The *Staphylococcus aureus* Epidermal Cell Differentiation Inhibitor Toxin Promotes Formation of Infection Foci in a Mouse Model of Bacteremia

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Inactivation of the host GTPase RhoA by staphylococcal epidermal cell differentiation inhibitor (EDIN) exotoxins triggers the formation of large transcellular tunnels, named macroapertures, in endothelial cells. We used bioluminescent strains of *Staphylococcus aureus* to monitor the formation of infection foci during the first 24 h of hematogenous bacterial dissemination. Clinically derived EDIN-expressing *S. aureus* strains S25 and Xen36 produced many disseminated foci. EDIN had no detectable impact on infection foci in terms of histopathology or the intensity of emitted light. Moreover, EDIN did not modify the course of bacterial clearance from the bloodstream. In contrast, we show that EDIN expression promotes a 5-fold increase in the number of infection foci produced by Xen36. This virulence activity of EDIN requires RhoA ADP-ribosyltransferase activity. These results suggest that EDIN is a risk factor for *S. aureus* dissemination through the vasculature by virtue of its ability to promote the formation of infection foci in deep-seated tissues.

Defining how pathogenic bacteria interact with the endothelium is of major relevance to evaluating the risk of severe forms of infections (21). Recent findings show that the epidermal cell differentiation inhibitor (EDIN) exotoxins trigger the formation of large transcellular tunnels, termed macroapertures, in endothelial cells of various vascular beds (5). EDINs belong to a large group of virulence factors produced by human pathogenic bacteria that target host Rho proteins (2, 4). As Rho GTPases are master regulators of the host cell actin cytoskeleton, they play a central role in controlling cell adhesion, migration, and phagocytosis (18). Moreover, Rho proteins control the formation and integrity of both intercellular adherence and tight junctions (11) and are thus major regulators of the endothelial barrier function (27, 36). Rho proteins also play essential roles in the transcellular or intercellular modes of diapedesis of leukocytes through the endothelium (6).

Staphylococcal colonization of the skin and mucosa is a risk factor for bacterial translocation to underlying tissues and the bloodstream. Failure to contain the initial infection can lead to sepsis or invasion of deeper tissues, leading to endocarditis, septic arthritis, and osteomyelitis (34). Staphylococcal infections involve the combined actions of a large panel of virulence factors, promoting bacterial colonization, destruction of tissues, and immune evasion (9, 12). These bacterial virulence factors comprise secreted exotoxins and cell surface-associated factors (22). Pathogenic *Staphylococcus aureus* can produce enzymes such as proteases, nucleases, hyaluronidases, lipases, and cytolytic toxins. These factors participate in the destruction of host tissues and may favor bacterial dissemination in the host tissues.

Several pathogenic strains of *S. aureus* produce EDIN type A (EDIN-A), EDIN-B, or EDIN-C (3, 15, 37–39). Whereas the gene encoding EDIN-B is located on the chromosome within a pathogenicity island (15, 26, 38), genes encoding EDIN-A and EDIN-C are plasmid borne (37, 38). Strains carrying the exfoliative toxin type D gene (*edt*) also harbor the *edin-B* gene (3, 15, 38). These strains correspond to the sequence type 80 clone of Panton-Valentine leukocidin-positive community-acquired (CA) methicillin-resistant *S. aureus* (MRSA), which continues to spread through Europe (39) and Tunisia (3). Although genes encoding EDIN have a higher prevalence in pathogenic isolates of *S. aureus* (10), the contribution of EDINs to bacterial virulence remains to be defined.

EDIN exotoxins translocate into the host cell cytosol from acidic compartments following macroinocytosis or after internalization into phagosomes (24). Upon reaching the cytosol, EDINs preferentially mono-ADP-ribosylate RhoA, with this modification occurring at asparagine-41 (8, 31, 35). Posttranslational modification of RhoA by ADP-ribosylation induces its tight association with RhoGDI, producing its release from membranes, where RhoA transduces signals (16, 17). Inactivation of RhoA blocks a major pathway responsible for both actin filament elongation and assembly into contractile actomyosin cables (8, 18, 28). In endothelial cells,activation of RhoA controls the formation of intercellular gaps by inducing contractile actomyosin fibers, which pull on intercellular borders (23). Thus, inactivation of RhoA by ADP-ribosylation reinforces the cohesion of adherence junctions in the endothelium.
lrium (36). Nevertheless, recent advances show that inhibition of RhoA impairs endothelium barrier function by producing transcellular tunnels, referred to as macroapertures (5). Indeed, endothelial cell intoxication either by any of the three isoforms of EDIN or by infection by EDIN-producing S. aureus triggers the formation of these transcellular tunnels. Macropores are also formed in the endothelium lining rat arteries infected ex vivo by EDIN-producing S. aureus (5). Macropores unmask the fibril matrix underneath the endothelium where bacteria adhere.

The capacity of EDIN to open macropores in vitro and ex vivo raised the possibility that this family of exotoxins might favor S. aureus hematogenous spread to deeper tissues (21). We investigated this by using bioluminescent strains of S. aureus in a mouse model of infection. Here we show that EDIN expression confers on S. aureus a greater capacity to form disseminated infection foci during bacteremia.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The staphylococcal S25 strain was previously isolated from a bacteremic patient suffering from spondylodiscitis (5). This strain was previously cured of its edin-bearing plasmid, using the plasmid-associated cadmium resistance, and transformed with either pMK4-pPROT expressing the EDIN wild type (S25-EDIN-Wt), pMK4-pPROT expressing catalytically inactive EDIN_{RHEX} (EDIN-{RE}) (S25-EDIN-{RE}), or the pMK4-pPROT empty vector (S25-EV) (5). Bioluminescent S25 was generated by transformation with pXen5 (pAUL-A Tn4001 luxABCDE Km') (14). Stable bioluminescent transformants were selected as described previously (19).

The Xen36 bioluminescent strain (Caliper Life Sciences Inc.) was derived from S. aureus ATCC 49525 (Wright), a clinical isolate from a bacteremic patient. We established by PCR that this strain does not express the edin-A, -B, or -C gene. The Xen36 strain was transformed with pMK4-pPROT expressing the EDIN wild type (Xen36-EDIN-Wt), pMK4-pPROT expressing catalytically inactive EDIN_{RHEX} (Xen36 EDIN-RE), or the pMK4-pPROT empty vector (Xen36-EV), as described previously (5). The strains were grown at 37°C in brain heart infusion (BHI) medium (Xen36) or BHI medium supplemented with 20 μg/ml chloramphenicol (Xen36 EDIN-Wt, Xen36 EDIN-RE, Xen36-EV). All Xen36-derived strains had similar growth rates in BHI broth and luminescence intensities.

Animals and experimental model of infection. Six- to 8-week-old female BALB/c mice were purchased from Charles River (L’Arbresle, France). The mice were maintained and handled according to the regulations of the European Union and the French Department of Health. For infections, overnight cultures of each strain of S. aureus were centrifuged and washed three times with phosphate-buffered saline (PBS). The pellets were diluted in PBS to obtain the desired bacterial concentrations. The lateral tail vein of each mouse was injected with 100 μl of bacterial suspension. For determination of bacteremia, blood was collected in a heparin-containing syringe from the tail vein, serially diluted in PBS, and plated on BHI medium plates, which were incubated for 24 h at 37°C.

Infection monitoring by bioluminescence imaging. The lateral tail vein of each mouse was injected with 100 μl of a suspension of bioluminescent S. aureus strains. Imaging was performed at different time points after inoculation, using an In Vivo imaging system, according to the manufacturer’s instructions (Caliper Life Sciences Inc.). Briefly, groups of five mice were anesthetized with 2.5% (vol/vol) isoflurane and placed in the IVIS100. Photons were counted over 5 min, and data analysis was performed using the Living Image (version 3.0) program (Caliper Life Sciences Inc.).

Histopathology. After identification of infection foci by bioluminescence imaging, the mice were euthanized and organs corresponding to the infection foci were collected, fixed in formalin, and embedded in paraffin. Consecutive 3-μm paraffin sections were stained with hematoxylin-eosin-saffron (HES). In parallel, Gram staining was performed to visualize the presence of bacteria.

Statistics. Analysis of variance with the Bonferroni post hoc test was used for statistical analyses, unless indicated otherwise in the figure legends. Analyses were performed with Prism (version 5.0b) software. Values were considered significant if P was <0.05.

RESULTS

EDIN-positive S. aureus strains produce disseminated bioluminescent foci during bacteremia. Induction of macropores in endothelial cells by EDIN exotoxins represents a potential virulence mechanism that may favor the dissemination of staphylococci in tissues via a hematogenous route. We investigated this by monitoring infection in living mice using a previously described bioluminescence technology (13). Isolation of clinical strain S. aureus S25 from the blood of a patient suffering from a spondylodiscitis was described previously (5). We transformed this strain with a plasmid containing a Photorhabdus luminescens lux operon (luxABCDE) and selected clones on the basis of their capacity to emit light. This allowed us to generate bioluminescent strain S25lux. We verified that S25lux triggered actin cable disruption and induction of macropores in endothelial cells, as previously observed for S25 (5) (Fig. 1A). Mice were infected by intravenous inoculation of 5 × 10⁷ CFU of S25lux. The light emitted was recorded 10 min after infection and then 3 h and 24 h after infection. These data indicate that bioluminescent foci formed 24 h after infection (Fig. 1B). Figure 1C shows two examples of histology analysis of bioluminescent foci that confirm the inflammation associated with the presence of bacteria. This revealed the capacity of S25lux staphylococci to produce disseminated bioluminescent infection foci as early as 24 h after induction of bacteremia.

Clearance of S. aureus from the bloodstream. We suspected that EDIN might favor bacterial dissemination in tissues by delaying the clearance of S. aureus from the bloodstream. We used previously described recombinant strains engineered from S25 to test this hypothesis (5). Briefly, S25 was cured of the native edin-bearing plasmid and transformed with the empty vector (S25) or plasmids encoding either wild-type EDIN (S25-EDIN-Wt) or a catalytically inactive mutant of EDIN (S25-EDIN-RE). The recombinant strains had similar kinetics of growth and the stable retention of plasmids 24 h after the onset of bacteremia (data not shown). Mice were infected by intravenous inoculation with 10⁸ CFU, delivered through the tail vein. Survival of the bacteria in the bloodstream was assessed 3 h and 24 h after infection. The staphylococcal load was enumerated by plating blood samples on agar plates and measuring colony formation. For all strains, we measured at 3 h a dramatic decrease (at least 4 log units) in the number of viable bacteria in the blood (Fig. 2). No significant differences in the recovery of CFU were measured between these strains 3 h and 24 h after infection (Fig. 2). Similar results were obtained with mice intravenously infected with 2 × 10⁷ CFU (data not shown). Collectively, this shows an absence of a detectable effect of EDIN on the kinetics of clearance of staphylococci from the bloodstream.

Formation of infection foci. Our findings prompted us to investigate the role of EDIN in the formation of disseminated bioluminescent foci during staphylococcal bacteremia. Owing to technical limitations associated with introducing the Lux operon in the genomic DNA of S25 and S25 cured of the edin-bearing plasmid, we made use of staphylococcal isolate Xen36, which was initially recovered from a patient with bacteremia and previously engineered to express luxABCDE (Caliper Life Sciences Inc.). We determined by PCR that Xen36 is
negative for edin-A, -B, and -C (data not shown). We thus addressed the question of whether acquisition of an edin-bearing plasmid confers to the bacterium a higher capacity to form disseminated infection foci during bacteremia. Mice were first infected intravenously with $10^8$ CFU of Xen36. The light emitted was recorded prior to infection (0 h) and then 3 h and 24 h after infection. Bioluminescent foci appeared 24 h after infection (Fig. 3A). Bioluminescent tissues were next analyzed after

FIG. 1. Induction of disseminated bioluminescent foci by EDIN-producing S25. (A) S. aureus bioluminescent S25 strain (S25lux) triggers the disruption of actin cables and the formation of macroapertures (inset). Human umbilical vein endothelial cells were treated overnight with the S25lux supernatant (dilution, 1/50). The supernatant was prepared from bacteria grown to an optical density at 600 nm of 1. The actin cytoskeleton was visualized using tetramethyl rhodamine isocyanate-conjugated phalloidin. Bar, 20 μm. (B) Bioluminescence imaging of infection. Five BALB/c mice were injected intravenously with $5 \times 10^7$ S25lux bacteria. Dorsal and ventral imaging of the mice was performed at 10 min, 3 h, and 24 h postinfection. The color scale indicates the signal intensity in numbers of photons/second/cm². (C) Histopathology of infection foci. Pictures show hematoxylin-eosin-saffron staining and the corresponding Gram staining of selected foci (arrows) in the heart at a high magnification ($\times400$). BALB/c mice were injected intravenously with $5 \times 10^7$ CFU/animal of S25lux. The color scale indicates the signal intensity in numbers of photons/second/cm².
hematoxylin-eosin-saffron staining and Gram staining of thin sections. Figure 3B shows two examples of histology analysis of infection foci that confirm the inflammation associated with the presence of clusters of bacteria. Taken together, these data show the capacity of Xen36 to produce bioluminescent infection foci.

Expression of EDIN favors formation of disseminated infection foci. We next examined whether acquisition of the edin gene might favor the formation of infection foci. Xen36 was transformed with the empty pMK4-pPROT vector (Xen36-EV) or vectors encoding wild-type EDIN (Xen36 EDIN-Wt) or catalytically inactive EDIN (Xen36 EDIN-RE). All recombinant strains of S. aureus showed similar growth kinetics and levels of light emission (data not shown).

FIG. 2. Bacterial clearance from the bloodstream by enumeration of bacteria in the blood of mice infected by S. aureus S25 derivatives. Three groups of 8 to 10 BALB/c mice were infected with 10^6 CFU/animal of S25, S25-EDIN-Wt, or S25-EDIN-RE. Enumeration of the bacteria was performed by measuring the numbers of CFU on LB agar plates in the absence of antibiotics. Values for each mouse are shown. Statistical analysis shows no significant differences (ns; P > 0.05).

FIG. 3. Monitoring of infection in a mouse model of Xen36 bacteremia. (A) Five BALB/c mice were injected intravenously with 10^6 CFU/animal of Xen36-EV. Dorsal and ventral imaging was performed on noninfected (0 h) mice and at 3 h and 24 h postinfection. The color scale indicates the signal intensity in numbers of photons/second/cm^2. (B) Histopathology of infection foci. Pictures show hematoxylin-eosin-saffron staining and the corresponding Gram staining of selected foci (arrows) in the kidney and muscle at high magnification (×400). BALB/c mice were injected intravenously with 10^6 CFU/animal of Xen36(pMK4-pPROT). The color scale indicates the signal intensity in numbers of photons/second/cm^2.
We also verified that expression of wild-type EDIN specifically triggered disruption of the actin cables and formation of macroapertures in endothelial cells (Fig. 4A, arrows). We next investigated the effect of EDIN in a mouse model of bacteremia. Mice were intravenously infected with \(5 \times 10^7\) CFU/animal of Xen36 EDIN-Wt or Xen36 EDIN-RE. Control experiments confirmed the stability of the edin-expressing plasmids over the 24 h following infection (data not shown). Under these conditions, we visualized the formation of disseminated bioluminescent foci 24 h after infection (Fig. 4B). Foci were defined for light intensities 2-fold higher than the background (\(>10^4\) photons/s). Most foci of the different bioluminescent strains were detected at the level of the sacral part of the spinal column and the leg joints. Importantly, we measured differences in the number of foci between Xen36 EDIN-Wt- and Xen36 EDIN-RE-infected animals (Fig. 4C). Xen36 EDIN-Wt produced up to six infection foci per mouse. The mean value was \(\sim 2.5\) foci/mouse for Xen36 EDIN-Wt, whereas the mean value was \(\sim 0.5\) focus/mouse for Xen36 EDIN-RE (Fig. 4C). Thus, expression of catalytically active EDIN in Xen36 significantly increases the rate of formation of disseminated bi-
oluminescent foci during bacteremia (P < 0.01). We next recorded and quantified the light signals emitted at the foci. These measurements revealed that the intensities of light emitted at the foci were similar under all conditions (Fig. 4D). We verified that the light emitted by infection foci at the level of the sacral part of the spinal column or leg joints between the EDIN-Wt and RE conditions were also of similar intensities (data not shown). This suggested that EDIN had no detectable effect on the growth and survival of the bacteria in tissues under these conditions. Thus, acquisition of an edin-expressing plasmid confers to Xen36 a greater capacity to form disseminated infection foci.

Finally, we conducted histopathological analysis of the bioluminescent foci. This analysis was conducted on mice infected by Xen36 EDIN-Wt (six samples), Xen36 EDIN-RE (four samples), and Xen36 containing the empty vector (four samples). Representative examples of the infection foci analyzed are shown in Fig. 6. Microscopic examination of samples revealed acute inflammatory processes characterized by a localized infiltrate of polymorphonuclear leukocytes with scattered macrophages. Infection foci localized at different sites, i.e., renal interstitium and tubules (pyelonephritis), as well as sacral fibrocartilage (spondylodiscitis) and femoral bone marrow (osteomyelitis). No microscopic differences between infection foci produced by either strain were observed. Moreover, we noticed a good correlation between the intensity of the fluorescent signal and the extent of the inflammatory reaction observed by histopathological analysis.

Together these observations show that expression of EDIN confers to S. aureus a greater capacity to form disseminated infection foci during bacteremia.

DISCUSSION

Determining the mechanisms of dissemination of S. aureus in host tissues is of major interest, given the high incidence and rate of mortality from staphylococcal infections. In order to address this challenge, we used a bioluminescence technology to investigate the role of EDIN in S. aureus-induced bacteremia. We show that expression of catalytic active EDIN in clinical strain Xen36 specifically increases the capacity of bacteria to induce disseminated infection focus formation. The bioluminescent foci produced by Xen36 EDIN-Wt and Xen36 EDIN-RE were of similar light intensities. Moreover, histopathological analysis revealed no differences between the infection foci produced by either strain. Finally, we show that EDIN plays no role in bacterial survival in the bloodstream. Collectively, these data show that EDIN favors the formation of disseminated infection foci during S. aureus-induced bacteremia. We propose that EDIN is a risk factor for deep-seated staphylococcal tissue infections following bacteremia.

Several potent virulence factors found in human pathogenic bacteria target Rho proteins or actin itself (2, 4). Rho proteins are implicated in a large number of key cellular processes, by virtue of their ability to control the dynamics of the actin cytoskeleton. The targeting of Rho proteins by bacterial virulence factors thus likely confers multiple advantages to pathogenic bacteria for host colonization and invasion. Several cellular biology studies have clearly established that EDIN exotoxins and their close homologues, the C2 exoenzymes of Clostridium botulinum, can inhibit immune cell migration and complement receptor phagocytosis (2, 7). Rather than triggering direct invasion of tissues by bacteria, it seems possible that EDIN might favor the formation of infection foci by blocking immune cell chemotaxis and function. This hypothesis is unlikely, considering that both EDIN-Wt- and EDIN-RE-expressing S. aureus strains have similar kinetics of clearing from the bloodstream. Moreover, the infection foci triggered by EDIN-Wt- and EDIN-RE had similar light intensities and histopathologies and were recorded early after infection. Together our data support the hypothesis that EDIN might favor the exit of bacteria from the bloodstream rather than bacterial survival.

S. aureus can colonize the skin and mucosa of healthy individuals (22). This gives to S. aureus a high propensity to reach the bloodstream when these physical barriers are breached. S. aureus is frequently isolated from patients with bacteremia. These infections can evolve to cause endocarditis or infections of the bones, joints, kidneys, and lungs. Various clinical strains of S. aureus produce EDIN exotoxins (3, 10, 26, 39). Epidemiological studies related to EDIN have not yet shed light on its virulent function in infection (15). Contrasting with this, cell biology studies of EDIN and the highly homologous C2 exoenzyme of Clostridium botulinum indicate that these factors can play roles that range from hijacking immune cell responses to compromising the integrity of the epithelium and endothelium barriers (1, 25). Indeed, we recently reported that EDIN, through inhibition of RhoA, specifically triggers the formation of large transcellular tunnels in endothelial cells (5). We have shown that infection of endothelial cells with S. aureus producing catalytically active EDIN also triggers the formation of macroapertures. Our analysis of the effect of EDIN in a model of rat arteries infected ex vivo with S. aureus producing EDIN...
revealed the formation of large macroapertures (5). This un-masks the subendothelial matrix where *S. aureus* binds (5). Here we provide the first demonstration that expression of EDIN favors invasion of tissues by *S. aureus* and that this virulence activity of EDIN requires RhoA ADP-ribosyltransferase activity.

An animal study showed that following bacteremia, *S. aureus* cells preferably adhere to capillaries and postcapillary venules (20). Postcapillary venules form a zone with a high level of exchange between the blood and tissues. They are particularly enriched in fenestrae and large discontinuities or gaps (20). By exposing matrix proteins, these gaps may offer to *S. aureus* cells both a surface to which they can bind and a way to disseminate into the surrounding tissue. Other ways of endothelium crossing, such as transcytosis, might also participate in bacterial dissemination. *S. aureus* can bind to endothelial cell α5β1-integrin via fibronectin and the bacterial fibronectin-binding proteins (32). Nevertheless, especially in light of the fact that bacteria entering endothelial cells can be killed on a massive scale by the bactericidal activity of lysosomes, the efficiency of *S. aureus* transcytosis remains unclear. Rather than providing a mechanism of cell invasion, it has been proposed that this phenomenon dramatically increases the mobility of bacteria at the endothelial cell surface and postpones their endocytosis (30). It was suggested that this might give bacteria enough time to produce cell-damaging toxins in order to survive and/or disseminate (29). It is possible that EDIN plays a key role at this step, allowing bacteria to dig transcellular tunnels and

FIG. 6. Histopathology of bioluminescent foci. Selected renal (A and B), osseous (C and D), and sacral (E and F) infection foci are shown. BALB/c mice were injected intravenously with $5 \times 10^7$ CFU/animal of Xen36-EV (control; A and C), Xen36 EDIN-Wt (B, D, and F), or Xen36 EDIN-RE (E). Pictures of mice show the localization of infection foci analyzed (arrows), as well as their relative intensities. Microscopic examination of selected foci shows the acute inflammatory process. Magnifications: ×200 (A and B), ×40 (C and D), and ×100 (E and F).
allowing them to access the endothelium basement membrane directly. In addition, EDIN might enlarge preexisting pores present in postcapillary venules. Consistent with this, it has been shown that the inhibition of RhoA in necrotic hepatic sinusoidal endothelial cells leads to a dilatation and fusion of fenestrae into large gaps (40).

Together, our data ascribe to EDIN an invasive virulence property during staphylococcal bacteremia.

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