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Phase-Variable Lipopolysaccharide Structures Enhance the Invasive Capacity of *Haemophilus influenzae*

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Genes necessary for the expression and phase variation of lipopolysaccharide epitopes of a virulent *Haemophilus influenzae* type b isolate (RM.7004) are contained within two chromosomal loci designated *lic-1* and *lic-2*. Mutations were introduced into both *lic-1* and *lic-2*, and the virulence of the double mutant was compared with that of the wild type in infant rats. These mutations in RM.7004 resulted in a significantly reduced incidence of bacteremia following intranasal inoculation, although nasopharyngeal colonization was similar for the mutant and wild-type strains. In contrast, no differences in bacteremia were observed when the mutant and wild-type strains were inoculated intraperitoneally.

Haemophilus influenzae is commonly found in the human upper respiratory tract as a commensal. Some strains are able to cause systemic infection following colonization of the nasopharynx (1). The capsular polysaccharide, identified on the majority of isolates associated with systemic (bacteremic) infections, has been shown to contribute to the intravascular survival and pathogenicity of the organism (12). Other factors that may contribute to the virulent phenotype of potentially invasive *H. influenzae* strains are less well understood. However, it has been demonstrated that differences in lipopolysaccharide (LPS) structure affect the virulence of the organism in an infant rat model (8, 21).

The LPS of *H. influenzae* has a core of oligosaccharide but lacks the O-antigen side chains characteristic of the enterobacteria (3). The surface-exposed oligosaccharides of this glycolipid demonstrate inter- and intrastrain heterogeneity which can be shown by using oligosaccharide-specific monoclonal antibodies or electrophoretic analysis (7, 8, 15, 17). This structural heterogeneity is due, at least in part, to a pattern of antigenic switching referred to as phase variation (8). We have recently indicated a possible molecular mechanism controlling this high-frequency spontaneous gain and loss of epitopes (18). *H. influenzae* isolates have several (two to five) genomic loci with multiple tandem repeats of the tetramer CAAT. Variation in the number of CAAT sequences can generate a translational switch, which could regulate expression of oligosaccharide structures by downstream genes. Currently recognized phase-variable LPS epitopes, which are defined by monoclonal antibodies, require genes in two of these loci (*lic-1* and *lic-2*) containing tandem repeats of CAAT (19). These two loci, found in all capsulated and noncapsulated strains tested to date, have been cloned and characterized from a serotype b strain, RM.7004, obtained from the collection of Loek van Alphen, Department of Medical Microbiology, University of Amsterdam, Amsterdam, The Netherlands (16).

The aim of this study was to determine the relative contribution of phase-variable LPS structures in the pathogenesis of systemic infection by using genetically defined strains in an animal model.

Mutations in both *lic-1* and *lic-2* were introduced into a *H. influenzae* strain (RM.7004) isolated from the cerebrospinal fluid of a child with meningitis. This strain expresses six distinct phase-variable LPS epitopes (19). The genome of RM.7004 also contains a third locus with multiple tandem repeats of CAAT (*lic-3*) (19), but the function(s) of this locus is unknown and was not examined in this study. Since *lic-1* and *lic-2* encode tertiary gene products (oligosaccharides) whose expression is subject to variation, the identification of phenotypic mutants, as opposed to phase variants, was not straightforward. Therefore, different selectable markers were incorporated into site-specific deletions within each locus in order to generate a mutant that constitutively lacks all six phase-variable epitopes (Fig. 1). A pUC18 plasmid containing an insert encompassing *lic-2* was digested with *Xba*I to release a 3.1-kilobase (kb) fragment spanning all open reading frames in *lic-2* but leaving flanking sequences intact. A kanamycin resistance gene derived from Tn*903* (13) was ligated in place of the *Xba*I fragment after the protruding ends were blunted with the Klenow fragment of *Escherichia coli* polymerase I (Amersham). The plasmid construction conferring resistance to ampicillin (50 μg/ml) and resistance to kanamycin (40 μg/ml) in *E. coli* host strain DH5α (Bethesda Research Laboratories, Inc.) was linearized and introduced by transformation into competent RM.7004 (6). Colonies of transformants were selected on brain heart infusion (Oxoid)–1% agar supplemented with 10% Levinthal base (1) and 10 μg of kanamycin per ml. The acquisition of kanamycin resistance correlated with the loss of the *Xba*I fragment of *lic-2* from the chromosome, as confirmed by Southern hybridization experiments (data not shown). The introduction of a mutation in *lic-1* into the *lic-2* mutant, RM.7004-XP1, followed a similar procedure. A pUC19 plasmid containing *lic-1* was digested with *Cla*I and *Eco*RV, releasing a 2.7-kb fragment which included sequences from all four *lic-1* genes (17, 18). A tetracycline resistance gene obtained from plasmid pHVT1, which confers tetracycline resistance when harbored in *H. influenzae* (2), was ligated in place of the *Cla*I-*Eco*RV *lic-1* fragment. The resulting plasmid construction could be selected by its ability to confer resistance to 15 μg of tetracycline per ml and 50 μg of ampicillin per ml. *H. influenzae* transformants were resistant to 10 μg of kanamycin per ml and 4 μg of tetracycline per ml and, on Southern hybridization, lacked the *Cla*I-*Eco*RV *lic-1* fragment in the chromosome. The *lic-1 lic-2* mutant, RM.7004-XP1-AH1-3, did not react with any of six monoclonal antibodies (4C4, 5G8, A1, A3, 12D9, and 6A2) that

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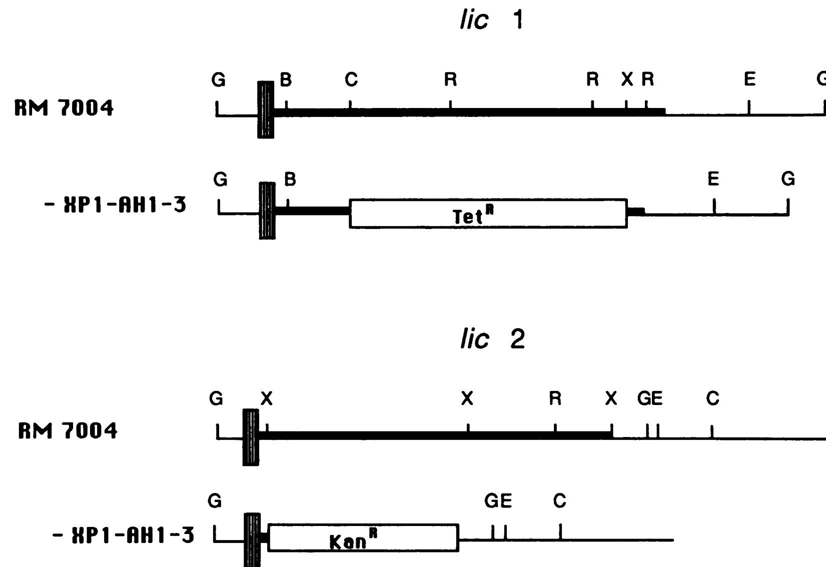

FIG. 1. Restriction maps of two chromosomal loci, *lic-1* (3.7 kb) and *lic-2* (3.3 kb), in a virulent clinical isolate, RM.7004. The multiple CAAT repeat sequences are indicated by striped boxes, and sequences shown to be necessary for expression of phase-variable LPS structures are indicated by solid bars. Deleted fragments were replaced with a tetracycline resistance marker (Tet^R in *lic-1*) and a kanamycin resistance marker (Kan^R in *lic-2*) to give the mutant, RM.7004-XP1-AH1-3, used in virulence experiments. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; R, *Eco*RV; X, *Xba*I.

identify several independent, phase-variable oligosaccharide epitopes (4, 5, 17) expressed by RM.7004. Electrophoresis of LPS extracted from RM.7004 and RM.7004-XP1-AH1-3 on 15% sodium dodecyl sulfate-polyacrylamide gel with silver staining provided further confirmatory evidence of the differences of the respective LPS.

The pathogenicity of RM.7004-XP1-AH1-3 was compared with that of RM.7004 in an infant rat model following intraperitoneal (i.p.) or intranasal (i.n.) inoculation. The inocula were prepared and quantitated as previously described (14) and adjusted to the desired concentration for i.p. or i.n. challenge. For each experiment, natural litters of 5-day-old Sprague-Dawley rats, reduced to 12 rats at birth and randomized, were challenged i.p. or i.n. as previously described (11, 14). Animals that received different strains were housed separately. Ten microliters of blood was removed from a tail vein and plated onto supplemented brain heart infusion agar to detect and quantitate bacteremia. The LPS phenotype of blood-derived organisms was confirmed by colony immunoblotting. Nasopharyngeal colonization was assessed by washing one nostril with 50 μl of sterile phosphate-buffered saline and then withdrawing 10 μl from the opposite nostril and plating it onto supplemented brain heart infusion agar with bacitracin (Sigma Chemical Co.) added to a final concentration of 1 mg/ml. Colonies obtained from nasopharyngeal washings were confirmed as *H. influenzae* type b if they agglutinated in the presence of type-specific antisera (Wellcome Diagnostics, Inc.).

There was no significant difference in the incidence or level of bacteremia between the two strains following i.p. administration. Inocula of 3×10^2 CFU of either strain given i.p. consistently produced bacteremia (RM.7004, 12 of 12 [100%] rats bacteremic and mean ± standard deviation bacteremia [5 days after inoculation] of 325 ± 194 CFU/10 μl of blood; RM.7004-XP1-AH1-3, 11 of 12 [93%] rats bacteremic and mean bacteremia of 390 ± 225 CFU/10 μl of blood). To compare the virulence of the strains following challenge with a limiting inoculation, rats were challenged i.p. with 0.3×10^1 CFU. This challenge caused a high-level bacteremia in the majority of animals inoculated with either strain (RM.7004, 16 of 24 [67%] rats bacteremic and mean ± standard deviation bacteremia [5 days after inoculation] of 400 ± 224 CFU/10 ml of blood; RM.7004-XP1-AH1-3, 18 of 24 [75%] rats bacteremic and mean bacteremia of 459 ± 211 CFU/10 μl of blood).

The ability of each strain to colonize the nasopharynx following i.n. administration was also similar (Table 1). However, i.n. inoculation with the LPS mutant resulted in a significantly lower incidence of bacteremia compared with that from inoculation with the parent strain, although the magnitude of bacteremia (number of organisms per 10 μl of blood) was similar. These results indicate that the mutant differed in its capacity to invade the bloodstream from the nasopharynx, although it was similar to the parent strain in its ability to colonize the nasopharynx and to cause a similar level of bacteremia in the few animals that developed systemic disease.

The LPS mutant was compared with its parent strain for some other characteristics which might affect their relative virulence. The two strains showed similar rates of growth in vitro. The quantities of extractable capsular polysaccharide detectable by enzyme-linked immunosorbent assay (10) were not significantly different, and the outer membrane protein profiles were similar. Both RM.7004 and RM.7004-XP1-AH1-3 were resistant to the bactericidal activity of either 20% pooled fresh human serum or pooled infant rat serum (20).

Thus, these studies demonstrated that phase-variable LPS structures encoded by *lic-1* and *lic-2* enhance the invasive capacity of *H. influenzae* type b. We have shown that during infection of humans, organisms possessing certain phase-variable LPS structures are selected for among a heterogenous population (18). Results from these animal studies indicate that this selection may occur during events following colonization and prior to bloodstream invasion.

TABLE 1. Relative virulence of genetically defined LPS mutant (RM.7004-XP1-AH1-3) and parent strain (RM.7004) in infant rats following intranasal challenge with 10^7 CFU

Strain	Days after inoculation	No. of bacteremic animals/no. challenged (%)[a]	Magnitude of bacteremia (mean CFU/10 μl of blood ± SD)	No. of animals colonized and sampled[b]	Magnitude of colonization (mean CFU/10 μl of nasal washings ± SD)
RM.7004	2	14/50 (28)	197 ± 129	16	117 ± 115
	5	22/49 (44.9)	160 ± 125	23	145 ± 151
RM.7004-XP1-AH1-3	2	5/51 (9.8)[c]	480 ± 349	22	106 ± 104
	5	6/51 (11.8)[c]	202 ± 41	22	122 ± 107

[a] From five independent experiments.
[b] From two independent experiments.
[c] $P < 0.008$ compared with parental strain (Fisher's exact test).

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