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Summary

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The brain and meningeal spaces are protected from bacterial invasion by the blood brain barrier, formed by specialized endothelial cells and tight intercellular junctional complexes. However, once in the bloodstream, *N. meningitidis* crosses this barrier in about 60% of the cases. This highlights the particular efficacy with which *N. meningitidis* targets the brain vascular cell wall. The first step of central nervous system invasion is the direct interaction between bacteria and endothelial cells. This step is mediated by the type IV pili, which induce a remodeling of the endothelial monolayer, leading to the opening of the intercellular space. In this review, strategies used by the bacteria to survive in the bloodstream, to colonize the brain vasculature and to cross the blood brain barrier will be discussed.

Overview

Neisseria meningitidis (meningococcus) is a Gram-negative coccus restricted to humans that is responsible for two major diseases, cerebrospinal meningitis and/or *purpura fulminans* (*i.e.* extensive necrotic *purpura* with massive vascular leakage and multiple organ failure) an often-fatal condition due to the associated septic shock. Paradoxically, *N. meningitidis* is a common inhabitant of the human nasopharynx, and as such is a normal, saprophytic organism that is transmitted from person to person by direct contact. The mechanisms responsible for nasopharyngeal colonization and crossing of the nasopharyngeal mucosa remain mostly unexplained. In a small proportion of colonized subjects *N. meningitidis* invades the bloodstream where circulating bacteria can colonize human vessels to cause meningitis and *purpura fulminans*. It has been demonstrated that *N. meningitidis* shows a tropism for microvessels. This indicates that *N. meningitidis* adhere to microvessels endothelial cells throughout the body.

Invasive meningoccal diseases can be classified in distinct clinical presentations (Jensen et al. 2003; de Greeff et al. 2008; Parent du Chatelet et al. 2011; Brandtzaeg and van Deuren 2012): **(i)** Meningitis without septic shock is the most frequent presentation that concerns about 45% of infected people (mortality rate from 1% to 5%); **(ii)** Meningitis associated with septic shock are observed in about 15% of the patients; **(iii)** Shock without meningitis is seen

in about 15% of the cases, mostly associated with the development of *purpura fulminans*. *Purpura fulminans* has a mortality rate of about 25%. Interestingly, there is a close relationship between the level of endotoxemia or circulating bacterial DNA and the clinical presentation of the disease. Indeed, patients suffering from meningitis only have less than 10^3 DNA copies/mL and less than 0.5 IU/ml circulating lipooligosaccharide, whereas patients suffering from septic shock have a median of 2.10^7 DNA copies/mL and 43 IU LPS/mL (Ovstebo et al. 2004). From these data, it can be speculated that the clinical forms of meningococcal disease mostly reflect the level of bacteremia.

In this review we will describe specific features of the blood brain barrier (BBB) and discuss the several steps of meningococcal invasion of the meninges that include (i) proliferation into the bloodstream, (ii) adhesion to the brain endothelium and formation of the colony, (iii) signaling to the cells, (iv) crossing of the endothelium and colonization of the surrounding tissues.

I. The Blood Brain Barrier

The blood brain barrier is a unique structure that tightly regulates the exchange of nutrients, solutes and water between the central nervous system (CNS) and the blood (Ballabh et al. 2004). As front line between the blood and the CNS, the CNS microvasculature is highly specialized and accounts for this particular BBB features (Reese and Karnovsky 1967). However, the brain endothelium is not an isolated structure and the blood brain barrier specificities of CNS endothelial cells are induced, and maintained by the surrounding cellular structures. It is therefore referred to as the neurovascular unit (Abbott et al. 2006). Endothelial cells from the CNS microvessels are surrounded by pericytes, which are themselves encased by the basal lamina (composed of laminins, collagen type IV, heparan sulfate proteoglycans, and nidogens). Pericytes, which are abundant along brain microvessels, are perivascular cells with multifunctional activities including maintenance of vascular homeostasis. The basal lamina of cerebral endothelium is also continuous with astrocytic end-feet that ensheath the brain microvessels. Astrocytes are considered to be

supporting cells in the brain providing trophic, metabolic, and structural support for neural networks (for review see: (Nag 2011)).

The cerebral endothelium forms a continuous cellular layer without any fenestration. The paracellular barrier is formed by interendothelial junctional complexes that restrict the free movement of hydrophilic compounds between adjacent cells. Junction complexes in the blood brain barrier comprise adherens and tight junctions that are concentrated at the apical side of endothelial cells (Weiss et al. 2009). Adherens junctions, described to initiate endothelial cell-to-cell contacts and to promote their maturation and maintenance, are composed of a cadherin–catenin complex and its associated proteins. Tight junctions are mainly composed of three transmembrane proteins (claudins, occludin, and Junction Adhesion Molecules) and a number of cytoplasmic accessory proteins including Zonula Occludens-1, -2, -3, cingulin, and others. Cytoplasmic proteins link membrane proteins to actin, which is the primary cytoskeleton protein for the maintenance of structural and functional integrity of brain continuous endothelium (Weiss et al. 2009).

The BBB controls exchanges between the blood and the cerebral compartment by preventing passive diffusion of hydrophilic solutes, mediating active transport of nutrients to the brain (Bickel et al. 2001). It also regulates transendothelial migration of circulating immune cells and prevents blood-borne pathogens to invade the brain (Engelhardt and Wolburg 2004).

In addition to the BBB, a second blood/CNS interface is formed by the epithelial cells of the choroid plexus facing the cerebrospinal fluid (CSF), which constitute the blood CSF barrier (Ghersi-Egea and Strazielle 2002). A third interface is provided by the arachnoid epithelium, underlying the dura, and completely enclosing the CNS. It completes the seal between the extracellular fluids of the CNS and that of the rest of the body. Nevertheless, their relatively small surface area means that it does not represent a significant surface for exchange between the blood and the CNS (Abbott et al. 2010).

II. Where to enter into the CSF?

Due to the lack of a reliable animal model, most hypotheses regarding the pathogenesis of meningococcal infections are derived from postmortem studies of patients who died from meningococcal meningitis or *purpura fulminans* or from biopsies of skin purpuric lesions (Pron et al. 1997; Faust et al. 2001; Harrison et al. 2002; Dupin et al. 2012). In the brain, data obtained from a patient who died of fulminant meningococcemia, at the time bacteria were invading the CSF, revealed that *N. meningitidis* adheres onto endothelial cells and forms small colonies in the lumen of the microvasculature. Bacteria are also found inside cells and in intercellular spaces (Pron et al. 1997; Mairey et al. 2006). These data suggest that *N. meningitidis* crosses the BBB through a direct interaction between bacteria and endothelial cells and do not need a Trojan horse such as leukocytes to cross the BBB. It has been thought that bacteria may preferentially enter the CSF via the choroids plexus. However, in this case, meningitis should theoretically be associated with ventriculitis, which is not corroborated by clinical and experimental data. Because of their proximity to the subarachnoidal space and their "leaky" interendothelial structure, the brain postcapillary venules and veins of the subpial and subarachnoid spaces located at Virchow-Robin spaces (Ransohoff and Engelhardt 2012) may be the site of passage of bacteria into the CSF (for review see Join-Lambert et al. 2010).

III. The blood phase

III.1 Proliferation into the bloodstream

To reach the brain microvessels *N. meningitidis* have first to survive in the bloodstream. Therefore, the bacterial attributes involved in growth and/or survival in the extracellular fluids are playing an essential role in meningeal invasion by *N. meningitidis*. Studies aimed at identifying factors responsible for bacterial survival, multiplication or adaptation in the bloodstream were based on the screening of libraries of transposon mutants using either an infant rat model (Sun et al. 2000) or survival in complemented serum (Geoffroy et al. 2003) as readouts. More recently, a transcriptomic analysis of bacteria grown in human whole

blood has been performed (Echenique-Rivera et al. 2011). These studies pointed out the major role of the virulence factors commonly observed in most extra-cellular pathogens. Beside iron chelation systems that are essential for pathogenic bacteria to obtain the necessary ferric iron *in vivo*, the polysaccharidic capsule, the lipooligosaccharide (LOS) and the recently described factor H binding protein participate in the prevention of bacterial killing by the complement.

III.2 Adhesion: an unknown cellular receptor for an unknown bacterial factor

The particular tropism of bacteria for endothelial cells is a major pathophysiological issue in meningococcal infection. The three main surface components that have been described to allow adhesion of *N. meningitidis* to human cells are type IV pili (Tfp), Opa and Opc proteins (Virji et al. 1993; Unkmeir et al. 2002; Virji 2009). However, in the bloodstream Opa and Opc are likely to be hidden under the polysaccharidic capsule and may not be able to interact with their receptors (Carcinoembryonic antigen-related cell adhesion molecules / CD66 ; and vitronectin or fibronectin that bind to cellular integrin, respectively). This is confirmed *in vitro* by the observation that a capsulated and non piliated strain that expresses Opa proteins is not able to adhere efficiently to endothelial cell in static or flow condition ((Hardy et al. 2000) and personal observation, respectively). Thus, in the bloodstream, Tfp are the main bacterial attributes capable of promoting adhesion. Type IV pili are polymeric filaments found on many Gram-negative bacteria (Wolfgang et al. 2000). These structures consist of the multimeric assembly of the major pilin PilE which are continuously assembled into fibers from a platform in the inner-membrane (for review see (Carbonnelle et al. 2009)). Three other minor pilins are localized into the fiber: ComP, PilX and PilV. Each minor pilin allow a specific phenotype *i.e.* competence for DNA transformation, bacterial aggregation and signaling to human cells, respectively (Winther-Larsen et al. 2001; Helaine et al. 2005; Mikaty et al. 2009; Brown et al. 2010).

Initial attachment of *N. meningitidis* adhesion requires both Tfp and adequate flow shear stresses. *In vivo*, blood flow generates mechanical forces that vary depending on the

vessels. Only a very low blood flow, which is present in capillaries and microvessels, will enable the adhesion of *N. meningitidis* to the endothelial cells (Mairey et al. 2006). This highlights the efficacy of *N. meningitidis* to colonize capillaries in the brain and the skin (Pron et al. 1997; Faust et al. 2001; Harrison et al. 2002; Dupin et al. 2012). To date, the cellular receptor responsible for pilus mediated adhesion remains unknown. The CD46 receptor was proposed as being the adhesion receptor for both *Neisseria gonorrhoeae* and *N. meningitidis* Tfp. However, this finding has not been confirmed by subsequent studies (Kallstrom et al. 1997; Kirchner et al. 2005). The Laminin receptor was also described as a potential receptor for *N. meningitidis*. Two bacterial ligands for this receptor have been reported, the PilQ secretin and the PorA protein (Orihuela et al. 2009). However considering that PilQ is expressed in non piliated mutants and that non-piliated non-capsulated strains are unable to interact with endothelial cells, its role remains to be determined. The I-domain-containing integrins were described to be essential for *N. gonorrhoeae* adhesion to primary urethral epithelial cells (Edwards and Apicella 2005), but similar data have not been reported for *N. meningitidis* and endothelial cells.

Type IV pili mediated adhesion is dependent on a Tfp associated protein *i.e.* PilC. It was suggested that PilC directly interact with the cellular receptor (Rudel et al. 1995). However, non-adhesive non-piliated isolates of a serogroup B strain with high PilC expression have been described, as well as piliated adhesive isolates with barely detectable PilC expression (Virji et al. 1995). This raises doubts about the role of PilC as an adhesin. The minor pilin PilV was also described to be important for adhesion of gonococci. Interestingly, a PilC mutant is not able to incorporate PilV into the Tfp (Winther-Larsen et al. 2001). It is therefore likely that the cell binding domain on the Tfp remains to be identified.

III.3 Colonization of the endothelium

To further grow on top of the apical surface of endothelial cells the bacteria must resist shear stress. This is dependent on both bacterial aggregation and signaling to the endothelial cells that lead to the formation of microvilli surrounding the colony, protecting it from the flow. The

minor pilin PilX is essential to promote inter-bacterial interactions. Thus, *pilX* mutants are unable to form aggregates or colonies on endothelial cells (Helaine et al. 2005), while a hypo-aggregative strain is also unable to form large colonies (Nassif et al. 1993). Measurements using optical tweezers showed that retraction of a single Tfp generates forces of up to 110 pN, in a transient manner for each fiber. Bundles of Tfp, which result from the association of 8 to 10 pili, act as coordinated retractable units. Thus, bundles can generate retraction forces in the nanonewton range (Biais et al. 2008). These forces allow the transition of the tfp into a new conformation, longer and narrower than the usual structure (Biais et al. 2010). This transition reveals new epitopes like the SM1 epitope (residues 49-53 of mature pilin: EYYLN), previously shown to be buried into the fiber. Brissac *et al* have recently shown that upon adhesion to endothelial cells, the SM1 epitope is revealed and this correlates with Tfp induced signaling to the cell. Interestingly, PilX is required for this transition to occur (Brissac et al. 2012). A strain lacking the minor pilin PilV is also defective in cell signaling and cannot resist shear stress *in vitro* (Mikaty et al. 2009). To detach from the growing colonies and colonize new niches, bacteria can modulate their aggregative properties by adding a phosphoglycerol molecule, onto the major pilin protein, that inhibits the formation of bundles of pili (Chamot-Rooke et al. 2011).

IV. How to penetrate the meninges?

Following the interaction of *N. meningitidis* with endothelial cells, the bacteria have at least four strategies to cross the brain endothelium: (i) transcellular transport, (ii) paracellular passage through opened tight junctions, (iii) cytoxic effect on the barrier and (iv) trojan horse strategy (i.e. infected phagocytes used to be transported across the barrier). Extracellular pathogens, such as *N. meningitidis*, do not use leukocytes as vehicles to cross the blood-CNS barrier. A breakdown of the blood-CNS barrier due to apoptosis or bacterial cytotoxity is unlikely, since tissue lesions, such as hemorrhages in the subarachnoidal space, are uncommon during bacterial meningitis. *In vitro,* Tfp dependent adhesion of *N. meningitidis* to endothelial cells promotes a cellular signaling responsible for the formation of microvilli and

the opening of the intercellular space between cells. Interestingly, a recent study of skin biopsies indicated that *N. meningitidis* is able to open the paracellular route *in vivo* (Dupin et al. 2012). Thus *N. meningitidis* is likely to hijack endothelial signaling pathways and open the paracellular pathway.

Independently of adhesion, the β2-adrenergic receptor was shown to be recruited to the site of bacterial cell interaction and was identified as an important signaling receptor for *N. meningitidis* (Coureuil et al. 2010; Lecuyer et al. 2012). Desensitization of the β2-adrenergic receptor in endothelial cells inhibits meningococcal induced signaling, while the expression of the β2-adrenergic receptor in an incompetent cell line for signaling is sufficient to promote a *N. meningitidis* induced cell response (Coureuil et al. 2010). The β2-adrenergic receptor is a G protein coupled receptor (GPCR) that signals via the heterotrimeric Gαs protein and β–arrestins. This adrenaline receptor is also known for its role in vascular homeostasis and disease. It has been shown that PilE and PilV directly interact with the extracellular N-terminal domain of the β2-adrenergic receptor to transmit the signal (Coureuil et al. 2010). This interaction is believed to modify the conformation of the receptor, resulting in the activation of β-arrestin-mediated signaling without activating the heterotrimeric Gαs protein and the downstream adenyl cyclase/cAMP pathway, a property referred to as biased activation (Drake et al. 2008; Coureuil et al. 2010). β-arrestins are scaffolding proteins involved in many cellular processes such as receptor internalization, MAP Kinase activation and actin polymerization (Scott et al. 2006; DeWire et al. 2007). Accumulated β-arrestins underneath microcolonies should play a major role in the sequestration of these signaling molecules.

IV.1 The cortical plaque: accumulation of proteins at the site of bacterial adhesion

Following this initial event of signaling, β2-adrenergic receptor and β-arrestins will accumulate at the site of adhesion, leading to the formation of a “raft-like” membrane domain

enriched in cholesterol and PIP2 (Mikaty et al. 2009). Production of PIP2 is critical during meningococcal induced signaling ((Doulet et al. 2006) and S. Bourdoulous, unpublished observations). Nevertheless, how PIP2 is produced at the site of bacterial adhesion is unknown. Two pathways may be involved downstream of the β2-adrenergic receptor and β-arrestins pathway (β2AR-βarrs), the recruitment of the phosphoinositide 5-kinase (PIP5K) which catalyze the formation of PIP2 from PIP (Oude Weernink et al. 2007), and the recruitment of PTEN, via the β-arrestins, which catalyze the production of PIP2 from PIP3 (Lima-Fernandes et al. 2011). Subsequently, the local production of PIP2 is necessary for the recruitment of ezrin (Fievet et al. 2004). Once activated by the β2AR-βarrs pathway, (Cant and Pitcher 2005; Coureuil et al. 2010) phosphorylated ezrin sequesters transmembrane receptors, organizes actin filaments and links the cortical actin network to the membrane (Doulet et al. 2006; Coureuil et al. 2010; Fehon et al. 2010), thus leading to the accumulation of many factors in a structure referred to as the cortical plaque (Merz et al. 1999).

IV.2 The activation of the Rho-GTPases controls actin polymerization and opening of the paracellular pathway.

The formation of the cortical plaque is accompanied by the activation of small GTPases of the Rho family *i.e.* Cdc42, Rac1 and RhoA (Eugene et al. 2002; Lambotin et al. 2005). GTPases control diverse cellular functions such as polarity, migration, endocytosis, and cell cycle progression. GTPases activate a robust stimulation of actin polymerization that leads to the elongation of membrane protrusions around bacteria (Eugene et al. 2002). Interestingly, the formation of such protrusions was also observed *in vivo* (Pujol et al. 1997). The pathophysiological role of these projections is believed to be associated with both the increase of the cell membrane surface to favor adhesion of the bacteria, and the shielding of growing microcolonies against shear stresses in the bloodstream (Mairey et al. 2006).

Actin polymerization and the formation of membrane protrusions rely on the recruitment of the polarity complex Par6/Par3/PKCζ that relocalizes the Cortactin-Arp2/3 complex at the site of bacterial adhesion (Coureuil et al. 2009). Cortactin (or cortical actin binding protein), is a perinuclear cytoplasmic protein that is involved in the reorganization of the cortical actin cytoskeleton. Cortactin interacts directly with the Arp2/3 complex (*i.e.* an actin nucleation complex) to promote actin branching and polymerization (Uruno et al. 2001; Coureuil et al. 2009). In addition to its recruitment, cortactin has to be activated. This activation is controlled by the tyrosine kinase Src that is sequestered in the cortical plaque. Interestingly, Src recruitment is independent of the Cdc42-Par6/PKCζ pathway. After infection, Src is recruited by direct interaction with the β-arrestins (Luttrell et al. 1999; Coureuil et al. 2010) and regulated by ErbB2 tyrosine kinase receptor that subsequently regulates cortactin phosphorylation (Hoffmann et al. 2001). The ErbB2 tyrosine kinase receptor belongs to the family of the epidermal growth factor (EGF) receptors. The interaction of *N. meningitidis* with human endothelial cells leads to ErbB2 activation in the cortical plaque most likely via homo-dimerization. This is an example of a secondary signaling activated by the accumulation of a cellular receptor at site of bacterial adhesion that contributes to the formation of the cortical plaque.

Another consequence of the recruitment of the polarity complex Par6/Par3/PKCζ is the opening of the inter-endothelial junctions allowing the transmigration of bacteria through the paracellular route (Coureuil et al. 2009). The Cdc42-Par3/Par6/PKCζ pathway is usually involved in the organization of the cellular junctions (*i.e.* adherens and tight junctions). Here, the ectopic activation of the polarity complex Par3/Par6/PKCζ leads to abnormal recruitment of adherens junction proteins (such as VE-cadherin, p120-catenin) that are then sequestrated underneath bacterial colonies through their interaction with β-arrestins (Coureuil et al. 2009; Coureuil et al. 2010). Importantly, proteins of cellular junctions are recruited from the existing pool of proteins present at the cell-cell contact. Thus, these

molecules are depleted at the intercellular junctions causing endothelial leakage. Adhesion of *N. meningitidis* also promotes the cleavage of occludin (a component of the cellular junction) by the metalloproteinase MMP-8 (Schubert-Unkmeir et al. 2010), thus altering further the intercellular junctions.

Conclusion

Recent exciting findings have considerably expanded our understanding of the cellular events involved in meningococcal interaction with cerebral endothelial cells. However, much remains to be discovered about the recruitment of the initial adhesion receptor and signaling receptor by Tfp. Binding to these receptors must be a common feature to the invasive strains, thus it seems important to map the domain of interaction between specific component of the Tfp and cellular receptors. This will lead to the development of potential "anti-invasive strain" peptide or vaccine. Another major drawback in the field is the lack of animal model to study the vascular colonization by *N. meningitidis*. The development of a suitable *in vivo* model would allow to confirm the *in vitro* observations and to develop new strategy against meningococcemia.

FIGURE LEGEND

Interaction of *Neisseria meningitidis* with endothelial cells

Step 1: *Neisseria meningitidis* adheres to microvascular endothelial cells through the interaction between Type IV pili and an unknown adhesion receptor. **Step 2:** Following initial bacterial adhesion, type IV pili mediate the recruitment and the activation of the β2-adrenoceptor and β-arrestins thus leading to the organization of a specific cytoplasmic molecular complex, referred to as cortical plaques. The formation of cortical plaques results from the local accumulation of ezrin, βarrestins, Cdc42 and the polarity complex Par3/Par6/PKCζ. **Step 3:** This leads to the formation of actin containing microvilli that will help the bacteria to resist shear stress in the bloodstream. In the meantime, proteins from cell-cell junctions accumulate at the site of bacterial adhesion. This induces the retraction of cell and the opening of the paracellular pathway by which the bacteria can invade the brain. Subsequently, bacteria will modulate their aggregative properties by adding a phosphoglycerol molecule on pilins and detach from the growing colonies to colonize new niches.

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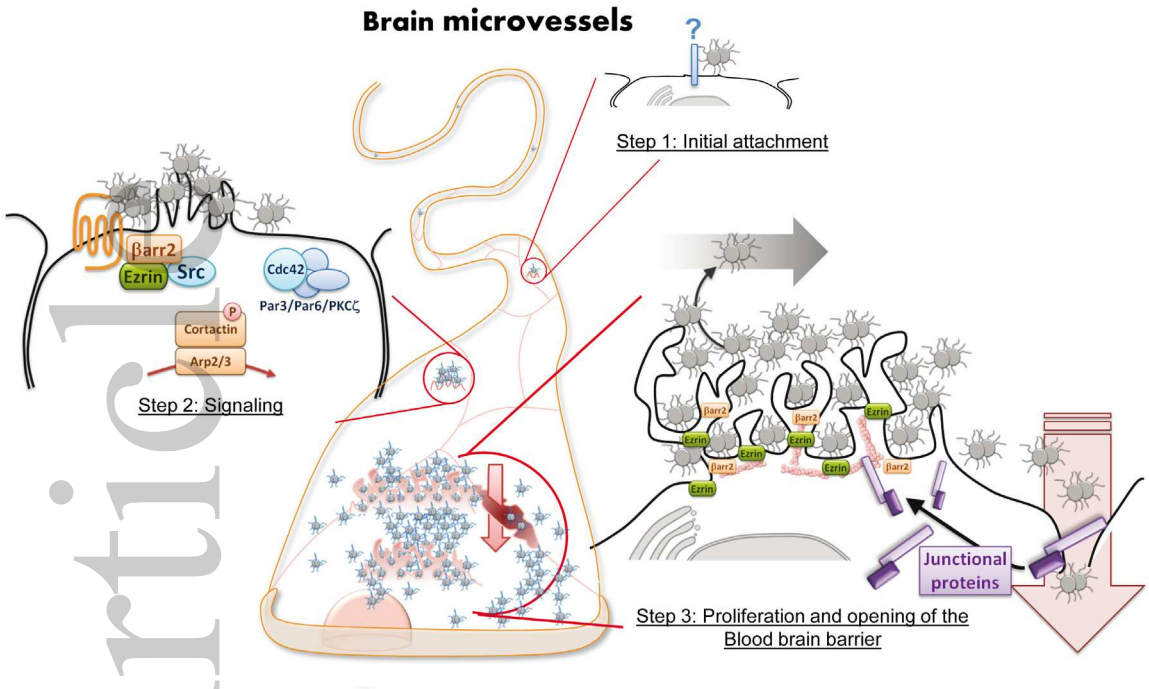

figure 1