Meningococcal Interaction to Microvasculature Triggers the Tissular Lesions of Purpura Fulminans

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Neisseria meningitidis is a strict human pathogen that closely interacts with human endothelial cells via type IV pili in vitro. To decipher whether this interaction plays a role in vivo, we set up an experimental model of fulminant meningococcemia in human skin grafted SCID mice using the wild-type strain 2C4.3. Human skin and mouse tissues were sampled 24 hours after bacterial challenge for histopathology, immunohistochemistry and ultrastructural analysis. In all infected mice, N. meningitidis targeted the human vasculature, leading to bacterial and blood thrombi, infectious vasculitis and vascular leakage. Mouse vessels, including brain vessels, remained unaffected by the infectious and thrombotic process, and a nonpiliated Δ pilE derivative of 2C4.3 failed to target human graft vessels and to induce vascular damages. These data demonstrate that N. meningitidis targets human endothelial cells in vivo and that this interaction triggers the vascular damages that characterize purpura fulminans.

Keywords. Neisseria meningitidis; purpura fulminans; infectious diseases; vascular infection; endothelial cells; xenograft model; SCID mice.

Neisseria meningitidis is a strict human pathogen that is responsible for 3 major clinical syndromes, including chronic meningococcemia, the indolent form of the disease, and 2 life-threatening infections: cerebrospinal meningitis and purpura fulminans [1]. The latter is a septic shock with disseminated intravascular coagulation and extensive dermal thrombosis leading to skin necrosis [2]. Although various bacterial infections have been associated with purpura, these lesions are highly specific of meningococcemia as they are observed in a vast majority of patients, independently of the clinical severity of the disease [3–6].

The pathophysiology of meningococcal invasive diseases is a complex multistep process. N. meningitidis is a natural inhabitant of the human oropharynx, and the reason why this commensal has the ability, in some circumstances, to disseminate into its host remains mostly unknown. Once into the bloodstream, specific virulence factors allow N. meningitidis to survive in the extra-cellular fluids, such as the capsule, the lipo-oligosaccharide, the iron chelation systems, and the newly identified factor H binding protein [7, 8]. These bacterial attributes are essential to resist polymorphonuclear cell phagocytosis and complement mediated lysis and to control the level of bacteremia. Other bacterial attributes are believed to be essential for meningococcal invasion of peripheral tissues. Postmortem samples and biopsies sampled in patients with meningococcemia have shown that N. meningitidis interacts with endothelial cells of the microvasculature throughout the body, including the brain and the skin, and forms colonies on the apical surface of endothelial cell [9–13]. These findings suggest that bacterial adhesion to endothelial cells is
the first step of peripheral tissues invasion by N. meningitidis [9–11, 14]. The only mean by which encapsulated meningococci can interact with human cells is via type IV pili (tfp) [15]. This adhesion is specific for human cells and can occur on both endothelial and epithelial cells [16]. In vitro, following their adhesion via tfp, bacteria multiply on the apical surface of endothelial cells, forming small colonies [17] that are similar to those observed in human samples. These colonies induce signaling events in endothelial cells that lead to the recruitment of signal transducing proteins and cortical actin, and to the formation of microvilli-like structures below bacterial colonies. In vitro, this strong bacterial adhesion confers resistance to shear stress as observed in the bloodstream [14, 18].

However, the human specificity of N. meningitidis pathogenicity still hampers our understanding of the pathogenesis of meningococcal invasive diseases. In some circumstances, mice and infant rats have been used to assess the ability of the bacteria to survive in the extracellular fluids [19–21]. Unfortunately, these models do not replicate the peripheral meningeal and skin lesions observed during human meningococccemia. Hence, the exact role of bacterial-endothelial cell interactions in the formation of the peripheral lesions associated with meningococccemia, and particularly skin purpuric lesions, remains not fully understood.

To address this issue, we used for the first time to our knowledge in bacterial pathogenesis studies an experimental model of meningococccemia in severe combined immunodeficiency (SCID) mice grafted with human skin [22]. In this work, we demonstrate that N. meningitidis binds specifically to human endothelial cells in vivo in a type IV pilus dependent manner, and that this interaction is responsible for graft tissue lesions that are similar to those observed in humans meningococccal purpura.

**MATERIAL AND METHODS**

**Bacterial Strains**

We used the serogroup C meningococcal strain designated 2C4.3, and a nonpiliated Δ pilE deleted isogenic mutant (Nm Δ pilE) that was constructed by inserting a kanamycin resistance cassette in the pilE gene locus [23]. The N. meningitidis strain 2C4.3, also known as clone 12 of the clinical N. meningitidis strain LNP8013, is a naturally occurring pilin antigenic variant of the original clinical isolate LNP8013, which expresses a pilin mediated high adherence to human cells. N. meningitidis strains were stored frozen at −80°C in Giolitti-Cantoni broth (GCB) supplemented with 30% glycerol.

**Mice**

Six week-old CB17/1cr-Prkdcscid/lcrLc0Crl SCID female mice were purchased from Charles River Laboratories (Saint Germain sur l’Arbresle, France). Experimental procedures were performed in sterile conditions, and in accordance with the guidelines of the Institut National de la Santé et de la Recherche Médicale (INSERM). The experimental protocol was approved by the Animal Experimentation Ethics Committee of the Université Paris Descartes (study registered under number CEEA34.OJ.L.039.12).

**Graft Protocol**

We used the experimental model described by Yan et al [22] that was initially designed to study the expression and role of endothelial cell adhesion molecules for white blood cell migration. Skin tissues were obtained from adult patients undergoing plastic surgery in the Saint-Joseph Hospital (Paris, France). In accordance with French legislation, human skin was obtained from patients who were informed and did not refuse to participate in the study. Mice were engrafted using a skin flap procedure [24]. Briefly, mice were prepared for transplantation by shaving the hair of the back and abdominal areas after an intraperitoneal injection of ketamine 100 mg/kg and xylazine 10 mg/kg. A skin flap was created and a full thickness human skin graft was placed onto the wound bed. The transplants were held in place with 6–0 nonabsorbable monofilament suture materials, and the flap was then sutured above the transplant. Grafted mice were used for N. meningitidis infection experiments 1 month after human skin transplantation.

**Infection Protocol and Bacterial Counts**

In vivo, iron-binding proteins such as transferrin and lactoferrin restrict the amount of ferric iron available in body fluids to a level that does not support meningococcal growth [25]. To counteract these iron-deficient conditions, N. meningitidis has several iron acquisition systems, such as transferrin binding proteins A and B that are induced when bacteria are grown under iron-deficient conditions [26]. These proteins are specific of human transferring. Therefore, bacterial strains were grown under iron-deficient conditions before each infectious challenge, and human holotransferrin was added to the bacterial inoculum to optimize bacterial growth in vivo [27, 28]. Briefly, N. meningitidis strains were grown overnight at 37°C on GCB agar plates prepared without iron and supplemented with deferoxamine (Desferal, Novartis) at a final concentration of 15 µM. Kanamycin (100 µg/mL) was added to the medium to grow the kanamycin resistant Δ pilE strain. Bacterial colonies were harvested and cultured in RPMI with 1% bovine serum albumin medium and 0.06 µM deferoxamine with gentle agitation to reach the exponential phase of growth. Bacteria were then resuspended in physiological saline containing 20 mg/mL of human holotransferrin to promote bacterial growth in vivo (2914HT, R&D systems) [28]. Preliminary experiments showed that a 100-fold-higher inoculum was necessary to obtain a sustained bacteremia by intravenous injection of bacteria, as compared to intraperitoneal route (data not shown). Mice were therefore infected intraperitoneally with 0.5 mL of this bacterial
suspension to minimize the inoculum required to obtain a reproducible high bacteremia in grafted mice.

To assess bacteremia in infected animals, 10 µL of blood was sampled using a heparinized hematocrit glass tube after puncture or the lateral tail vein, or at the time of death by intracardiac puncture after intraperitoneal injection of a lethal dose of ketamine and xylazine. We used a dermatological punch-biopsy system to assess human skin bacterial load. Biopsies were then homogenized in RPMI medium. Bacterial counts were performed by plating serial dilutions of blood or of skin graft homogenates on GCB agar plates. Results were expressed in colony-forming units (CFU) per mL of blood or per skin punch biopsy.

Histopathology and Immunohistochemistry

Mice were killed 24 hours after infection. Mouse organs (liver, spleen, brain) and skin grafts were carefully removed, fixed in 10% buffered formalin and embedded in paraffin.

Hematoxylin, eosin, and safranin staining and immunohistochemistry were performed on 5-µm tissue sections using the Leica BOND-MAX automated system (Leica-microsystems, Wetzlar, Germany). The following primary antibodies were used: monoclonal mouse anti-human CD31 (1:50, clone JC70A, Dako M0823), monoclonal mouse anti-human CD31, and a rabbit polyclonal serum anti N. meningitidis 2C4.3 strain (1:3000). Noninfected graft sections were used as controls. Epitope retrieval was performed using Bond ER1 (Leica Microsystems, AR9961). Primary antibodies were revealed using horseradish peroxidase and DAB (Leica Microsystems, DS9390 and DS9800). The preparations were counterstained with hematoxylin. Images were visualized with a Nikon microscope and were processed using the Adobe Photoshop software (Adobe, Mountain View, California).

Semithin Sections and Transmission Electron Microscopy of Infected Human Skin Grafts

Skin graft biopsies were fixed with 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4), rinsed in Sorensen buffer, postfixed in 2% aqueous OsO4, dehydrated in graded ethanol solution, and embedded in Epon. Semithin sections were stained with 1% toluidine blue and 2% safranin. Ultrathin sections were counterstained with uranyl acetate and 2% lead citrate. Examination was performed with a 1011 transmission electron microscope (Jeol Inc, Japan). Acquisition and processing were performed with an Erlangshen CCD camera (Gatan Inc, California) and Digital Micrograph software (Gatan, California).

Statistical Analysis

Statistical analyses were performed using Student t-test. A P value < .05 was considered statistically significant.

RESULTS

N. meningitidis Specifically Interacts With Human Skin Graft Vessels

We first determined the optimal inoculum to induce a sustained bacteremia in SCID mice with the wild-type N. meningitidis strain 2C4.3. Mice infected with >10^4 CFU developed a bacteremia as early as 1 hour after bacterial challenge, and the death of animals occurred 36–48 hours thereafter (Figure 1A). In humans, the level of bacteremia strikingly correlates with the severity of the disease, and a high level of bacteremia is associated with purpura fulminans and meningococcal septic shock [29, 30]. Consequently, all subsequent infectious experiments in human skin grafted SCID mice were performed using 10^6 CFU of bacteria, as this inoculum allowed to obtain a reproducible immediate high and sustained bacteremia in all infected animals.

Then we determined whether N. meningitidis targeted the skin grafts microvasculature. Twenty-four hours after the infectious challenge, animals were killed, and human skin grafts as well as mouse organs were sampled to detect N. meningitidis by immunohistochemistry. In these experimental conditions, no macroscopic lesions of human skin grafts were observed (Figure 1B). However, extracellular bacteria adhering to the intimal surface of endothelial cells of capillaries and post capillary venules were seen in all human skin graft sampled from infected animals (9/9 skin grafts obtained from 3 different experiments). These colonies usually underlined the entire endoluminal surface of vessels, or in some circumstances completely occluded the vascular lumen (Figure 1C). Immunostaining of sections containing both human skin graft and the subcutaneous mouse panniculus carnosus demonstrated that N. meningitidis only colonized graft vessels that were exclusively from human origin as demonstrated by a positive specific human CD31 antigen staining (Figure 1D). Accordingly, in other mouse organs including skin, brain, liver, and spleen, N. meningitidis antigen could only be detected within the cytoplasm of phagocytic cells (Supplementary Figure 1). These data demonstrate that N. meningitidis targets endothelial cells in vivo, and that this interaction is specific of human tissues.

N. meningitidis Endovascular Colonization Induces the Vascular Damages Observed in Purpuric Lesions of Patients With Meningococcemia

We next investigated the pathological consequences of N. meningitidis colonization of human skin graft vessels. Vascular lesions were similar to those described in patients, including thrombosis of dermal vessels, perivascular infiltrates, and vascular leakage (Figure 2A) [3, 10, 12, 31]. Inflammatory cells, including mononuclear cells and neutrophils, surrounded capillaries located in the superficial and mid dermis and infiltrated their walls (Figure 2A and 2B). These vessels showed endothelial swelling and sometimes fibrinoid necrosis of their walls.
Bacteria were often seen in the cytoplasm of recruited phagocytic cells (Figure 2C). Massive endovascular bacterial colonization was associated with cell wall disruption and blood thrombus formation (Figure 2D through 2G). Vascular occlusion predominated in the deep layers of the dermis and no cellular inflammation was paradoxically observed within the lumen of massively infected vessels and in the surrounding dermis as reported in early purpuric lesions in humans [8, 10]. Extravasation of red blood cells associated to hemosiderin deposits could be observed in the vicinity of intravascular infection, reflecting the first step of purpuric lesions (Figure 2G). It should be pointed out that in mouse organs, no histological lesion was observed (Figure 2H) as assessed by HES staining. Semithin sections of infected human skin grafts demonstrated that Neisseria meningitidis colonization of endothelial cells did not lead to the recruitment of inflammatory cells in vessels with preserved architecture (Figure 2I through 2K). Alternatively, recruitment of phagocytic cells was observed in vessels associated with the presence of extravascular bacteria (Figure 2L), suggesting that vasculitis lesions probably occur secondary to bacterial release into the dermis.

**N. meningitidis Disrupts the Endothelial Wall**

We further investigated the ultrastructural features of infected grafts by transmission electron microscopy (Figure 3). Single cell adhesion of *N. meningitidis* to endothelial cells induced a localized remodeling of the apical cell membrane, and no internalized bacteria could be observed, thus mimicking observations obtained after short incubation times (4 hours) in vitro [17, 32]. In that case, the endothelial basal membrane, the basal lamina, and pericytes were not altered (Figure 3A). In those vessels partially colonized or occluded by bacteria, more pronounced endothelial cell alterations were observed including the formation of vesicular structures (Figures 3B and 3C) and vacuolization of the cytoplasm (Figure 3D), leading to complete disruption of the endothelial cell wall (Figure 3E). Extracellular bacteria (Figure 3F) and bacteria eventually localized within the

**Figure 1.** *Neisseria meningitidis* targets human skin graft endothelial cells. A, Susceptibility of SCID mice to *N. meningitidis* after intraperitoneal injection of *Nm* 2CA.3 wild-type strain (3 animals per group). All mice infected with >10⁴ CFU developed systemic meningococcemia and died within 36 to 48 hours. B, Human skin graft as observed at the time of infection. C and D, Meningococcal colonization of human skin grafts vessels as assessed 1 day after bacterial challenge (10⁸ CFU) by immunohistochemistry. C, *N. meningitidis* (red) colonizes human skin graft vascular structures (brown, pericytes) including capillaries and venules, sometimes leading to complete vascular occlusion. D, Transversal section including both human skin graft and mouse muscular subcutaneous tissue (D1, magnification: ×400). Arrows show colonized vessels (red). D2 through D7: High magnification (× 1000) of mouse and human graft vessels after double immunostaining against meningococcal antigens (rabbit *N. meningitidis* antiserum, red) and human CD31 (brown). D2: Human CD31⁺ mouse vessels are not colonized by *N. meningitidis*. D3: Human CD31 staining on an uninfected human vessel. Infected graft vessels (D4–D5) are all positive for human CD31 antigen. Abbreviation: L: Vascular lumen.
cytoplasm of phagocytic cells (Figure 3G) could also be observed in the dermis along with red blood cell, demonstrating vascular leakage. Finally, bacterial adhesion to endothelial cells was seen in association with thrombus formation as demonstrated by the presence of fibrin strands (Figure 3H).

**N. meningitidis Adhesion to Human Endothelial Cells and Vascular Lesions Require Type IV Pili**

Considering the role of tfp in bacterial endothelial cells interaction in vitro, we next aimed at defining the role of type IV pilus in the pathogenesis of these lesions. We subsequently tested the ability of a capsulated nonpiliated Δ pilE derivative of *N. meningitidis* 2C4.3 to target the graft vasculature (Figure 4). Infection with 10⁶ CFU of the nonpiliated strain resulted in a similar level of bacteremia as compared to that of the wild-type strain, demonstrating that the pilE deletion was not associated with a decreased virulence in grafted SCID mice. However, the nonpiliated strain failed to target the human skin graft as demonstrated by a significant lower bacterial load within the grafted skin (Figure 4A). Immunohistochemistry revealed sparse intravascular circulating *Nm* antigen-positive phagocytic cells within human skin grafts (Figure 4B–D), such as those observed in

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**Figure 2.** *Neisseria meningitidis* colonization of the human skin grafts vasculature reproduces the pathological findings of meningococcal purpura. A, Typical pathological findings of infected human skin grafts (HES staining), including vessel thrombosis (T) and vasculitis (V). B, Vessels with vasculitis features were lined by swollen endothelial cells, showed fibrinoid necrosis of their walls and were surrounded and penetrated by inflammatory cells. C, Vasculitis lesions were associated with the presence of bacteria, often within the cytoplasm of phagocytic cells (arrows show bacteria). D–F, *N. meningitidis* vascular colonization led to the formation of bacterial thrombi (D, vessel disruption [E] and blood thrombi [F]). G, Release of red blood cells into the surrounding dermis (arrowheads) and hemosiderin pigments (hs) demonstrate vascular leakage. H, Thrombus formation and vascular damage was not observed in mouse organs of infected mice. I–K, Semithin sections of infected human skin grafts demonstrate that meningococcal vascular colonization occurs without recruitment of inflammatory cells (arrows show bacteria). L, Recruitment of phagocytic cells was only observed in vessels with damaged architecture and associated with the presence of extravascular bacteria (arrows: extravascular bacteria; arrowheads: phagocytic cells). Abbreviation: RBC: red blood cells.
mouse organs. Furthermore, no tissue or vascular lesions could be observed within the human skin grafts. Altogether these data strongly suggest that bacterial-endothelial cell interactions are required to initiate meningococcal associated skin lesions in vivo.

**DISCUSSION**

In this work, we show for the first time that *N. meningitidis* targets the human vasculature in vivo and confirm both the human specificity and the critical role of ttp for interaction of capsulated *N. meningitidis* isolates with endothelial cells [33, 34]. Experimental histopathological findings strikingly correlated with those observed in the skin lesions of humans [3, 10, 12, 31], demonstrating the clinical relevance of this xenograft model of invasive meningococcal disease. In our experimental conditions, meningococcal colonization of skin grafts vessels and related vascular damages were easily observed by histopathology in all infected animals. However, purpuric lesions were not visible at the time of death. In our view, this finding reflects the fact that the histological lesions we observed are early events in the genesis of purpuric lesions. Most of them were located in the deep dermis, thus without superficial expression.

The new insight revealed by our model is that skin lesions associated with meningococcemia are induced by the colonization of the microvasculature by *N. meningitidis*. Indeed, although intense research efforts accomplished during the last 2 decades revealed the mechanisms and consequences of *N. meningitidis*-endothelial cells interactions in vitro, the clinical relevance of these data was still poorly established [2]. It was first postulated that these pathological findings could be a direct consequence of tissular invasion by *N. meningitidis* as bacteria have been found often in large numbers within skin purpuric lesions [31]. The pathogenesis of meningococcal-associated purpura was later attributed to a local Sanarelli-Shwartzman reaction, an endotoxin- and cytokine-primed vasculitis mediated by adhesion and degranulation of activated neutrophils on endothelial cells [35, 36]. This concept was drawn from experiments performed in nonhumanized models of meningococcemia. In these conditions, likely because of the human specificity of *N. meningitidis*-endothelial cells interactions, a preliminary intradermal injection of bacterial components was necessary to induce

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**Figure 3.** *Neisseria meningitidis* adhesion to human graft endothelial cells induces vascular damages. A, Single cell adhesion of *N. meningitidis* to endothelial cell induces a localized remodeling of the endothelial cell endoluminal membrane in a vessel with conserved architecture, including the basal lamina and pericytes. *N. meningitidis* appears as round shaped structures of 0.5–0.7 µm in size with a peripheral electron dense layer. B–E, Severe vessel colonization induces vascular damages. The cytoplasm of endothelial cells is fragmented into vesicular structures (white arrows, B, transversal section, C, oblique section) or vacuolated (D, frontal section, black arrows), leading to complete disruption of the endothelial cell wall (E). F and G, Free bacteria and red blood cells can be observed without any residual vascular structures, demonstrating severe vascular damage (F) and within recruited phagocytic cells (G). H, Adhesion of *N. meningitidis* to endothelial cells can be associated with localized thrombus formation as demonstrated by the presence of fibrin. The endothelial cell on the left is highly vacuolated. Black and white arrowheads: basal lamina. Abbreviations: Coll, collagen fibers. EC, endothelial cell. M, macrophages. *Nm*: *Neisseria meningitides*; PN, polymorphonuclear cells. RBC, red blood cells.
intradermal vessel thrombosis associated with a dense vascular and perivascular infiltration of neutrophils [36]. Interestingly, De Voe et al [8] highlighted that the recruitment of inflammatory cells in human skin biopsies is not observed at the early stage of the disease. In our view, our results provide the missing piece of the puzzle, reconciling these two historical hypotheses and demonstrating that the first event that triggers purpuric lesions is meningococcal vascular colonization.

Our results also provide data on the kinetics and pathogenesis of these lesions. As demonstrated by semithin sections and transmission electron microscopy, single cell adhesion did not induce significant vascular damage. In addition, vascular colonization did not lead to intravascular recruitment of phagocytes, thus permitting an intravascular accumulation of bacteria that could lead to thrombus formation and necrosis of the vascular wall. These data are in agreement with the results obtained by Doulet et al [37], who showed that bacterial adhesion to human endothelial cells in vitro impairs the recruitment of human leukocytes by preventing the formation of endothelial docking structures. Hence, colonized vessels may be a protected site for bacterial survival in vivo.

Our data also demonstrate that meningococcal vascular colonization is required to initiate the thrombotic process that characterizes purpura fulminans and is usually only considered as a consequence of disseminated intravascular coagulation and sepsis [38, 39]. In our experimental model, thrombus formation could either result from blood flow arrest in those vessels occluded by bacteria or result from the cytopathic effect of bacteria on the endothelial wall, thus exposing the subendothelial tissue factor to the vascular lumen. In vitro, the meningococcal lipopolysaccharide (LPS) exerts a cytotoxic effect on endothelial cells that is increased by tdp dependant adhesion, suggesting increased local delivery [32, 40, 41]. Bacterial adhesion also induces a rapid prothrombotic response similar to that obtained using high concentrations of purified endotoxin [42]. Finally, vascular disruption led to extravasation of erythrocytes and bacteria within the dermis and was associated with vasculitis. These findings provide an explanation for the fact that purpuric lesions occur independently of the severity of the disease [2, 10]. In patients with purpura fulminans that are characterized by a high level of bacteremia [7], the unusual severity of skin lesions may be due to massive vascular colonization.

Altogether this study demonstrates the tropism of N. meningitidis for human vessels in vivo, and that pilus-mediated interaction with the microvasculature is responsible for the specific clinical cutaneous presentation of systemic meningococcal diseases. The use of this chimeric model will allow a detailed molecular understanding of the tissular events that lead to thrombus formation, vasculitis, and subsequent vascular damages. A better understanding of the pathogenesis of these lesions will open the path to specific treatments of what remains a dreadful epidemic disease.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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