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Role of exposed sialic acid in the interaction between meningococci and neuronal cells in the invasive meningococcal disease

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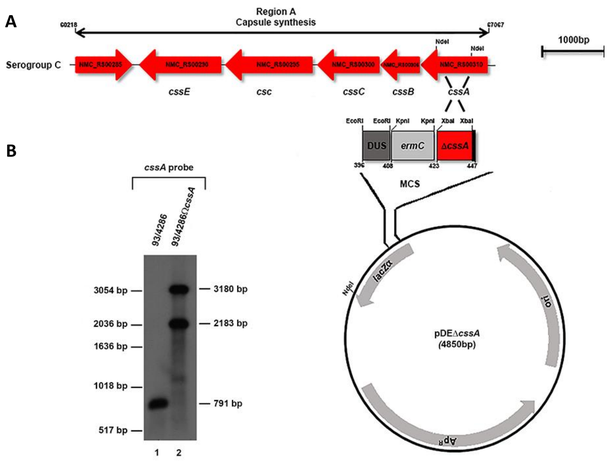
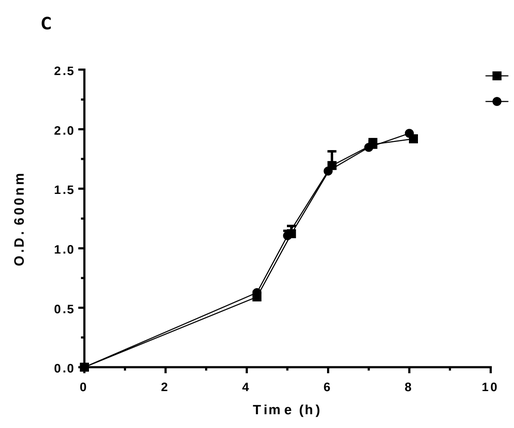
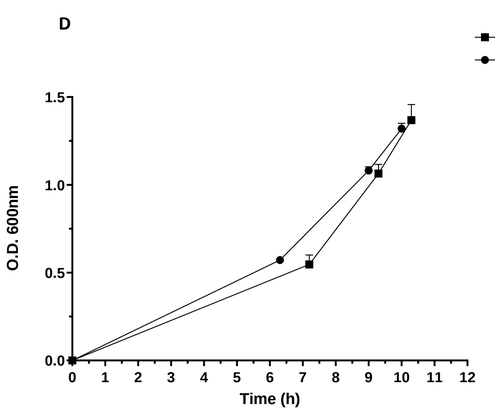
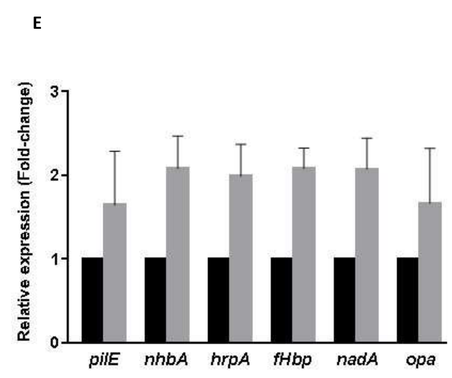
INTRODUCTION: *Neisseria meningitidis* is a leading cause of sepsis and meningitis worldwide in humans. Invasive disease is preceded by asymptomatic nasopharyngeal colonization occurring in up to 18% of the normal population. In some individuals this common transitory colonizer is able to breach the mucosal barrier, get into bloodstream and multiply uncontrollably, and finally cross the blood-brain barrier (BBB) to cause meningitis. Both host and bacterial factors seem to be involved in this switch from harmless transitory colonization to devastating disease. Among its virulence factors surface-exposed sialic acids occupy a prominent position. Four serogroups responsible for epidemics (B, C, Y and W-135) carry sialic acids in their capsular polysaccharides, sialic acid is also found as a modification of the meningococcal LOS in these serogroups. The large abundance of surface-exposed sialic acids is associated with virulence and serum resistance to both phagocytosis and complement-mediated killing via alternative pathway activation, resulting in enhanced survival in the bloodstream and central nervous system (CNS). There is also evidence that the meningococcal polysialic acid capsule is important for bacterial survival within human cells, that it mediates the interaction of bacteria with host cell microtubules during cell infection, and that it protects the bacteria against cationic antimicrobial peptides (CAMP), including human cathelicidin LL-37. On the other hand, expression of the polysialic acid capsule hinders colonization and invasion of the nasopharyngeal barrier by masking adhesins/invasins. In the past, our research group has developed a model of meningococcal meningitis (MM) based on intracisternal (i.cist.) infection of adult mice. Survival and clinical parameters of infected mice and microbiological and histological analyses of the brain demonstrated the establishment of meningitis with features comparable to those of the disease in humans. Meningococci were also found in the blood, spleen, and liver of infected mice, and bacterial loads in different organs were dependent on the infectious dose. The aim of the present study was to evaluate the role of surface-exposed sialic acids in the establishment of meningitis and meningoencephalitis in BALB/c mice when the bacteria are directly injected i.cist using the MM model. To this purpose, we have used the reference serogroup C meningococcal strain 93/4286 and an isogenic *cssA* knockout mutant defective in UDP-N-acetylglucosamine 2-epimerase that catalyzes the first step of sialic acid biosynthesis. The 50% lethal dose (LD_{50}) of these strains were determined as well as their abilities to replicate in the brain and other organs. To investigate the infectious dynamics and histopathological correlations of the disease in the MM mouse model, histological evaluation, cerebral bleeding analysis, and localization of bacteria in brain structures were carried out.

Construction of a serogroup C cssA-defective isogenic mutant and its characterization under in vitro conditions









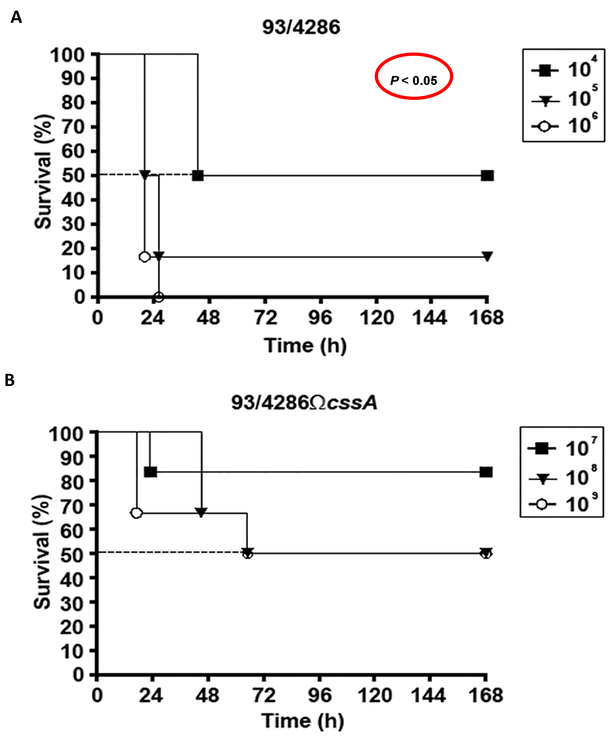
The isogenic mutant 93/4286Ω*cssA* of the serogroup C reference strain 93/4286 was obtained by insertional inactivation of the *cssA* gene, coding for the UDP-*N*-acetylglucosamine 2-epimerase, that is the first gene of the region A capsule synthesis locus (**A**). Southern blot analysis confirmed disruption of the *ccsA* gene, by using a *cssA*-specific probe (**B**). In order to exclude any differences during bacterial replication, the growth rates of wild-type strain 93/4286 and its derivative *cssA*-mutant were preliminarily analyzed in gonococcus (GC) broth at 37°C (**C**). The *cssA* mutant exhibited growth curves comparable to those of the wild-type strain, with a growth rate (μ=0.97±0.08) comparable to that of the reference strain (μ=0.85±0.06) without any statistically significant difference. Moreover, the *cssA* mutant had a growth curve and colony morphology similar to those of the wild-type strain even in Dulbecco's modified Eagle's medium (DMEM) (**D**). In addition, the *cssA* mutant, grown in GC broth, exhibited a slight upregulation in the expression of virulence-associated surface adhesins such as pilin (pilE; 1.64±0.63-fold change) and nonfimbrial adhesins such as: opacity protein (opa; 1.66±0.65-fold change), neisserial heparin-binding antigen (nhbA; 2.08±0.38-fold change), neisserial adhesin A (nadA; 2.07±0.36-fold change), adhesin/invasin (hrpA; 1.98±0.38-fold change), and factor H binding protein (fHbp; 2.08±0.23-fold change) (**E**). The difference in the expression levels of all analyzed genes was not, however, statistically significant.

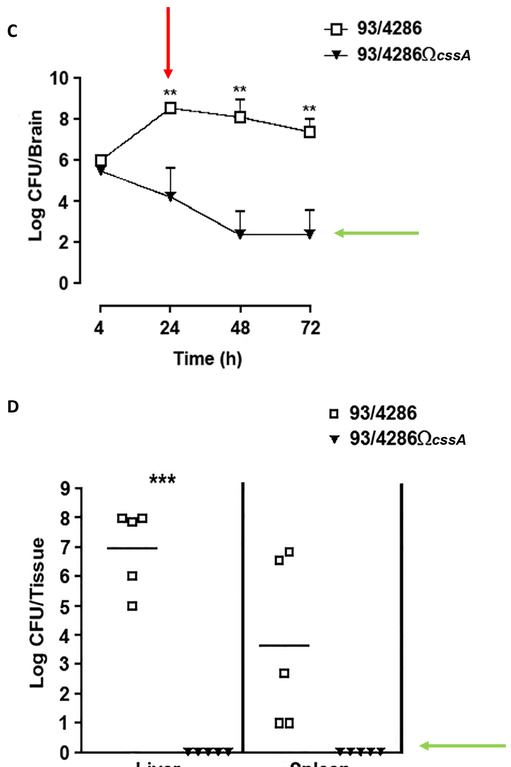
The survival rate of mice infected with the cssA-mutant is significantly increased and the cssA-mutant replication is severely impaired in the murine host

The virulence of the *cssA*-defective strain was assessed in the MM model by analyzing animal survival at different bacterial doses. Three groups of mice were infected by i.cist. injection of 10^4, 10^5, and 10^6 CFU of the wild-type strain 93/4286 or 10^7, 10^8, and 10^9 CFU of the *cssA*-mutant strain. Mouse death, weight loss, and temperature drop generally occurred within the first 72 h after meningococcal inoculation, results with the wild-type strain 93/4286 indicated that 50% and 16.6% of rodents survived meningococcal challenge with 10^4 and 10^5 CFU, respectively, while all mice died at the dose of 10^6 CFU (**A**). A significant difference was observed between the three groups (log rank test, P<0.05). In contrast, at the lowest dose of 10^7 CFU, there was 83.3% survival in the group infected with the mutant strain, while 50% survival was recorded in mice inoculated with 10^8 CFU (**B**), indicating a 10,000-fold-increased LD_{50} of the *cssA*-defective mutant.

Than, to determine the number of meningococci in the brain at different stages of disease, animals were injected i.cist. with $5x10^5$ CFU of the 93/4286 or 93/4286Ω*cssA* strain and sacrificed at different time points after challenge (**C**). A rapid increase in CFU counts was observed for wild-type bacteria that reached the highest numbers 24 h after inoculation. In contrast, bacterial loads in the brain of mice challenged with the *cssA*-defective mutant progressively dropped over time, reaching 2.026±1.774 log CFU at 72 h post-infection (**C**). Bacterial clearance from the infection site occurred in 33.3% of subjects challenged with the mutant, whereas infection was never eradicated from the brain of mice that had received the wild-type strain.

In addition, to evaluate clearance of bacteria from infected mice, two groups of animals were inoculated with $5x10^5$ CFU of both strains, and bacterial viable counts in the spleen and liver were determined (**D**). Systemic meningococcal infection caused by the *cssA*-defective mutant was entirely cleared within 48h from i.cist. challenge, whereas none of the animals inoculated with the wild-type had eliminated bacteria from peripheral organs. Two days after inoculation, mean CFU counts of the wild-type strain in the spleen and liver were still 3.212±3.354 log CFU and 6.949±1.37 log CFU, respectively. Differences in bacterial loads in the liver between the two animal groups were statistically significant (P<0.001).





Wild-type meningococci induced severe MM in mice with preferential localization in the corpus callosum

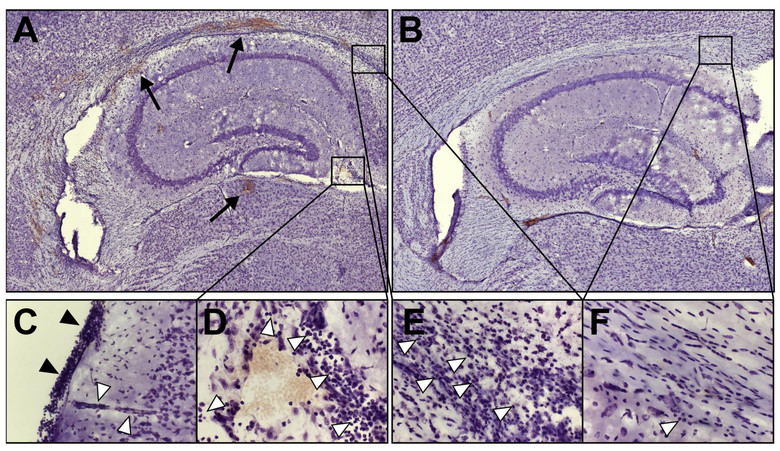


Fig. 1-Cresyl violet stained sections of brains from infected animals

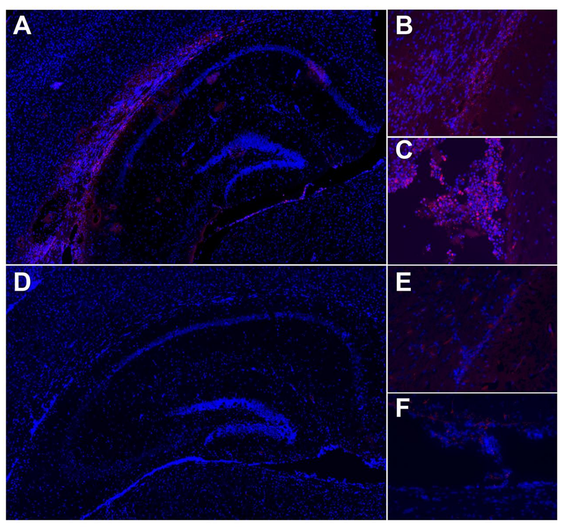


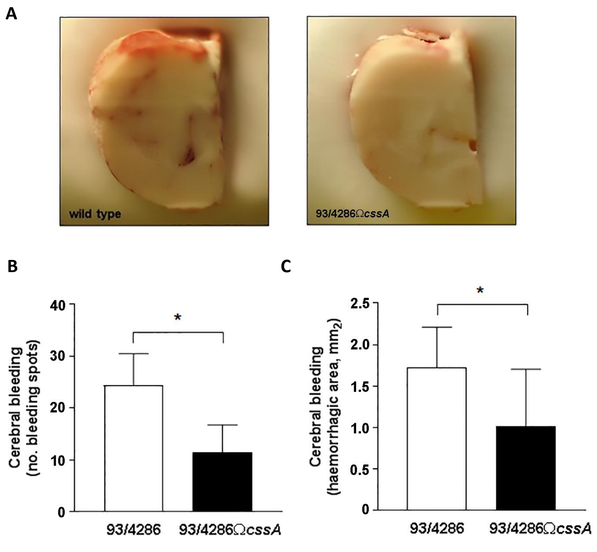
Fig. 2-Immunofluorescence analysis of brain sections

To compare the disease induced by the wild-type 93/4286 and *cssA*-defective mutant, histological analysis and bacterial immunostaining were performed on brain slices from infected mice 48 h after infection.

MM was considerably more severe in animals infected with the wild-type (**1A**) than in those challenged with the *cssA*-defective strain (**1B**). Histological analysis showed the typical features of bacterial meningitis, including the presence of inflammatory cells in the subarachnoid (**1C**) and perivascular and ventricular spaces (**1D**). Vasculitis (**1C**) and hemorrhages (**1A**) were observed mainly in animals infected with the wild-type strain. Interestingly, inflammatory infiltrates were detected in the *corpus callosum* (**1E**). Indeed, 80% of mice infected with the wild-type presented with severe inflammation in the *corpus callosum* (**1E**). In contrast, no massive evident inflammatory infiltrates, but only few immune cells, could be observed in the *corpus callosum* of animals infected with the mutant strain (**1F**).

The presence and localization of bacteria were further investigated by immunofluorescence. In animals infected with the wild-type 93/4286 strain, immunoreactivity with a meningococcal antiserum was mostly detected in the *corpus callosum* (**2A,B**), in association with neutrophils in the ventricles (**2C**). In contrast, immunostaining of meningococci revealed no signal in the *corpus callosum* of animals infected with the *cssA*-defective mutant (**2D,E**). A weak immunoreaction was detected in association with cells in the ventricles (**2F**).

Mice infected with the cssA-defective mutant showed reduced intracerebral hemorrhages



To perform a quantitative analysis of brain bleeding, the number and area of cerebral bleedings were determined in mice infected by the wild-type or the *cssA*-defective strain. In accordance with histological data, results showed a significant reduction in macroscopical assessment of cerebral hemorrhages (**A**), in the number of bleeding spots (**B**) (P=0.01), and in the hemorrhagic area (**C**) (P=0.048) in mice challenged with *cssA*-defective bacteria.

CONCLUSIONS

- ✓ **In the present study, we first aimed at validating the meningococcal meningitis mouse model by using a reference serogroup C strain and its attenuated isogenic *cssA*-mutant unable to produce sialic acids. Then, comparison of the virulence levels of the two strains was also instrumental to further explore the pathogenesis of meningococcal disease and subsequent cerebral damage by analyzing possible interactions between meningococcal surface-exposed sialic acids and brain structures. The LD_{50} of the wild-type strain 93/4286 was about four orders of magnitude lower than that of the 93/4286Ω*cssA* mutant and compared to the wild-type strain, the ability of the mutant to replicate in the brain and spread systemically was severely impaired.**
- ✓ **Histological analysis and bacterial immunostaining on brain slices confirmed higher disease severity with more pronounced inflammation, vasculitis, and hemorrhages in mice infected with the wild-type strain than in those challenged with the *cssA*-mutant. Interestingly, 80% of mice infected with the wild-type strain presented with severe inflammation in the *corpus callosum*, and most of the immuno-positive signal was localized in this brain structure and meningococci were also detected on the meninges, in the ventricles, and in the *choroid plexus*.**
- ✓ **Massive presence of bacteria in the vessels as well as in the epithelium of the *choroid plexus* and ventricular system is a very common finding in histopathological examination of patients with MM. Indeed, the *choroid plexus* is considered an important gateway for meningococcal traversal from the bloodstream into the CNS during meningitis in humans. It is very likely that the bacteria utilize this highly vascularized site to spread systemically from the CNS in the intracerebral mouse model of MM by using a reverse route.**
- ✓ **The localization of meningococci in the *corpus callosum* is unexpected, suggesting a certain tropism of *N. meningitidis* for this brain structure. From a theoretical point of view, in order to accumulate within the *corpus callosum*, in the intracerebral mouse model of MM the bacteria have to leave the cerebrospinal fluid space, survive in the bloodstream, and reenter the brain. Our data seem to suggest the *corpus callosum* as a major site of bacterial reentry in the intracerebral mouse model. This could be due to a high concentration of adhesion molecules relevant to meningococcal-host cell interactions at the level of the cerebral vessels or other structures in the *corpus callosum*.**

In conclusion, results of histological analysis and bacterial immunostaining indicate surface-exposed sialic acid as a main determinant for meningococcal intracellular growth/survival and also as a possible mediator in the interaction between meningococci and neuronal cells, suggesting a new role of these microbial structure in the interplay between *N. meningitidis* and the host in the pathogenesis of meningococcal disease that, however, should be further explored.

ACKNOWLEDGMENTS: **This research was supported in part by PRIN 2012 (grant number 2012WJSX8K), "Host-microbe interaction models in mucosal infections: development of novel therapeutic strategies."**

Colicchio et al., 2019; Colicchio et al., 2015; Ricci et al., 2014; Hill et al., 2010; van Deuren M, 2000; Estabrook et al., 1992.