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ORIGINAL INVESTIGATION

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Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitis* serogroup B are prerequisites for virulence of meningococci in the infant rat

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Abstract We investigated the contribution of the polysialic acid capsule and of terminal lipooligosaccharide (LOS) sialylation to the pathogenicity of *Neisseria meningitidis* in vivo using a set of defined isogenic mutants of the *N. meningitidis* strain B 1940 deficient in either capsule synthesis or LOS sialylation. Furthermore a spontaneous capsule-deficient variant was investigated, which was capable of switching on the capsule synthesis at a frequency of 3×10^{-3} in vitro. Infection of infant rats with the wild-type strain revealed a high potential to cause bacteremia. This potential was attenuated in the capsule-phase variable mutant (LOS sialylation^{+}). However, using a mutant irreversibly deficient in capsule synthesis, but expressing a sialylated LOS, bacteremia could only be achieved using 10^6 times higher numbers of bacteria when compared to the wild-type. The unencapsulated bacteria were located extracellularly upon examination of blood smears, suggesting that defense mechanisms, i. e. phagocytosis, directed against unencapsulated meningococci were exhausted using very high infecting doses. Interestingly, when infant rats were infected with encapsulated meningococci which were unable to sialylate the LOS, bacteremia could never be achieved, even with an infective dose as high as 10^8 colony forming units (CFU). Despite the presence of capsular polysaccharide this mutant was phagocytosed by peritoneal phagocytes, as was the unencapsulated, LOS-sialylated mutant, suggesting that the inability to cause bacteremia was due to a higher susceptibility to the action of the complement system, which is virtually unsaturable. We conclude that in the infant rat model of meningococcal infection both forms of sialic acid on the bacterial cell surface are indispensable for systemic survival.

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Introduction

Neisseria meningitidis, a gram-negative diplococcus, causes sepsis and meningitis. Despite the availability of potent antimicrobial therapies meningococcal disease remains a focus of interest among infectious diseases due to its high mortality rate of 10% and frequently occurring sequelae, such as deafness (Edwards and Baker 1981). Despite the lack of well-defined animal models, clinical observations and in vitro investigations have elucidated the host's defense mechanisms against this pathogen. The importance of the complement system for the elimination of meningococci became evident in patients with late complement component deficiencies, who tend to have recurrent neisserial infections, probably due to failure of the membrane attack complex formation (Figueroa et al. 1993; Platonov et al. 1995). Phagocytosis by polymorphonuclear leukocytes (Estabrook et al. 1992; Rest and Frangipane 1992; Ross et al. 1987; Schlesinger et al. 1994) and monocytes (McNeil et al. 1994; Sjursen et al. 1992) could be demonstrated in vitro and clinical evidence for the importance of opsonophagocytosis in the defense against meningococcal disease came from the observation that patients with fulminant septic shock frequently expressed only low IgG 2-binding allotypes of Fcγ receptors on their polymorphonuclear leukocytes (Bredius et al. 1994). Specific antibodies present in vaccinated volunteers or patients at risk for meningococcal disease enhanced both the neutrophil response, i. e. respiratory burst and opsonophagocytosis, and the bactericidal effect of serum (Aase et al. 1995; Schlesinger et al. 1994; Sjursen et al. 1992), thus underlining the importance of specific antibodies for the host defense despite the extensive ability of *Neisseria* species to vary their antigenic profile (Meyer et al. 1990).

Among the various factors contributing to neisserial pathogenicity, sialic acids as α-2,8 linked long-chain homo-

polymers in the capsule of serogroup B meningococci or as a terminal modification of the lipooligosaccharide (LOS) are essential components of the neisserial surface required for systemic spread in the blood stream. The meningococcal capsule is known to inhibit adhesion to monocytes and neutrophils and subsequent opsonophagocytosis (Klein et al. 1996; McNeil et al. 1994). In the case of serogroup B meningococci, the predominant serogroup in the Northern hemisphere, the composition of the α-2,8 linked polysialic acid capsule serves as a molecular mimicry and inhibits the host immune response, since α-2,8 linked polysialic acid is also found in human glycoproteins, e. g. the neural cell adhesion molecule (NCAM; Finne et al. 1987). A unique role of the capsule in inhibiting the action of the complement system has been doubted since evidence was reported that meningococci are capable of the terminal sialylation of their LOS (Mandrell et al. 1991). Using genetically defined mutants of the meningococcal strain B 1940 we recently demonstrated that only LOS sialylation contributed to protection against serum bactericidal effects mediated by the alternative pathway (Hammerschmidt et al. 1994).

A major pitfall for the analysis of neisserial pathogenicity in vivo is the lack of an appropriate animal model due to the restriction of *Neisseria* to the human host. The infant rat model is most frequently used to induce bacterial sepsis and meningitis (Nassif et al. 1992; Saukkonen 1988). Intraperitoneal infection of the animals leads to systemic spread in the blood stream and consecutively to meningitis. However, the importance of meningococcal surface-bound sialic acid for the ability to spread systemically in the infant rat model has not been studied in detail, although this would be most desirable in order to confirm the results obtained in vitro. In this study we used a spontaneous capsule deficient variant and defined isogenic mutants of the serogroup B meningococcal strain B 1940 deficient in either capsule synthesis or LOS sialylation in order to define the role of each component in resistance to the host's defense mechanisms during systemic spread in the infant rat model.

Materials and methods

Bacterial strains

Neisseria meningitidis strain B 1940 (serogroup B, LOS immunotype L 3, 7, 9) is a clinical isolate and was obtained from U. Berger, Institute of Hygiene, Heidelberg, Germany. The strain is piliate and expresses Opa and Opc (Hammerschmidt et al. 1996 a). The characteristics of the B 1940 mutants used in this study are summarized in Table 1. Mutant strain B 1940 *siaD*::Tn*1725* (capsule$^-$, LOS sialylation$^+$) is a Tn*1725* knock-out mutant defective in the expression of polysialyltransferase (Hammerschmidt et al. 1994). B 1940 *galE*$^-$ (capsule$^+$, LOS sialylation$^-$) was established by replacing the *galE* and *rfb* genes by a chloramphenicol resistance cassette resulting in expression of a truncated LOS (Hammerschmidt et al. 1994). The capsule-deficient derivative B 1940*siaD*$_{SSM}$ was obtained by infection of epithelial cells with the wild-type strain and varies its capsule expression by use of a slipped strand mispairing mechanism in the *siaD* gene (Hammerschmidt et al. 1996 b). The frequency of reversion to the capsule$^+$ phenotype is 3×10^{-3}. All strains and mutants used were passaged by infection of Hep-2 laryngeal carcinoma cells 4–7 times as described recently (Hammerschmidt et al. 1996 a). The potential to exogenously sialylate the LOS, the LOS immunotype, expression of pili and Opc and Opa were unaffected by this procedure (Hammerschmidt et al. 1996 a). Bacteria were stored at –70 °C until use.

Experimental infection of infant rats

Infant Wistar rats were obtained from Charles-River (Sulzfeld, Germany). At the age of 5 days the infant rats were infected intraperitoneally with *N. meningitidis* strains and mutants, which were grown overnight on chocolate agar, resuspended in phosphate buffered saline (PBS) and diluted appropriately according to the optical density at a wavelength of 600 nm. The definite infecting dose was determined by plating serial tenfold dilutions. The animals were kept with their mother. After 9 h, the infant rats were decapitated to obtain blood for determination of the number of colony-forming units (CFU) per milliliter of blood by serial tenfold dilutions. The limit of detection was 20 CFU/ml. Meningococci in blood samples were demonstrated microscopically by preparing blood-smears from bacteremic animals using citrated (3.8%) blood. The blood smears were stained with Diff-Quik (DADE, Switzerland). The staining set allows rapid staining, e. g. of blood smears, yielding results comparable to those of the Pappenheim technique. Peritoneal lavage was performed with 1 ml PBS follwing decapitation. Meningococci recovered from the lavage fluid were analyzed for the presence of capsular polysialic acid as described below. The number of peritoneal cells in non-infected animals was determined by counting of cells in the lavage fluid using a Neubauer's chamber. Phagocytosis of meningococci by peritoneal cells was analyzed by microscopic examination of Diff-Quik-stained cells from 300 µl lavage fluid centrifuged onto glass slides at 1200 rpm for 10 min in a Shandon Cytospin 3 (Life Sciences International, UK). All experiments were performed at least twice.

Colony blot

For the detection of the meningococcal capsule colony blots using the monoclonal antibody 735 specific for α-2,8-linked polysialic acid (Frosch et al. 1985) were performed as described previously (Hammerschmidt et al. 1996 a).

Statistics

Data are presented as means of two (for dose titration curves) or means ± standard deviation of three infant rats per group. Differences between the groups compared were accepted as being significant if $P<0.05$, as determined by the two-tailed Student's *t*-test.

Table 1 *Neisseria meningitidis* strain B 1940 variants and mutants used in this study

Strain/mutant	Capsule	LOS sialylation	Reference
B 1940	+	+	Hammerschmidt et al. (1996 a)
B 1940*siaD*$_{SSM}$	–/(+)	+	Hammerschmidt et al. (1996 b)
B 1940*siaD*::Tn*1725*	–	+	Hammerschmidt et al. (1994)
B 1940*galE*$^-$	+	–	Hammerschmidt et al. (1994)

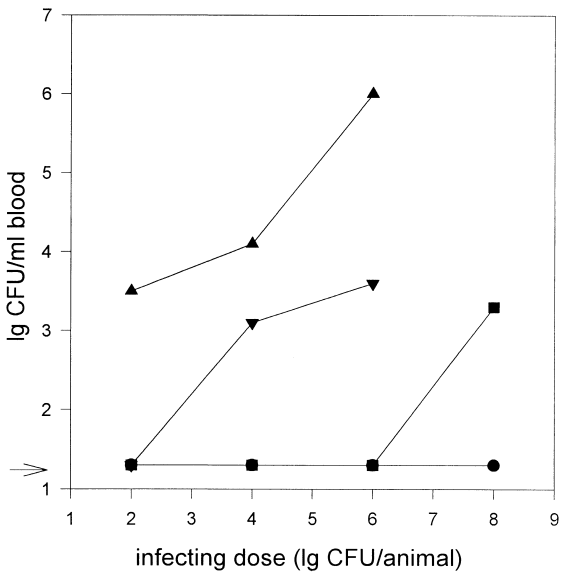

Fig. 1 Infant rats were infected intraperitoneally with *Neisseria meningitidis* strains and mutants using various infecting doses. Blood was obtained after 9 h by decapitation and the numbers of bacteria per milliliter of blood determined. The *arrow* indicates the detection limit (1.3 lg CFU/ml). ▲ Wild-type strain B 1940, ▼ B 1940*siaD*$_{SSM}$, ■ B 1940*siaD*::Tn*1725*, ● B 1940*galE*$^-$

Results

Bacteremia in infant rats caused by infection with *N. meningitidis* strain B 1940 and its isogenic mutants

Infant Wistar rats were infected intraperitoneally with 2×10^6 CFU of the respective meningococcal strain or mutant per animal. The degree of bacteremia was assessed after 9 h by sampling of the blood following decapitation. As can be seen in Table 2 the wild-type strain (capsule$^+$, LOS sialylation$^+$) exhibited the highest degree of bacteremia, 6.8±1.3 lg CFU/ml blood, which was significantly higher than the infection observed with the capsule-deficient variant B 1940*siaD*$_{SSM}$ (LOS sialylation$^+$), which switches to a capsule$^+$ phenotype in vitro at a frequency of 3×10^{-3}. This variant gave rise to bacteremia of 4.0±0.4 lg CFU/ml blood. The mutants B 1940*galE*$^-$ (capsule$^+$, LOS sialylation$^-$) and B 1940*siaD*::Tn*1725* (capsule$^-$, LOS sialylation$^+$) were not able to spread into the blood stream. This suggests that both encapsulation an LOS sialylation are indispensable for systemic spread of the meningococci.

Comparative infections of the infant rats using the wild-type strain, the variant B 1940*siaD*$_{SSM}$ or the mutants B 1940*siaD*::Tn*1725* and B 1940*galE*$^-$, applying different doses ranging from approximately 10^2 to 10^8 CFU/animal (Fig. 1) revealed that ≤100 CFU of the wild-type strain were sufficient to cause bacteremia; 10^8 CFU were lethal during the 9-h observation period. Interestingly, this infecting dose was also lethal for the variant B 1940*siaD*$_{SSM}$, although only 0.03% of the population were capsule-positive switch variants. Using the highest infecting dose of 10^8 CFU a small amount of bacteria could also be re-isolated in the case of the mutant B 1940*siaD*::Tn*1725*. However, 10^6 CFU were not sufficient to cause bacteremia after 9 h of infection. The lowest potential for systemic spread was observed for the LOS sialylation-negative, but capsule-positive mutant B 1940*galE*$^-$, in which case no bacteria could be re-isolated from the blood even in animals which had received 10^8 CFU.

Table 2 Infant rats were infected intraperitoneally with approximately 2×10^6 CFU of *Neisseria meningitidis* strains and mutants. The animals were decapitated after 9 h and the numbers of bacteria per milliliter of blood were determined. Data are presented as means of three infected animals ± standard deviation

Strain/mutant	Infecting dose (Ig CFU/animal)	Ig CFU/ml
B1940	6.4	6.8 ± 1.3
B1940*siaD*$_{SSM}$	6.1	4.0 ± 0.4
B1940*siaD*::Tn*1725*	6.5	<1.3
B1940*galE*$^-$	6.0	<1.3

Colony blots for detection of the capsule

In order to determine the encapsulation status of the meningococci re-isolated from the blood and the peritoneal cavity colony blots were performed using the monoclonal antibody 735 (Frosch et al. 1985) specific for α-2,8-linked polysialic acid. Representative re-isolates were examined from all groups of the experiments depicted in Table 2 and Fig. 1. In the case of strain B 1940 and variant B 1940*siaD*$_{SSM}$ all re-isolates obtained from the blood and the peritoneal cavity were encapsulated. Thus, encapsulation was necessary for survival not only in the blood stream, but also in the peritoneal cavity, suggesting in the case of variant B 1940*siaD*$_{SSM}$ a selection for capsule-positive meningococci already present in the inoculum or reverted from the capsule-positive to the capsule-negative phenotype in vivo soon after infection. Therefore, the >100-fold lower degree of bacteremia with B 1940*siaD*$_{SSM}$ using an infecting dose of 10^6 CFU when compared to the wild-type strain corresponded well to the lower input of encapsulated variants. Selection for encapsulated bacteria could also be demonstrated when animals were inoculated with a mixture of the wild-type strain and the unencapsulated mutant B 1940*siaD*::Tn*1725* at a ratio of $1:3\times10^3$ (data not shown). Again, only the encapsulated wild-type strain could be re-isolated from the blood after 9 h of infection. However, clones re-isolated from animals infected with 10^8 CFU of the mutant B 1940*siaD*::Tn*1725* were not encapsulated. This was not surprising, since the mutation in the *siaD* gene was a stable event. The isolation of unencapsulated meningococci only after application of very high doses suggested that the mechanisms of resistance inhibiting the proliferation of unencapsulated bacteria, i. e. phagocytosis, can be exhausted.

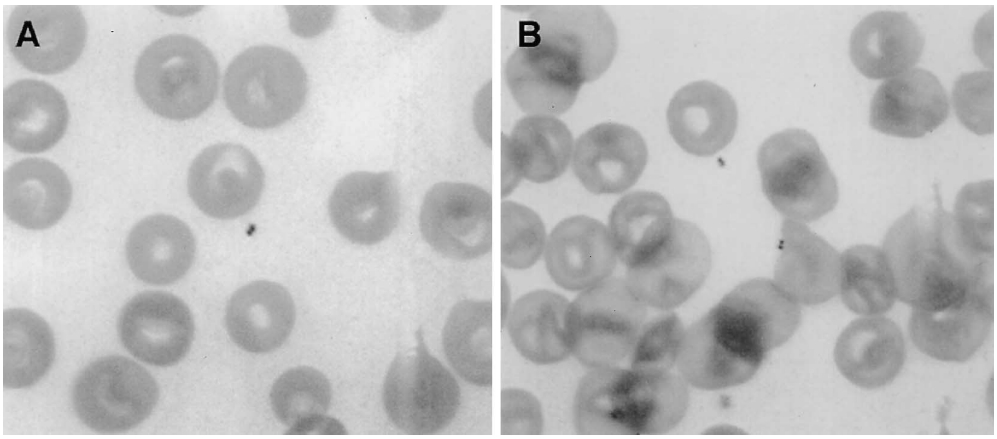

Fig. 2 Diff-Quik-stained blood smears of infant rats infected with 10^6 CFU of the wild-type strain B 1940 (**A**) or 10^9 CFU of the mutant B 1940*siaD*::Tn*1725* (**B**). Blood was obtained 9 h after infection

Microscopic analysis of blood smears of bacteremic animals

The meningococcal capsule is believed to inhibit phagocytosis by monocytes and neutrophils (Klein et al. 1996; McNeil et al. 1994) and therefore allows systemic spread of meningococci into the blood stream. The unencapsulated mutant B 1940*siaD*::Tn*1725*, however, when given to the infant rats at high doses (10^8 CFU/animal) could be recovered from the blood after 9 h of infection, suggesting that defense against unencapsulated meningococci, i. e. phagocytosis, can be exhausted. We therefore analyzed blood smears from bacteremic animals microscopically in order to determine whether systemically spread unencapsulated meningococci were phagocytosed. First, we examined Diff-Quik-stained blood smears prepared from infant rats infected intraperitoneally with 10^6 CFU of the wild-type strain. As expected, wild-type meningococci were not associated with phagocytic cells in the blood stream (Fig. 2 A). Infection of infant rats with 10^8 CFU of the mutant B 1940*siaD*::Tn*1725* resulted in bacteremia below the detection limit in blood smears. We therefore increased the infective dose tenfold, which resulted in microscopically detectable bacteremia. As can be seen in Fig. 2 B, all unencapsulated meningococci (LOS sialylation$^+$) were found extracellularly and were not associated with phagocytic cells, suggesting that bacteremia occurs in animals infected with unencapsulated mutants if the capacity of the phagocytic system has been exhausted.

Phagocytosis of meningococci by peritoneal cells

The first line of defense against intraperitoneal meningococcal infection is provided by phagocytic cells of the peritoneal cavity. By peritoneal lavage of uninfected infant rats with 1 ml lavage fluid we determined the number of phagocytic cells released from the peritoneal cavity to be $10^{6.2\pm0.2}$ cells/animal ($n = 3$). Although it is improbable that all peritoneal cells can be mobilized by a lavage, this value provides us with the possibility to assess approximately the ratio of bacteria to peritoneal cells achieved at the beginning of infection with a defined inoculum of meningococci. For example, in the experiment demonstrated in Table 2 all infant rats were infected intraperitoneally with approximately 2×10^6 CFU, resulting in an initial ratio of bacteria to phagocytic cells of 1 : 1. Microscopic examination of Diff-Quik-stained peritoneal cells (Fig. 3 A) revealed that the proportion of polymorphonuclear leukocytes was less than 10%.

As a first step in the analysis of phagocytosis of meningococci by intraperitoneal phagocytes we conducted a microscopic examination of peritoneal lavages obtained after 1 h of intraperitoneal infection with 10^8 CFU/animal of the wild-type strain B 1940, the unencapsulated mutant B 1940*siaD*::Tn*1725* or the mutant deficient in LOS sialylation (B 1940*galE*$^-$). As can be seen in Fig. 3 B wild-type meningococci were barely found to be ingested by phagocytes after 1 h of infection. In contrast, the mutant strains were both heavily phagocytosed (Fig. 3 C, D). The results demonstrate that meningococci deficient in either capsule synthesis or LOS sialylation are not resistant to intraperitoneal phagocytosis, which is likely to contribute to their diminished pathogenic potential in the infant rat.

Discussion

In the present study the infant rat model was used to assess the role of the meningococcal capsule and of LOS sialylation for the survival of *N. meningitidis* group B in the blood stream. Our results indicate that both capsular and LOS-linked sialic acids are indispensable for the systemic spread of meningococci. All re-isolates of variant B 1940*siaD*$_{SSM}$, which is capsule-deficient, but switches in vitro to the capsule-positive phenotype at a frequency of 3×10^{-3}, were encapsulated, suggesting that all unencapsulated clones had been eliminated. Furthermore, the unencapsulated mutant B 1940*siaD*::Tn*1725*, comprising a

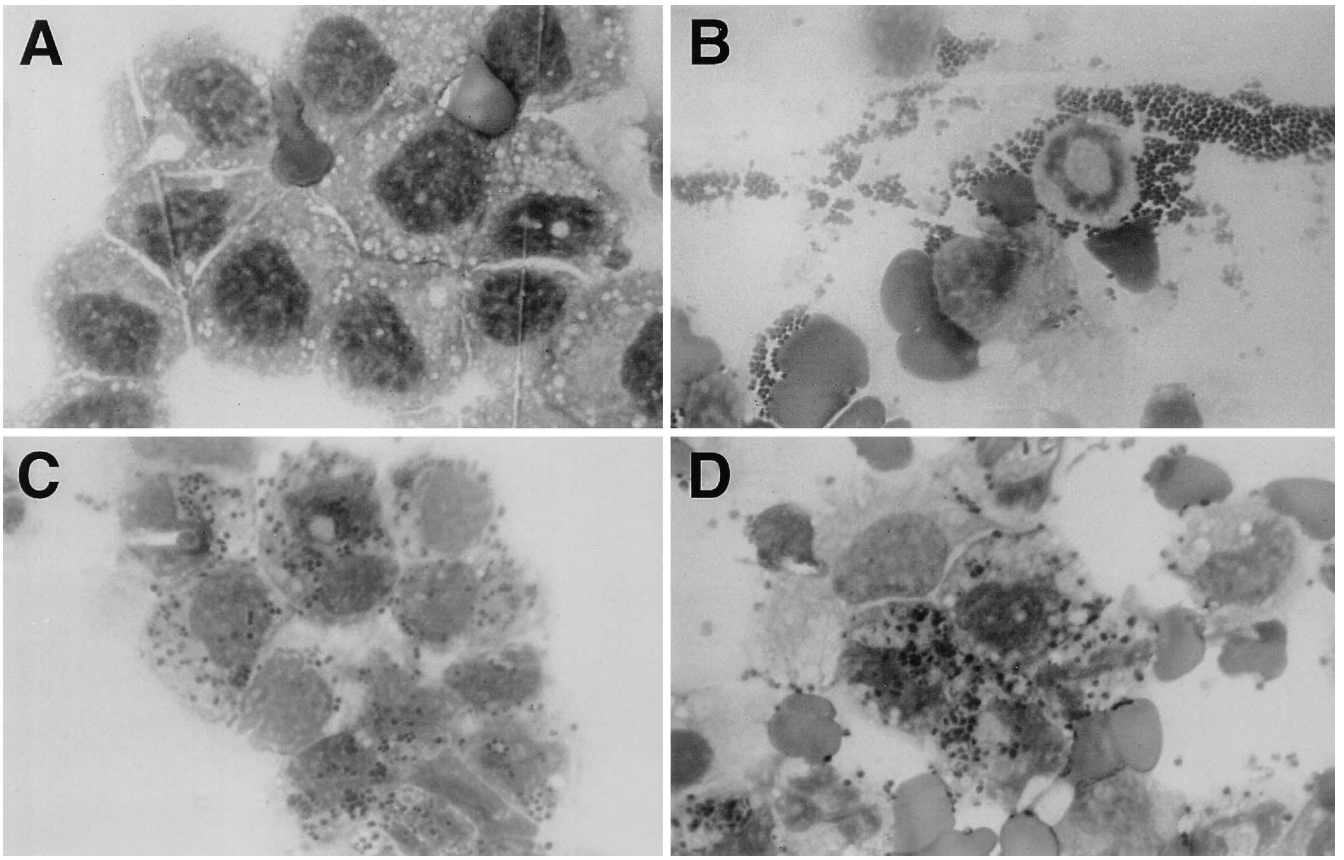

Fig. 3 Diff-Quik-stained samples of peritoneal lavages of uninfected infant rats (**A**) or of infant rats infected with 10^8 CFU of the wild-type strain B 1940 (**B**), the mutant B 1940*siaD*::Tn*1725* (**C**) or the mutant B 1940*galE*$^-$ (**D**). The lavage fluid was obtained 1 h after infection

stable knock-out mutation in the *siaD* gene, was avirulent. Meningococci lacking capsule expression could only be re-isolated in the case of the mutant B 1940*siaD*::Tn*1725* when applied in large numbers of CFU (approximately 10^8). Blood smears obtained from animals bacteremic with this unencapsulated mutant revealed that no meningococci were associated with phagocytic cells. Since the meningococcal capsule is considered to be the major determinant inhibiting phagocytosis (Klein et al. 1996; McNeil et al. 1994) our results demonstrate that the cellular defense mechanisms directed against unencapsulated meningococci appeared to be saturable. Support for this hypothesis comes from our recent observation that both encapsulated and unencapsulated variants of a serogroup C meningococcal strain could be obtained from blood cultures of a patient suffering from end-stage severe meningococcal disease (Hammerschmidt and Frosch, unpublished observation). Our results concerning the importance of the neisserial capsule for systemic survival in the infant rat rare in accordance with an early report by Masson et al. (1982), who demonstrated that unencapsulated serogroup B meningococci re-isolated from mice infected with large doses of the wild-type strain under systemic iron load were virtually avirulant in contrast to the isogenic wild-type strain. Interestingly, this unencapsulated variant was capable of switching back to the encapsulated state in vivo and carried sialic acid pools different from the capsule, probably the sialylated LOS. Thus, it resembled characteristics of the variant B 1940*siaD*$_{SSM}$ used in our study, which switches on and off expression of the polysialyltransferase, thus changing capsule phenotype without affecting LOS sialylation (Hammerschmidt et al. 1996b). Masson and Holbein (1985) further suggested that the protective role of the capsule in vivo depends on the amount of capsular polysaccharide, since they observed a correlation between virulence and sialic acid level, which was increased by growth of meningococci under conditions of iron or nutrient limitation and low pH. However, the authors did not rule out in their study that the increase in virulence was not at least partially due to changes in the iron uptake apparatus. Clinical observations showing that blood isolates of meningococci are usually encapsulated as well as the fact that gonococci lacking encapsulation are very rarely reported as causative agents of bacteremia (Del-Rio et al. 1989) underline the physiological relevance of our findings in the infant rat model for meningococcal survival.

The protective effects of the polysialic acid capsule only became evident when the bacteria were capable of sialylating their LOS. The mutant B 1940*galE*$^-$ was constructed by replacing the *galE* and *rfb* genes by a chloramphenicol resistance cassette which leads to deletion of the terminal three sugars of the LOS, making terminal sialylation impossible (Hammerschmidt et al. 1994). This mutant was

avirulent despite the presence of a capsule and did not spread systemically even in animals infected with 10^8 CFU. This corresponds to our previous report that this mutant was highly sensitive to the bactericidal effect of the alternative pathway of the complement system in contrast to LOS-sialylated strains (Hammerschmidt et al. 1994). We also showed recently that C3b deposition on neisserial surfaces during complement activation was enhanced in mutants not capable of sialylating their LOS (Vogel and Frosch, unpublished results). However, the contribution of endogenous and/or exogenous sialylation by meningococci to pathogenicity remains a matter of discussion (Estabrook et al. 1992; Jarvis 1994). Studies in gonococci revealed that LOS sialylation not only prevents complement-dependent killing by immune sera (de la Paz et al. 1995; Wetzler et al. 1992), but also opsonophagocytosis (Kim et al. 1992) and non-opsonic interaction with neutrophils (Rest and Frangipane 1992). In the infant rat, we demonstrated in this study that meningococci deficient in either capsule synthesis or LOS sialylation are sensitive to phagocytosis by peritoneal phagocytes. Therefore, neither the presence of capsular polysaccharide nor LOS sialylation alone rendered meningococci resistant to phagocytosis. However, the possibility of achieving bacteremia with unencapsulated, LOS-sialylated meningococci, but not with the capsulated *galE* mutant, suggests that the lytic activity of the complement system, which is virtually unsaturable, is more efficiently prohibited by sialylated LOS than by the presence of capsular polysaccharide.

In conclusion, our study using genetically defined meningococcal mutants demonstrates that both the capsular polysaccharide and sialylation of LOS are essential virulence factors in the infant rat model. Mutants defective in either component are sensitive to intraperitoneal phagocytosis. Analysis of our in vivo data suggests that meningococci protect themselves against the lytic action of the complement system primarily by expression of sialylated LOS. However, further studies are needed to clarify the efficacy and the mechanisms of phagocytosis and complement lysis in relation to the sialic acids on the surface of meningococci.

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