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Comparative Virulence of *Haemophilus influenzae* with a Type b or Type d Capsule

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To determine the importance of specific capsule type in the pathogenesis of invasive *Haemophilus influenzae* disease, we compared the virulence of type b and type d strains isolated from different children with the virulence of transformation-derived type b and type d organisms. In addition, the unencapsulated derivative of these strains was also examined. Virulence was assessed by determining the ability of the strains to produce bacteremia with intranasal or subcutaneous inoculation. Unencapsulated derivatives were unable to cause bacteremia by any route; all type b strains (whether natural or derived by transformation), a natural type d, and a type d derived by transformation were able to produce bacteremia with similar frequency (42 to 62%) when 10^7 colony-forming units was given intranasally. Subcutaneous inoculation of 10^3 colony-forming units of strains with the type b capsule produced bacteremia at a greater frequency than did the strains with the type d capsule ($P < 0.002$). The type d isolate was more virulent than a mutagenized derivative of the strain. We conclude that the type b strains are more virulent than type d when inoculated subcutaneously.

Haemophilus influenzae is a major cause of meningitis in infants. In 1931, Pittman showed that *H. influenzae* could be divided into two broad groups: one encapsulated and able to cause invasive disease, the other unencapsulated and not isolated from the blood or cerebrospinal fluid of patients with sepsis or meningitis (21). The encapsulated isolates were divided into six serological types, a through f, based on immunological specificity. This specificity was subsequently shown to be due to chemical differences in the soluble polysaccharide capsule (14). All six capsule types can be found in healthy carriers; however, the majority (95%) of systemic disease is caused by type b organisms (19, 27). Fewer than 100 cases of sepsis or meningitus due to capsular types a, c, e, or f or due to unencapsulated strains have been reported in immunologically normal individuals (19, 23, 25, 27). The reason for the predominance of type b in systemic disease is unknown.

In contrast, unencapsulated strains predominated (>95%) in surveys of nasopharyngeal carriage in healthy children; only 5% of the strains were capsulated (27). When capsulated strains were isolated in surveys of nasopharyngeal colonization, all six capsule types were found with equal frequency (27). This suggests that the predominance of type b in invasive disease is not due to a higher prevalence of nasopharyngeal carriage.

Type b capsule is composed of polyribose-ribitol phosphate (13, 14); in contrast, the other five capsule types contain a hexose instead of a pentose (13, 14). This has led to the hypothesis that the pentose sugar in the type b capsule confers some unique advantage on these strains resulting in greater virulence (13).

Unfortunately, little work has been done to support the notion that strains bearing type b capsule are uniquely virulent compared with the other five capsular types. To examine the role of the b capsule, we compared the virulence of a type b and a type d isolated from different children. Unencapsulated derivatives of each strain were also examined, as well as strains with the type b or type d capsule, derived by transformation. The type d capsule was chosen because it appears to be the least common of the five capsule types to cause sepsis and meningitis (19). In addition, a laboratory-mutagenized derivative of the type d capsule was available. This latter strain (Good) has been extensively characterized, including a partial genetic map depicting the general location of the capsule b (*cap B*) locus introduced by transformation (7, 16).

The infant rat model was chosen because it has been extensively studied, and three different routes of inoculation result in bacteremia and meningitus. Intranasal (i.n.) inoculation requires the cells to penetrate the mucosal barrier, elude cellular defense systems, and multiply in the blood. Subcutaneous (s.c.) inoculation requires that the cells evade local reticuloendothelial clearance and multiply in the blood. With intraperitoneal (i.p.) inoculation, the minimal infec-

tive inoculum is identical to that used for the intravenous route; it requires that the bacteria survive and multiply in the blood. All three routes can lead to sustained bacteremia in infant rats. The minimal infective inoculum of strain E_1 (a type b strain) producing sustained bacteremia in 100% of inoculated animals has previously been shown to be 1 colony-forming unit (CFU) i.p., 10^2 CFU s.c., and 10^8 CFU i.n. (17, 18, 22-24).

MATERIALS AND METHODS

Bacteria. Table 1 lists the strains used.

Media. The solid medium used to grow *H. influenzae* was modified GC medium base (Difco Laboratories) supplemented with 10 μg of hemin, 10 μg of L-histidine, and 2 μg of nicotinamide adenine dinucleotide per ml. In certain experiments with strain E_1, 250 μg of streptomycin per ml was added.

Antiserum agar plates were prepared as described by Bradshaw et al. (6). A 1:40 dilution of Burro 132 anti-type b serum (kindly donated by J. Robbins) or a 1:10 dilution of anti-type d serum (Difco) was used in supplemented GC medium plates. Grace Leidy kindly donated polyvalent antirough *H. influenzae* rabbit antisera.

The complete chemically defined media developed by Catlin et al. to grow the multiauxotrophic strain Good Rd (A8) was used (7). This medium, containing all compounds required for growth, and the differential medium of Catlin et al. lacking either L-methionine, L-leucine, L-proline, or L-alanine were prepared to verify the phenotype of the original strains before and after inoculation into animals, to determine the phenotype of the transformants, and to select for capsulated Good Rd transformants. Agarose (0.85% Seakem) was substituted for agar in the formulation of the defined medium. The liquid medium used was 3.5% brain heart infusion broth (BHI; Difco) supplemented with 10 μg of hemin, 10 μg of L-histidine, and 2 μg of nicotinamide adenine dinucleotide per ml. The competence medium M-IV was prepared as described by Herriott (11) and used to develop competence for transformation experiments.

Biotyping and auxotyping. The Minitech method of biotyping described by Back and Oberhofer (5) was used. Inocula for auxotype testing were taken from plates incubated overnight; the cells were suspended in PBS (phosphate-buffered saline: 0.13 M NaCl, 2.7 mM KCl, 3 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and the cell density was adjusted to allow a Steers replicator to deliver between 10^3 and 10^4 CFU to the plate. The differential medium lacking one amino acid was inoculated first, followed by a complete plate containing all compounds required for growth.

DNA source for transformation. Cell lysates containing transforming deoxyribonucleic acid (DNA) were prepared from Garf type d (Sd), Santo type b (Sb), and Santo type d (Sd). Each was grown to stationary phase (0.7 optical density units at 675 nm) and harvested by centrifugation. The cells were suspended in 1 M NaCl, 0.01 M ethylenediaminetetraacetic acid, and 0.15% sodium dodecyl sulfate, heated at 37°C for 15 min, cooled, and frozen at −20°C. The suspension was thawed by heating at 37°C for 15 min and refrozen. The heat-freeze cycle was done a total of three times to assure complete cell lysis of the cells. DNA for transformation was prepared by diluting the lysate 1:15 with sterile saline; both were kept frozen at −20°C.

Transformation to type b or type d capsule. Strains Good Rd, Santo Rb, and Garf Rd did not agglutinate with any of the six commercial typing sera (Difco) and did not have detectable halo formation after 48 h of incubation on antiserum plates containing either type b or type d antiserum (15). The strains were grown in supplemented BHI to an absorbance of 0.4 (675 nm), washed in M-IV competence medium, resuspended in M-IV, and incubated for 100 min at 37°C as previously described (11). Two milliliters of

TABLE 1. *H. influenzae strains used*

Strain	Capsule type	Biotype	Phenotypic characteristics[a]	Reference
Santo Sb	b	I	Met^-	10
Santo Rb	Nontypable	I	Met^-	10
Santo Sd	d (transformed by using Garf Sd)	I	Met^-	This paper
Garf Sd	d	IV		10
Garf Rd	Nontypable	IV		10
Garf Sb	b (transformed by using Santo Sb)	IV		This paper
Good Rd	Nontypable, derived from Garf Sd	IV	$Ala^- Val^- Lys^- Hyp^- Leu^- Pro^- Trp^-$	10
Good Sb	b (transformed by using Santo Sb)	IV	$Ala^- Val^- Lys^- Hyp^- Pro^- Trp^-$	This paper
Good Sd	d (transformed by using Garf Sd)	IV	$Ala^- Val^- Lys^- Hyp^- Pro^- Trp^-$	This paper
E_1	b	I	Str^r	21

[a] Typed by using Difco a through f antisera and anti-b or anti-d agar plates.

[b] Met^-, requires methionine; ala^-, requires alanine; val^-, requires valine; hyp^-, requires hypoxanthine; leu^-, requires leucine; pro^-, requires proline; trp^-, requires tryptophan; lys^-, requires lysine; Str^r, streptomycin resistant.

culture was incubated with 0.1 ml of cell lysate diluted to contain 0.1 to 0.5 μg of DNA for 30 min (20). Four milliliters of BHI was added, and the cells were allowed to grow for 3 to 4 h. At that time, the recipient strains Santo Rb and Garf Rd were diluted 1:10 in BHI and incubated overnight at 37°C in 5% CO_2 in a tube containing 1.0% gelatin and 1.0% antirough antisera (1–3). The following day the majority of cells had formed a pellet which was not easily dispersed by agitation. The supernatant was removed, diluted, and plated.

Since the capsulation locus (*capB*) in Good Rd (transformed to Sb) maps between the leucine and proline loci (7), such transformants were isolated by plating on selective medium. After 3 to 4 h of growth (0.6 optical density units at 675 nm), the cells were washed, suspended in PBS, diluted, plated on Catlin differential medium lacking L-leucine or L-proline, and incubated overnight at 37°C in 5% CO_2.

Each of the three strains was screened for iridescent colonies, which were purified and typed by using Difco typing sera (agglutination) and antiserum agar plates (halo production) (15). For slide agglutination testing and for agar plates, the a, b, c, d, e, and f antisera (Difco) were reconstituted as recommended. When quantitating type d capsular hexose by immunoprecipitation, the lyophilized antiserum was reconstituted in one-fourth the recommended volume. Each isolate was then biotyped and auxotyped to determine whether other phenotypic characteristics had been altered. Leucine and proline markers were chosen rather than biotin, since we could not consistently produce biotin-free medium.

Animals. Timed-pregnant pathogen-free albino Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Inc., and maintained pathogen-free by use of independently ventilated cage enclosures. Five-day-old rats were used for testing organism virulence.

Inoculation of animals. Strains were subcultured from frozen stocks 2 days before an experiment. A 100-ml amount of supplement BHI was inoculated with colonies taken from an overnight incubated plate and grown for 3 to 4 h at 37°C to an absorbance of 0.5 optical density unit at 675 nm. The cells were harvested and suspended in PBS with 0.1% gelatin (PBS-G), and the cell density was adjusted to 10^9 organisms/ml. Five-day-old rats caged with their mother in litters of 8 to 10 were inoculated with 10 μl of bacterial suspension (10^7 CFU) i.n. with a Hamilton syringe. In certain experiments, 0.1 ml of bacterial suspension (10^3 CFU) diluted in PBS-G was injected s.c. between the scapulae; in others, 0.1 ml of bacterial suspension (containing between 10^0 and 10^6 CFU) was injected i.p. The animals were housed in separate units after inoculation to prevent aerosol-mediated cross-infection. Forty-eight hours after inoculation, 10-μl blood samples were taken from the transected jugular vein of each animal, diluted in 0.5 ml of PSB-G, spread on a 150 by 15-mm supplemented GC agar plate, and incubated overnight at 37°C in a 5% CO_2 atmosphere. Ten microliters of cerebrospinal fluid was obtained by surgically exposing the cisterna magna of exsanguinated animals and puncturing it with a sterile needle. The fluid was placed directly onto an agar plate. Plates were incubated for 96 h (as described above) and reexamined before being discarded. Colonies isolated from blood or cerebrospinal fluid were tested for their ability to grow on 250 μg of streptomycin per ml or to grow on Catlin differential medium lacking either L-methionine, L-leucine, L-proline, or L-alanine; all strains recovered from animals were retyped with anticapsular serum.

Statistical analysis. Statistical analysis was done with the χ^2 test with Yates correction or Fisher's exact test (4).

RESULTS

Transformation to capsular type b or d. Santo Rb, Garf Rd, and Good Rd were selected as recipients. Santo Rb was transformed by using DNA from Garf Sd; Good Rd was transformed by using DNA from Santo Sd; Good Rd and Garf Rd were transformed by using DNA isolated from Santo Sb. The recipients were allowed to grow for 3 to 4 h after transformation to permit expression of the *cap* locus, and then they were incubated in antirough serum. When the cells were plated without prior agglutination with antiserum, no iridescent colonies were isolated. However, when plated after agglutination with antirough serum, between 10 and 40% of the cells were iridescent and subsequently shown to be capsulated. When the Good Rd strain was plated on differential medium lacking leucine or proline, 5 to 15% of the leucine prototrophs and 0.02 to 0.1% of the proline prototrophs were capsulated, a rate similar to that found by Catlin et al (7). The *capB* and *capD* loci both mapped between the leucine and proline markers.

All of the transformants resulting from Santo Sb DNA transformation had type b capsule; reactivity with other anticapsular antisera was not detectable. Similarly, those strains resulting from Garf Sd DNA transformation produced only type d capsule; they did not agglutinate with commercial sera to other types.

Transformants were nonselectively passed every 2nd day to determine the stability of capsule production. In all cases, the production of capsule was stable for 21 days: in most cases 100% of the colonies were still capsulated as determined by iridescence. These strains were used for the animal studies. No other measurable phenotypic trait (i.e., auxotype or biotype) was altered when the strains were transformed to capsule type b or type d.

Animal studies. The infant rat has been well characterized as a model infection in the past few years by using the type b strain E_1 (17, 18, 22–24). We therefore included this strain in the study as a positive control. Bacteremia, 48 h after inoculation, was chosen as the criterion of infection to reduce the number of animals re-

quired for statistical analysis (4). Intranasal inoculation of 10^7 organisms was chosen because in previous work with E_1, 50 to 70% of the animals were bacteremic at 48 h (17, 18, 23). Subcutaneous inoculation of 10^3 was chosen since this inoculum produces bacteremia in 100% of the animals with E_1. Intraperitoneal inoculation of 1 CFU of strain E_1 produces bacteremia in 100% of the animals. In contrast, i.p. inoculation of 10^6 CFU of an unencapsulated strain results in transient bacteremia; larger inocula, whether with viable or dead cells, result in death in certain animals, presumably due to endotoxin (23, 26).

Table 2 summarizes the results of the animal experiments. The number of organisms isolated in the 10-μl blood sample varied from 1 to 300 CFU. This variation occurred in infants within a single litter receiving an identical inoculum. This 2-log variation was seen with both the type b and type d strains. Therefore, only the presence or absence of bacteremia is tabulated. All three unencapsulated isolates, regardless of the route of inoculation, were not detected in the blood. Intraperitoneal administration of 10^6 organisms did not produce detectable bacteremia.

Fifty percent of the animals developed bacteremia when 10^7 E_1 was given i.n. Similar results were obtained for Santo Sb, Santo Sd, Garf Sd, Garf Sb, and Good Sb ($P = 0.1$). However, only 1 of 26 animals became bacteremic when Good Sd was used. Rates of i.n. carriage for Good Sd and Good Sb were identical, 11 of 18 (61%) and 13 of 21 (62%), respectively. Good Sd was significantly less ($P < 0.002$) able to cause bacteremia in infant rats than Santo Sd or Garf Sd after i.n. inoculation. Forty to 50% of the bacteremic animals developed meningitis. No difference was found between type b or type d organisms or between natural or transformed derivatives in the incidence of meningitis. Only bacteremic animals had organisms present in their cerebrospinal fluid (data not shown).

The two natural type b strains, E_1 and Santo Sb, were able to produce bacteremia in 100% of the animals inoculated s.c. with 10^3 CFU, whereas the two type d strains transformed to type b produced bacteremia in 90% (Good Sb) and 75% (Garf Sb) of the animals (a difference not statistically significant; $P = 0.1$). The type d strains produced bacteremia in 9% (Santo Sd) to 20% (Good Sd) (no significant difference) of the animals inoculated s.c. The rate of bacteremia by the s.c. route for the type d strains was significantly less ($P < 0.002$) in comparison with the rate found with the type b strains.

TABLE 2. *Number of animals bacteremic 48 h after inoculation*

Strain	No. bacterimic/total		
	i.n., 10^7 organisms	s.c., 10^3 organisms	i.p., 10^6 organisms
E_1	5/10	8/8 + 1 death[a]	7/7[b]
Santo Sb	13/21	8/8 + 1 death	—[c]
Santo Sd	11/19	1/21 + 1 death	—
Santo Rb	0/10	0/11	0/10
Garf Sb	8/19	21/27	—
Garf Sd	8/15	4/20	—
Garf Rd	0/10	0/17	0/15
Good Sb	8/15	9/10	—
Good Sd	1/26	4/22	—
Good Rd	0/20	0/20	0/9 + 1 death[d]

[a] Death presumed due to infection.
[b] Inoculation of one organism.
[c] —, Not done.
[d] Bacteremia not detectable; death presumably due to endotoxin.

DISCUSSION

All three unencapsulated strains were able to be transformed to type b or type d capsule production at similar frequencies and without regard to the original capsular type. Garf Rd and Santo Rd, when transformed and plated without antirough serum incubation, produced no detectable capsulated colonies; however, when antiserum was added and the transformants were incubated overnight, 10 to 40% of the colonies recovered were capsulated. Catlin et al. have mapped the *cap*B locus between the leucine and proline markers (7). We found that both *cap*B and *cap*D map in this area. Whether these are allelic or map in separate locations cannot be determined until more markers are identified in that region of the chromosome.

The only previous report studying virulence in animals by using the same strains carrying different capsular types was reported by Leidy et al. (13) in 1960; they used i.p. inoculation with hog gastric mucin in adult mice. They concluded that the natural type d strain (Garf) studied was more virulent than the type b strain (Santo), and the Garf Rd strain transformed to capsulation was more virulent than the similarly derived capsulated Santo Rd. However, in this model the unencapsulated derivatives were capable of causing death, a phenomenon not seen in the infant rat model unless the i.p. inoculum exceeds 10^6 CFU.

We used the same strains as did Leidy et al.; however, infant rats were used instead of adult mice. Infant rats have been shown to mimic the human disease pattern more closely; bacteremia and meningitis can occur after i.n., s.c., or i.p. inoculation without mucin (18, 22, 23, 26). However, a lower incidence of meningitis is found in bacteremic rats (60 to 80%) (18) than in infant

primates or humans (22). In our experiments, we found that the unencapsulated derivatives were unable to cause bacteremia. This agrees with the observation that these strains are rapidly cleared from the bloodstream of nonimmune animals and correlates with the clinical observation that they are unable to cause invasive disease (22).

Meningitis as measured by the presence of *H. influenzae* in the spinal fluid was found in 40 to 50% of the bacteremic animals, which is slightly lower (60 to 80%) than previously found with E_1 (18). Only bacteremic animals had meningitis, suggesting that bacteremia precedes the development of meningitis. Because of the relatively low number of animals developing meningitis coupled with the high correlation between bacteremia and meningitis seen in primates, we chose bacteremia as our criteria for defining virulence for these experiments.

With s.c. inoculations, a clear difference between type b and type d strains was seen. At an inoculum of 10^3 CFU, the two natural type b strains produced bacteremia in 100% of the animals; the transformants Good Sb and Garf Sb produced bacteremia in 90 and 75% of the animals, respectively, whereas the type d strains produced bacteremia in a lower percentage (9 to 20%) of the animals. The type d strains were statistically less virulent ($P < 0.002$) than the type b strains when given s.c. at the same inoculum. At a higher s.c. inoculum, 10^6 CFU, all 20 animals receiving Garf Sd were bacteremic, but only 50 to 60% of the animals inoculated with 10^6 CFU of Santo Sd or Good Sd developed bacteremia (data not shown).

When introduced by s.c. inoculation, type b strains appeared better able to survive the local host defenses and enter the bloodstream to multiply than the strains carrying the type d capsule. In vivo multiplication in these experiments is inferred, because a single organism isolated in a 10-μl sample corresponds to 2.5×10^2 organisms in the total blood volume (volume of infant rats is 2.5 to 3.0 ml of blood). The blood represents only 1.5% of the total bacterial population in the body fluids of infant rats (24). Thus, a single colony found in the blood sample represents a bacterial population of at least 10^4 organisms, which is 1 log greater than the 10^3 inoculum given s.c. In addition, 90% of the blood samples contained 10 or more colonies, which represents at least a 2-log increase over the inoculum; hence, in vivo multiplication is probable. This suggests that the type b capsule affords a survival advantage over the type d capsule when in vitro-grown cells are inoculated s.c. (22). The type b capsule may protect the cells from phagocytic clearance better than the type d capsule or in some other way give a survival advantage to the type b cells once they are in the body. However, this distinction is not evident between Garf Sb and Garf Sd or between Santo Sb and Santo Sd, all inoculated i.n. This suggests that both capsular types can penetrate the nasopharyngeal mucosa.

All of the type b strains, whether natural or derived by transformation, as well as Garf Sd (natural) and Santo Sd (transformed), were virulent and not statistically different in their ability to produce bacteremia when inoculated i.n. The Good Sd produced bacteremia in only 1 of 26 animals and was less virulent ($P < 0.002$) than either Santo Sd or Garf Sd.

To reduce the likelihood that a second factor such as changes in outer membrane or lipopolysaccharide is actually responsible for these results, sheared DNA derived from both Santo Sd and Garf Sd, with a maximum size of 6 megadaltons, was used to retransform Good Rd. Limiting amounts of DNA were used to ensure that each cell received only a single piece of DNA during transformation. This limits the number of genes transformed other than the capsule gene and ensures that we are comparing isogenic strains which differ in only a few genes. Capsulated transformants were selected by using antirough antiserum. The resulting Good Sb and Good Sd were inoculated i.n. and s.c. into infant rats. Good Sd was again significantly less virulent than Good Sb when given i.n. or s.c. with a bacteremia incidence of 9 and 15%, respectively, for Good Sd. All Good Sb and Sd transformants, regardless of the DNA source or length, showed similar abilities to produce bacteremia; i.e., Good Sb transformants were more virulent than Good Sd. This suggests that there is something intrinsically different between the strain Good and its parent, Garf. This difference is evident by a reduced ability to produce bacteremia after i.n. inoculated when both are carrying the type d capsule. This difference does not appear when both Good and Garf are carrying the type b capsule.

It is still possible that we are measuring something other than the effect of capsule in our animal assay, such as a regulatory gene which affects capsule and other virulence structures. However, one would have to postulate that this other gene is very closely linked to the capsule locus and is not expressed in the unencapsulated derivatives. The original unencapsulated derivatives of Garf and Santo were selected during in vitro passage of the strains. This dissociation of capsule production is found in most capsulated strains and correlates with the total absence of capsule or the presence of very small amounts of capsule around one pole of the cell as meas-

ured by fluorescent antibody (8). No other phenotypic characteristic has been correlated with the loss of capsule production other than the loss of virulence (8).

The Good strain, originally derived from Garf Sd, has been extensively passed in the laboratory. This particular derivative carries eight auxotrophic markers produced by *N*-methyl-*N*-nitroso-*N'*-nitrosoguanidine mutation (15). The difference in virulence between Good Sb and Good Sd when inoculated i.n. could be due to the altered ability to colonize the nasopharynx. However, carriage after i.n. inoculation is similar for both strains, 61 to 62%. Additionally, unencapsulated strains appear better able to adhere to human epithelial cells than encapsulated strains (J. M. Lewis and A. S. Dajani, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B53, p.26). Differences in amount or distribution of capsule around the cell could also explain the differences in virulence. However, preliminary data suggest that the amounts of capsule made (percentage of hexose or pentose) are similar for both transformed and natural type b and type d strains. Additionally, electron micrographs indicate no differences in capsule distribution between natural and transformed strains.

The apparent difference in virulence observed with the different routes of inoculation has been seen with other bacteria. Group C streptococci, when inoculated by aerosol into mice, are dependent on the presence of hyaluronic acid for virulence rather than M protein. When inoculated i.p., the presence of M protein is more important for virulence. The presence of M protein appears to be more important in human infections; thus, the i.p. inoculation of mice more closely correlates with group C streptococci virulence in humans (9). A similar situation may exist in *H. influenzae* carrying type d capsule when examined i.n. or s.c. Whether other capsule types (a, c, e, or f) show differences in virulence when different routes of inoculation are used is presently under investigation. Finding this difference in other types would suggest that when measuring virulence in capsulated strains other than type b, the use of the s.c. route may be preferred; this route may more closely correlate with the disease pattern in humans, since few strains other than type b have been associated in invasive disease.

The difference in virulence with the route of inoculation was not observed with the type b strains. All four strains examined appeared equal in their ability to produce bacteremia when inoculated i.n. or s.c. Whether the difference between type b and type d strains can be attributed to the capsule itself or whether other determinants which are closely linked or coregulated with the type b capsule are responsible cannot be determined until all the genes related to capsule production are identified and mapped. However, from the data it is clear that transformation, with small pieces of DNA, and selection for capsule production convert an avirulent, unencapsulated strain into a capsulated virulent strain capable of causing bacteremia and meningitis in infant rats. In addition, the experiments suggest that type b strains are more capable of surviving and multiplying in infant rats than the same strain carrying the type d capsule when inoculated s.c.

At the present time, experiments are under way to clarify the steps leading from i.n., s.c., or i.p. inoculation to the development of bacteremia in hopes of understanding the steps involved in virulence. In addition, the outer membranes of Good Sb and Good Sd are being examined to determine whether there are differences between these two transformants. We also hope to determine which route of inoculation most closely mimics the disease process in humans.

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