are high²³. While this is often attributed to exhaustion of the immune system, malnutrition, etc, enhanced virulence expression among the offending organisms could also play an important role. Emerging technologies that will allow for in depth analysis of virulence phenotype among recovered organisms will help clarify this issue. Once these mechanisms are uncovered, application of anti-virulence compounds might offer promise as an adjunctive measure to current treatment approaches in addition to antibiotics.

In summary, the gut of a surgically stressed host represents a complex ecology that has the potential to shape the virulence of opportunistic pathogens resulting in the expression of a lethal phenotype. Further elucidation of this new dimension in the host pathogen interaction may allow for a more discriminative analysis to explain the mechanisms by which surgical patients have higher infectious related morbidity and mortality beyond the operative site.

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Figure 1.

The experimental protocol consisted of two phases, beginning with intestinal inoculation of *P. aeruginosa* following either a 30% hepatectomy or sham laparotomy. After 24 hours, cecal contents from both groups were harvested and *P. aeruginosa* was isolated on selective media. Bacterial colonies from both groups were then admixed with sterile stool pellets and cross-transferred into the peritoneum of mice undergoing either sham laparotomy or 30% hepatectomy.

Figure 2.

Pyocyanin production (2A) was significantly increased in *P. aeruginosa* harvested from the ceca of mice following 30% hepatectomy versus sham laparotomy (controls). *P. aeruginosa* harvested from the cecum of mice subjected to 30% hepatectomy caused 100% mortality at 24 hours (2B) after peritoneal cross-transfer. *P. aeruginosa* harvested from the cecum of mice subjected to sham laparotomy (control) appeared healthy throughout the experimental period and did not demonstrate sepsis. * $P < 0.01$

Figure 3. Photon camera imaging of intraperitoneal bioluminescent strains at 6 and 12 hours post injection. Images demonstrate a typical spatiotemporal progression and distribution of the localized bioluminescent *P. aeruginosa* inoculum from 6 to 12 hours post exposure.

Figure 4.

Morphotypes identification of *P. aeruginosa* (4A) isolates demonstrating a shift toward a “wrinkled” surface morphotypes when isolates were harvested from mice subjected to 30% hepatectomy compared to the round/smooth appearing isolates harvested from the cecum of mice subjected to sham laparotomy (control). Distinct morphotypes were then subcultured and cross-transferred into the peritoneum (4B); wrinkled morphotypes produced high mortality rates at 24 hours compared to round/smooth morphotypes which cause no sepsis or mortality. * $P < 0.01$.

Figure 5. Cytokines were measured in serum (A-B) and peritoneal washings (C-D) of mice 24 hours after peritoneal cross-transfer of *P. aeruginosa*. A trend toward a more pro-inflammatory response was observed in mice inoculated with the wrinkled versus smooth/round morphotypes of *P. aeruginosa*.

GROUP	INTESTINAL INOCULATION		INTRAPERITONEAL CROSS-TRANSFER
I	Sham _{intestine}	→	Sham _{peritoneum}
II	Sham _{intestine}	→	Hep _{peritoneum}
III	Hep _{intestine}	→	Sham _{peritoneum}
IV	Hep _{intestine}	→	Hep _{peritoneum}