MAJOR ARTICLE

Multicellularity and Antibiotic Resistance in *Klebsiella pneumoniae* Grown Under Bloodstream-Mimicking Fluid Dynamic Conditions

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Background. While the importance of fluid dynamical conditions is well recognized in the growth of biofilms, their role during bacteremia is unknown. We examined the impact of physiological fluid shear forces on the development of multicellular aggregates of *Klebsiella pneumoniae*.

Methods. Wild-type and O-antigen or capsular mutants of *K. pneumoniae* were grown as broth culture in a Taylor-Couette flow cell configured to provide continuous shear forces comparable to those encountered in the human arterial circulation (ie, on the order of 1.0 Pa). The size distribution and antibiotic resistance of aggregates formed in this apparatus were determined, as was their ability to persist in the bloodstream of mice following intravenous injection.

Results. Unlike growth in shaking flasks, bacteria grown in the test apparatus readily formed aggregates, a phenotype largely absent in capsular mutants and to a lesser degree in O-antigen mutants. Aggregates were found to persist in the bloodstream of mice. Importantly, organisms grown under physiological shear were found to have an antibiotic resistance phenotype intermediate between that of fully planktonic and biofilm states.

Conclusions. When grown under intravascular-magnitude fluid dynamic conditions, *K. pneumoniae* spontaneously develops into multicellular aggregates that are capable of persisting in the circulation and exhibit increased antibiotic resistance.

Bloodstream infections are a frequent clinical occurrence, with an annual incidence in North America of 1200 cases per 100 000 general population [1]. While almost any local bacterial infection may result in bacteremia, infected medical devices are increasingly the most common identified source in both inpatient and outpatient populations [2]. As the pathophysiology of device infection is driven by bacterial adherence, biofilm formation, and surface shedding, bloodstream infections share an intuitive link with bacteria growing in multicellular structures.

Although biofilms are an area of great research interest, multicellularity among bacteria is not limited to surface-anchored structures, and this behavior may have importance in the development and persistence of bacteremia. In a recent report, our group demonstrated that the human pathogen *Klebsiella pneumoniae*, when growing planktonically, tends to form multicellular aggregates, some containing many dozens of cells, without the need for an associated anchoring surface [3]. A key finding in that work was that the size distribution of spontaneously formed aggregates was a function of the fluid dynamic conditions under which the cells were growing. In an orbitally shaken flask, multicellular structures formed as long as the Reynolds number (Re) of the flow regime in the flask was below the turbulent transition. In

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highly turbulent flow (ie, supercritical Re conditions), multicellular structures were uncommon, presumably because the kinetic energy imparted on aggregates from the surrounding media was sufficient to prevent or disrupt intercellular adhesion. An important limitation of that work was the inability to define more clearly the hydrodynamic environment in the flask. While a difference in aggregation was found between high- and low-kinetic energy systems, direct correlation to intravascular conditions was not possible.

K. pneumoniae is among the species most commonly isolated from human blood [1, 4–6]. As such, a link between fluid dynamic conditions and bacterial growth could represent an important and previously unstudied aspect of the host-pathogen interface. From a pathophysiologic perspective, certain fluid dynamic conditions in the bloodstream may permit or prevent multicellular bacterial growth and thus influence persistence or resolution of bacteremia. Aggregated, nonphagocytosed bacteria have been noted on routine light microscopy of peripheral blood from bacteremic patients [7]. Multicellular existence (either spontaneously occurring as flocs or shed from a parent biofilm) in this setting raises pivotal questions about the susceptibility of these structures to phagocytosis, microvascular removal, and even antibiotic susceptibility.

In the current study, we modified a Taylor-Couette flow cell—a fluid dynamics research instrument that creates well-characterized flow fields between 2 axially aligned cylinders—to reproduce a portion of physical features of blood flow in the human circulatory system. With this instrument serving as a bloodstream-mimicking bioreactor, we were able to assess the formation of aggregates by *K. pneumoniae* and address several clinically important questions. First, what is the size distribution of bacterial aggregates growing under shear forces as compared with those in the bloodstream? Once formed, do these aggregates persist when injected into the bloodstream? To what extent do 2 prominent pathogenic extracellular features of *Klebsiella* organisms, O-antigen and capsule polysaccharide, contribute to aggregation? Last, given that biofilm-type growth is known to impart antibiotic resistance to this species, is multicellularity at the scale of individual aggregates (ie, on the order of 10 μm) sufficient to convey antibiotic resistance against 2 commonly used agents for gram-negative bacterial sepsis, namely ceftriaxone and ciprofloxacin? Our findings indicate that an aggregative phenotype is supported in a physiologically relevant fluid dynamic regime, that this phenotype is driven largely by surface carbohydrates, and that multicellular aggregation is associated with antibiotic resistance.

MATERIALS AND METHODS

Organisms, Media, and Growth Conditions

K. pneumoniae 43816 (K2:O1) was obtained from ATCC. A previously characterized, isogenic O-antigen–deficient mutant (*ΔwbbO*) was created using a conditional replicating suicide plasmid [8–10]. Also, previously described *K. pneumoniae* LM21 (K35:O2ac) and a similarly constructed capsular mutant (Δ*cps*) were graciously provided by the laboratory of Christiane Forestier [11]. The bacterial strains were cryopreserved at −80°C in tryptic soy broth (TSB). For all other purposes, when TSB supplemented with 1% glucose was used, it increased the carbon to nitrogen ratio, a growth media feature known to encourage capsule production in *Klebsiella* species [12, 13]. Liquid batch cultures were prepared through next-day loop inoculation and were incubated at 37°C in an orbital shaker at 200 RPM until an optical density (at 600 nm) of 0.1 was obtained. These early log-phase growth liquid cultures were used as the starter culture for all other experiments.

Taylor-Couette Flow Cell

The Taylor-Couette cell designed and constructed by our group is shown in Figure 1 [14]. It consists of a 7-cm diameter stationary aluminum cup and a concentric 6-cm diameter motor-driven rotating bob. Energy consumption by the motor is monitored continuously by tracking the electrical current flowing into the controller (Model P4460, P3 International, New York, NY). The cup is water jacketed and plumbed to an adjacent circulating water bath to maintain media temperature. A laser tachometer measures the rotational velocity of the bob. The device holds 80 mL of growth media and is sterilized prior to each experiment by filling it with 100% ethanol for 1 hour.

The flow pattern in a Taylor-Couette cell is turbulent when using low viscosity operating fluids such as water or growth media [15]. Therefore, shear forces encountered by bacteria and aggregates are a function of their diameter relative to the length of the smallest turbulent eddies (ie, the eddies in which chaotic flow settles into viscous flow), the velocity of those eddies, and the viscosity of the growth media. We estimated values for these features in 2 ways. First, we directly measured the energy dissipation of the system to derive the energy dissipative microscales, using the method described by Kolmogorov, as follows:

$$\eta = \left(\frac{\nu^3}{\varepsilon}\right)^{\frac{1}{4}}, u = (\nu\varepsilon)^{\frac{1}{4}}, \tau = \mu\frac{u}{\eta},$$

where η is the mean length of the smallest eddies in meters, u is the mean velocity of those eddies in meters per second, ν is the kinematic viscosity of the media in meters squared per second, ε is the energy dissipation of the system as measured by electrical consumption of the motor in Watts, τ is the shear force exerted by the eddy in Pascals, and μ is the absolute viscosity of the media in Pascal · seconds [16]. Viscosity was measured using a Schott-Gerate automated, temperature-regulated Ubbelohde viscometer. The energy dissipation rate was determined by dividing the net energy consumption of

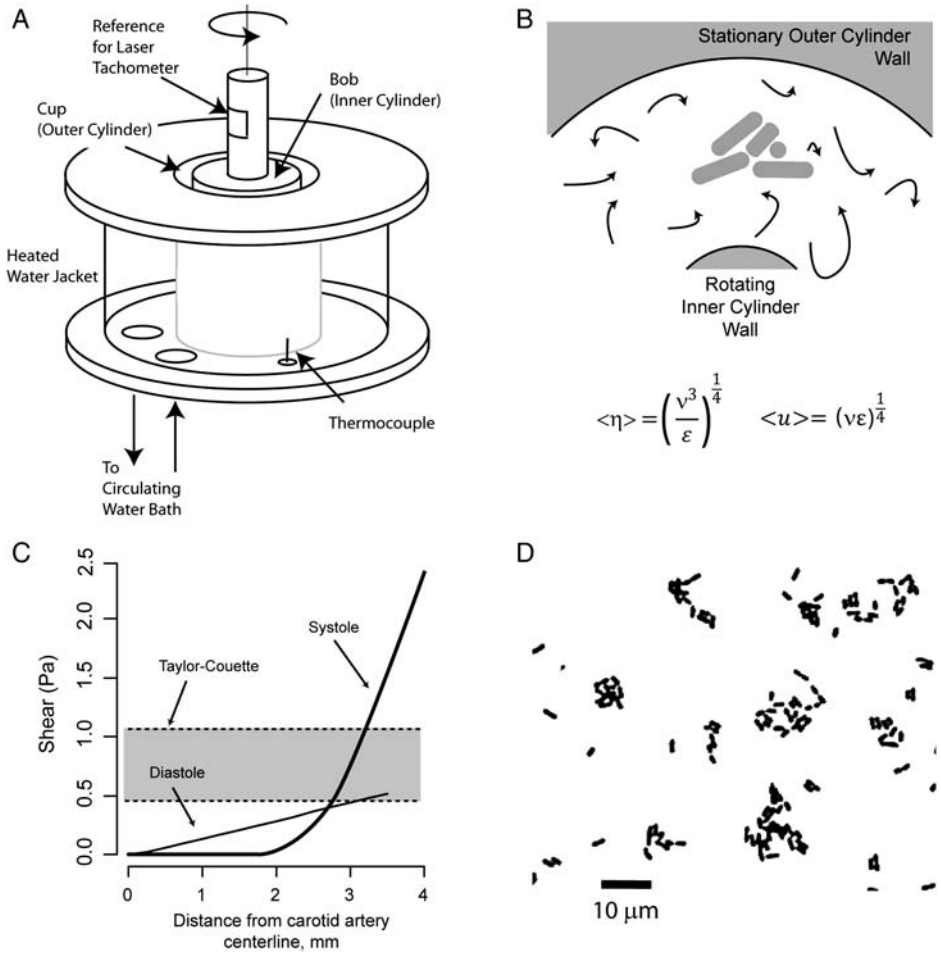

Figure 1. Use of a Taylor-Couette cell for bacterial growth under defined fluid dynamic conditions. *A*, The device used. Growth media is held in the gap between the cup and bob (outer and inner cylinders) as the bob rotates at a fixed rate. The device accommodates approximately 80 mL of media. *B*, Schematic representation of the flow field imparted by the system. When used with low viscosity operating fluids, the flow in the system is turbulent, but predictably so. The forces acting on an aggregate in this system are a function of the mean length (η) and velocity (υ) of the eddies in which flow transitions to chaotic to viscous. This transition is determined by the kinematic viscosity of the fluid ν and the energy imparted on the rotating bob ε. See Methods for details. *C*, Comparison of the shear used in the current study to shear forces encountered in a typical human medium-sized vessel, in this case the internal carotid artery. Systolic and diastolic shear as a function of radial location were determined from intra-arterial velocity measurements in the carotid arteries of healthy volunteers, as reported elsewhere [23]. The gray region is the estimated magnitude of shear force experienced by an aggregate in our apparatus. The region is bounded on the upper end by the estimate value arrived at through measurement of energy dissipation in the system and bounded on the lower end by the value arrived at computationally. *D*, Representative Gram stain of bacterial aggregates formed following 2 h of growth, modified to maximize contrast.

the apparatus (the energy consumption of the system when filled with media minus the consumption when operating the apparatus empty) by the mass of the media in the device.

Second, we adapted an analytical method recently proposed by Bäbler et al for modeling aggregate breakup in turbulent flows, which assumes multifractal scaling and permits estimation not only of mean length, velocity, and shear force, but also of the probability distribution around those values [17]. For the current work, we determined the mean value and 95% confidence interval for each feature.

Growth and Measurement of Aggregates

Bacteria at early log-phase growth were added to prewarmed media in the Taylor-Couette cell and incubated for 2 hours. This short time course was chosen for 2 reasons. First and most important, it is comparable to the duration of clinical episodes of bacteremia [18]. Second, cultures grown for longer periods outstripped the rate of oxygen diffusion into the top of the device and thus were associated with depressed dissolved oxygen content. Samples from the apparatus were taken immediately after inoculation and at 1 and 2 hours for size distribution determination, using a Coulter counter

(Z2, Beckman-Coulter, Fullerton, CA), as described elsewhere [3]. All experimental conditions were performed in triplicate, with each replicate occurring on a different day.

Persistence of Multicellular Aggregates in vivo

For animal experiments, 6–10-week-old outbred male ICR-mice (Harlan Sprague-Dawley, Indianapolis, IN) were used. Animal protocols were approved by the University of Michigan's Animal Use Committee. Mice were anesthetized using 2.5% inhaled isoflurane (Vedco, St. Joseph, MO) and injected with 200 μL of bacterial suspension into the deep dorsal vein of the penis [8]. Approximately 4 minutes after inoculation, blood was withdrawn for histological examination. In the context of bacterial clearance, the time scale of interest is the circulatory system transit time (the time required for a bacterium or bacterial aggregate to make 1 pass through the circulation), which is estimated by dividing the total blood volume by the cardiac output. This value is halved to account for at least 2 capillary bed transits (1 peripheral and 1 pulmonary) per trip. In humans, the capillary-to-capillary transit time is thus approximately 30 seconds. In mice, the transit time is approximately 3 seconds [19, 20]. In our protocol, blood was withdrawn after bacteria had the opportunity to encounter a capillary bed approximately 80 times, which would correspond to a human bacteremic episode lasting 40 minutes. Blood smear samples were stained using Diff-Quik (Dade-Behring, Switzerland), and bacteria and bacterial aggregates were counted by reviewing at least 50 high-power fields on 10 peripheral blood smears per animal.

Antibiotic Resistance of Aggregate Bacteria

Minimum bactericidal concentrations (MBCs) and minimum inhibitory concentrations of ceftriaxone and ciprofloxacin were determined for bacteria under 3 fluid dynamic conditions: during highly energetic orbital shaking at 200 RPM, in the Taylor-Couette cell, and as static biofilms. Static biofilms were grown on polystyrene pegs immersed in 96-well microtiter wells with TSB and incubated for 18 hours at 37°C without refeeding or shaking [21]. Biofilms were then centrifuged from their substratum pegs into new broth containing antibiotics. Taylor-Couette and shaking flask cultures were pipetted into new media containing antibiotics. MBC is reported as the concentration required to sterilize the culture, as determined by quantitative culture on agar plates at 24 hours.

Statistical Analysis

All analysis was performed in R, version 2.13.0 [22]. To compare particle size distributions between experimental conditions and strains, general additive modeling was used [3]. This technique is a modification of analysis of variance that uses splines to fit complex curves and has been used by our laboratory to perform comparisons of particle size distributions.

RESULTS

The measured and calculated physical features of the Taylor-Couette cell are provided in Table 1. Generally, while the flow in the system was turbulent, the estimated shear force within the smallest turbulent eddies (ie, the shear forces to which the bacterial aggregates were exposed) was comparable to those encountered in the arterial circulation (Figure 1*C*) [23].

Development of Multicellular Structures Under Bloodstream-Like Fluid Dynamic Conditions

During 2 hours of incubation in the Taylor-Couette cell, significant aggregative growth was observed in both wild-type *Klebsiella* strains. An example micrograph is shown in Figure 1*D*. The aggregate size distributions of cells growing in the Taylor-Couette cell and in highly turbulent shaking flask conditions are compared in Figure 2. In a flask orbitally shaken at 200 rpm, there was substantially less aggregate growth.

Importance of Surface Carbohydrate Polymers on Aggregate Growth

We hypothesized that the predominant surface polysaccharides of *Klebsiella* organisms, the O-antigen and capsule, were likely contributors to aggregate durability during growth in

Table 1. Measured and Calculated Characteristics of the Taylor-Couette Cell

Fluid Dynamic Feature	Value
Measured (37°C)	
Absolute viscosity, Pascal · sec	7.8×10^{-4}
Density, g/mL	1.0
Kinematic viscosity, m^2/s	7.9×10^{-7}
Energy dissipation, W	0.14
Calculated smallest eddy feature[a]	
Kolmogorov [16] method	
Length, μm	23
Velocity, mm/s	34
Shear rate, 1/s	1500
Shear force, Pa	1.2
Bäbler et al [17] method, mean (95% CI)[b]	
Length, μm	39 (28–57)
Velocity, mm/s	21 (14–28)
Shear rate, 1/s	560 (260–1027)
Shear force, Pa	0.4 (.2–.8)

[a] See Methods for calculations. The smallest eddy represents the length scale at which flow transitions from inertial, turbulent flow to flow dominated by viscous dissipation.

Abbreviation: CI, confidence interval.

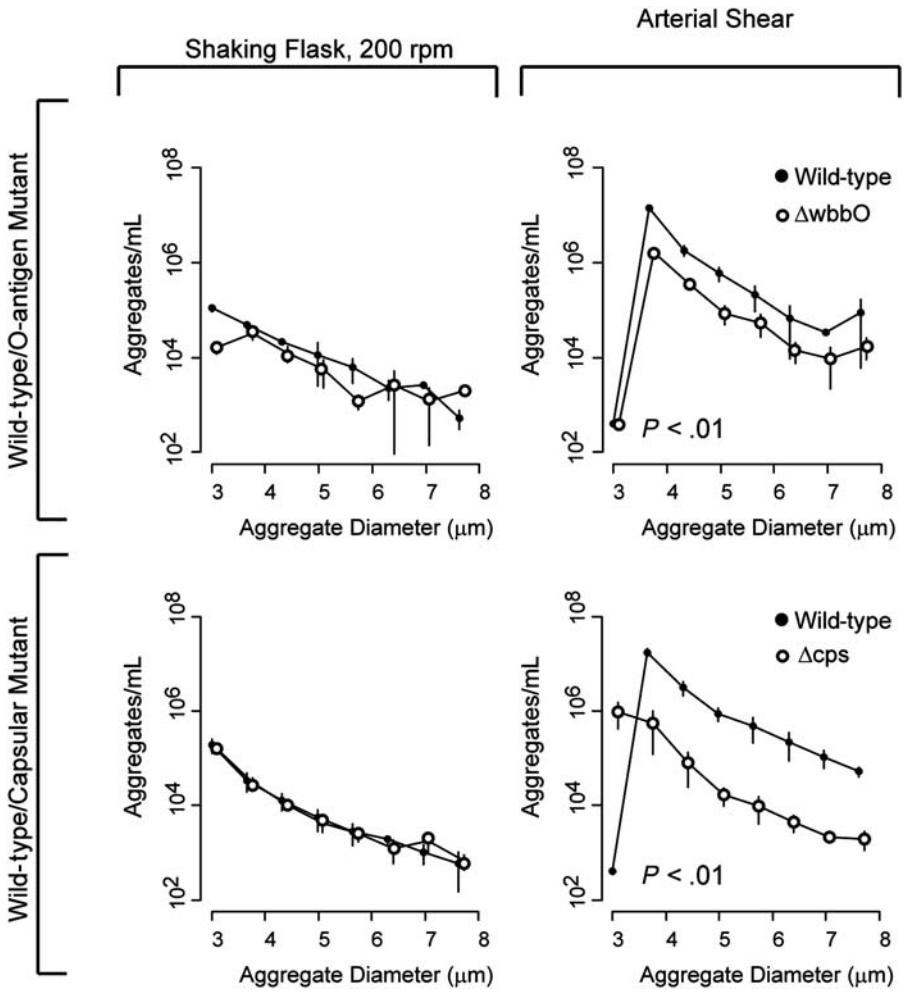

Figure 2. Aggregate formation as a function of growth conditions and extracellular polymeric substance production. The size distributions of cells grown either in highly turbulent shaking flasks (left column) or under controlled conditions in the Taylor-Couette cell (right) were measured using a Coulter counter. Two isogenic pairs of organisms were studied, wild-type and O-antigen–deficient strains (upper row) and wild-type and capsule-deficient strains (lower row). For all strains studied, bacteria grown under bloodstream-like flow conditions demonstrated significantly greater aggregation than those grown in a shaking flask. Loss of either O-antigen or capsule reduced the frequency of larger aggregates, with loss of capsule producing the more profound effect ($P < .01$ for each).

flowing media. As shown in Figure 2, both capsular and O-antigen polysaccharides contribute to this phenomenon, with the absence of either structure reducing the number of multibacterial aggregates ($P < .01$). Of the 2 structures, the capsule provided a larger contribution to aggregate formation.

Impact of Small-Scale Community Growth on Antibiotic Susceptibility

Klebsiella biofilms acquire antibiotic resistance when grown in stationary biofilms [24]. To determine whether suspended bacterial aggregates had similar protection, we determined the MBCs of ceftriaxone and ciprofloxacin for wild-type bacteria grown in shaking flasks or under bloodstream-comparable conditions. Both agents are common first-line therapy for suspected gram-negative bloodstream infections. As a positive control, each strain was also grown as a biofilm on a 96-well immersed-peg apparatus [21]. Bactericidal susceptibility to antibiotics is shown in Figure 3. For both wild-type strains and for each of the antibiotics tested, bacteria recovered from the Taylor-Couette cell demonstrated an MBC intermediate between shaking-flask-grown culture and biofilm, demonstrating that growth as small communities was sufficient to confer antibiotic resistance to a significant degree.

Ability of Cell Aggregates to Survive in the Bloodstream

To confirm that aggregates formed in vitro within our system were capable of surviving under similar conditions in vivo, mice were given an intravenous injection of bacteria grown under aggregative conditions. Animals were subsequently euthanized, and peripheral blood smears were prepared to

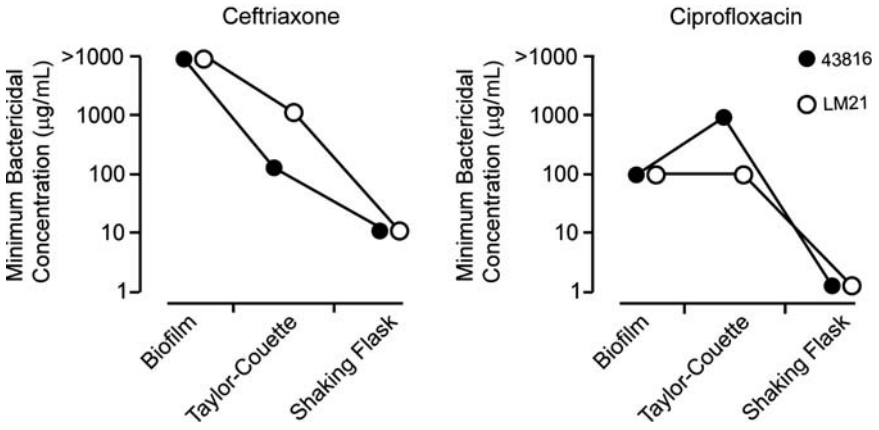

Figure 3. Enhanced antibiotic resistance in bacteria growing under proaggregative conditions. Shown are the minimum bactericidal concentrations of 2 clinically important antibiotics, ceftriaxone and ciprofloxacin, for *Klebsiella pneumoniae* grown under highly turbulent shaking flask, Taylor-Couette, and biofilm conditions. For the 2 wild-type strains studied (*K. pneumoniae* 43816 and LM21), 2 hours of aggregative growth in the Taylor-Couette cell produced antibiotic resistance intermediate between planktonic and biofilm conditions. Each plotted points reflects 8 experimental replicates. In these experiments, use of 10-fold changes in concentration resulted in no variation within replicates to allow for calculation of standard deviation.

measure the size distribution of persistent aggregates. This distribution is shown as a histogram in Figure 4*A* and indicates that, while single cells or cell pairs were common, multicellular structures were easily detected in the bloodstream after many passes through the microcirculation. In Figure 4*B*, an example aggregate is shown from 1 peripheral smear. In this particular example, the volume of the aggregate is nearly doubled by an adherent collection of platelets. It is important to note that this large structure was found circulating in the bloodstream—it had not been successfully filtered.

DISCUSSION

In the current work, we demonstrate that shear forces likely to be encountered by pathogens during a bacteremic episode permit the development of multicellular aggregates of a common blood-borne gram-negative pathogen, *K. pneumonia*, in a clinically relevant period. The process appears to be largely driven by extracellular polysaccharides (EPS), especially by the capsule. Aggregates grown in vitro persist when administered intravenously. Importantly, growth in this fashion

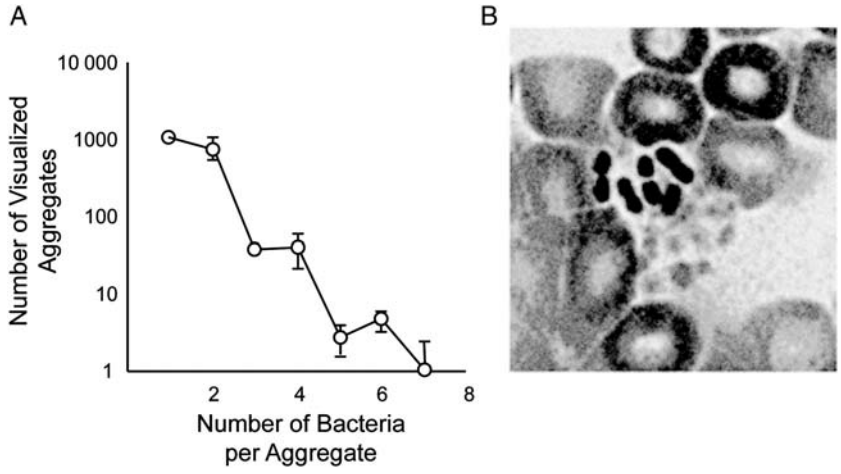

Figure 4. Persistence of multicellular bacterial aggregates in murine blood during bacteremia. Bacteria grown under aggregating conditions were intravenously inoculated into animals and allowed to circulate for approximately 80 microvascular transits (see Methods for details). Ten peripheral smears were prepared from each animal, and fifty 100× fields were examined for bacteria. *A*, Distribution of bacterial aggregates by number of organisms per aggregate, shown as mean value per bin ± SD. *B*, Example multicellular aggregate from a bacteremia mouse. The aggregate itself is comparable in size to an erythrocyte and appears to be attached to a similarly sized aggregate of platelets. Despite its large size, this structure was able to resist microvascular filtration dozens, if not hundreds of times during its intravascular lifespan. Diff-Quik stain, 100×.

conveys resistance to a broad-spectrum beta-lactam and a fluoroquinolone, 2 classes of first-line antibiotic therapy in suspected bacteremia.

We believe that these results justify careful attention to accurately reconstructing the "bacterial experience" during bacteremia and that better modeling the fluid mechanical features of this host-pathogen interface may, as has been the case with biofilms, continue to yield important insights into bloodstream infections. Here we address this point by characterizing the shear stress experienced by the bacteria in the Taylor-Couette flow. Although the flow regime established in our apparatus is grossly turbulent, it is important to consider that the structure of the turbulent flow is determined by the length scale under consideration. The Reynolds number, the usual metric for quantifying the amount of turbulence in a flowing fluid, is directly proportional to the characteristic length of the flow under study; when considering the environment around a 5–10-μm bacterial aggregate, even highly turbulent host environments (eg, at the outlet of a severely stenotic heart valve) are characterized by viscous rather than chaotic flow. For example, the scale of the smallest eddies in the Taylor-Couette cell used here is approximately 25 μm. Thus the device used in the current report provides important replication of intravascular conditions in an infected host, simulating viscous flow on the scale of the bacterial aggregates.

An issue unlikely to be resolved without in vivo experiments is determining to what extent aggregate formation in any bioreactor is a process of adhesive bacterial collisions rather than persistence of daughter cell adhesivity following cell division. We have previously considered this issue theoretically [25–27]. Opportunity for collisions between bacteria and bacterial aggregates would be expected to occur often in any type of homogenous liquid culture such as that studied currently. However, in the bloodstream, collisions of individual bacterial cells are highly unlikely at the density at which they are typically found. Given erythrocyte concentrations of approximately 10^9 cells/mL, bacteria are outnumbered by a ratio of 10^7:1. Published images of aggregation in ill humans therefore suggest cell division with daughter cell adhesivity as the primary source of aggregate growth.

Production of EPS (both capsule and O-antigen) was an important requirement for aggregate formation in our experiments. We believe it is likely that this is the case in vivo but would point out several limits of the current work in this regard. The growth media that we used (TSB with 1% supplemental glucose) is known to support the production of EPS, presumably via the ratio of carbon to nitrogen [12, 13]. To what extent this reflects growth in plasma, which is replete with potential carbon and nitrogen sources, is unknown. We are unaware of reports indicating which substrates *Klebsiella* organisms prefer while growing in plasma. Nutrient-mediated EPS production during actual bacteremia may influence the magnitude of the aggregative phenotype that we observed.

It is interesting to consider what the clinical impact of this behavior might be. Differences between standard in vitro and observed in vivo antibiotic susceptibility are directly suggested by our data. Additionally, hyperdynamic sepsis represents a potential experimental platform to further examine mechanical coupling between bacteria and the bloodstream. In a murine model of acute bacteremia, we have provided evidence that increased cardiac output may facilitate sterilization of the blood, possibly by increasing the number of times a bacterium or aggregate passes through a filtering microvascular bed [28]. Future work is warranted to better understand the implications of increases or decreases in kinetic energy imparted by the bloodstream onto blood-borne pathogens during serious infection.

Notes

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