

## A Virulent Nonencapsulated *Haemophilus influenzae*

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Nontypeable *Haemophilus influenzae* strain INT1 was isolated from the blood of a young child with clinical signs of meningitis following acute otitis media. No immunologic or anatomic predisposition of this child for invasive bacterial infection with an unusual organism was documented. Sensitive ELISA proved the absence of intra- or extracellular capsular polysaccharide production by INT1, and Southern blot analysis confirmed the lack of an intact capsulation (*cap*) gene locus within the chromosome. Nevertheless, INT1 established bacteremia and meningitis in infant and weanling rat models of invasive *H. influenzae* infection. High-molecular-weight DNA isolated from INT1 was shown to confer an invasive phenotype on transformation of a nonencapsulated, avirulent laboratory strain of *H. influenzae*. Together these findings imply the presence of one or more as-yet-undiscovered, noncapsular virulence factors of *H. influenzae* that are capable of mediating invasive disease and resistance to immunologic clearance.

Encapsulated *Haemophilus influenzae* type b (Hib) causes invasive disease in children, including septicemia and meningitis. Type b capsular polysaccharide has traditionally been regarded as the critical virulence determinant, since it has been shown to confer resistance to phagocytosis and complement-mediated host defenses [1–4]. Serum antibodies to type b capsular polysaccharide are protective against invasive Hib infection [5, 6], and the introduction of widespread immunization with Hib conjugate vaccines in 1988 has resulted in a 93% decrease in invasive Hib disease in American children <5 years of age [7, 8].

In contrast to Hib, nonencapsulated *H. influenzae* commonly colonize the upper respiratory tract of healthy persons and are implicated in mucosal infections such as otitis media, sinusitis, bronchitis, and conjunctivitis [9]. Meningitis or other invasive disease due to nonencapsulated *H. influenzae* has been reported rarely, typically in elderly patients with significant underlying disease [10–13], persons with HIV infection or other immunodeficiencies [14, 15], those with head trauma or neurosurgical complications [16–18], and premature neonates born to women with evidence of intrauterine infection [19–21].

In 1992, a US epidemiologic survey determined that ~5% of invasive isolates (blood, cerebrospinal fluid [CSF], synovia,

or pleura) in children <5 years of age may be nontypeable by standard serologic tests [22]. Data from the United Kingdom, including capsular genotyping, corroborated these prevalence figures, including 7 cases of bacteremic nontypeable *H. influenzae* infection in previously healthy children outside the neonatal period [23]. With universal implementation of conjugate Hib vaccination, the percentage of residual invasive *H. influenzae* disease attributable to nonencapsulated strains appears to be increasing. The Public Health Laboratory Service Communicable Disease Surveillance Centre in London reports that 46 (61%) of 76 cases of invasive *H. influenzae* disease occurring in the United Kingdom between October 1993 and September 1994 were due to nontypeable strains [24]. The specific microbial virulence factors that allow nonencapsulated *H. influenzae* to cause serious disease remain unknown, and to our knowledge, the ability of such organisms to cause invasive disease in an animal model has never before been demonstrated.

### Case History

A 30-month-old white boy was in his usual state of good health until 6 days before admission, when he developed a low-grade fever and abdominal pain. He was seen by his primary pediatrician, who noted right otitis media and prescribed amoxicillin, which was not taken because of vomiting. Three days before admission, he was seen in an emergency department, where he appeared lethargic and dehydrated with unresolved otitis media. There were no meningeal signs on examination. His peripheral leukocyte count was 21,000/mm<sup>3</sup> with 58% neutrophils and 18% band forms. A blood sample was obtained for culture, the patient received intravenous rehydration and 50 mg/kg ceftriaxone intravenously, and was discharged home to resume amoxicillin administration.

The child continued to be irritable and listless with fever to 39.2°C over the ensuing 48–72 h but was able to take liquids and amoxicillin. He was carefully examined daily by his pediatrician who, on day 6 of symptoms, noted the development of passive

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Received 23 May 1995; revised 15 August 1995.

Presented in part: 95th general meeting of the American Society for Microbiology, Washington, DC, 22 May 1995 (abstract B-134).

Financial support: National Institutes of Health (HD-07233, AI-29549, AI-30068); University of Missouri Research Board (C3-41583).

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resistance to neck motion. A lumbar puncture revealed 338 white blood cells/mm<sup>3</sup> (88% neutrophils) and 39 red blood cells/mm<sup>3</sup>. CSF protein and glucose content were not quantitated. Repeat blood culture was done, intravenous dexamethasone and ceftriaxone were administered, and the patient was transported to Children's Hospital for admission.

Blood cultures from both the day of admission and the earlier emergency department visit grew *H. influenzae*, nontypeable by testing with antibody-coated latex particles (Wellcogen, London) and by slide agglutination (Difco, Detroit). No organisms were isolated from spinal fluid. The *H. influenzae* isolate proved susceptible to ceftriaxone and chloramphenicol but was ampicillin-resistant by virtue of  $\beta$ -lactamase production. Over his 4 days of hospitalization, the patient had steady clinical improvement, completed a course of dexamethasone, and was discharged home to finish 14 days of intravenous ceftriaxone.

Factors that may have predisposed this child to this unusual infection were sought. A review of the child's medical history was remarkable only for recurrent otitis media during the first year of life. No Howell-Jolly bodies were noted on peripheral blood smear. He was fully immunized, including four *H. influenzae* type b conjugate vaccinations (PRP-CRM; Praxis Biologics, Rochester, NY) administered at 2, 4, 6, and 21 months of age. At discharge, he had an anti-Hib capsular polysaccharide titer of 935 ng/mL (standardized IgG EIA, Praxis antigen, done at Specialty Laboratories, Santa Monica, CA). Six weeks after his hospitalization, computed tomography with contrast of the head demonstrated fluid in the right middle ear and air and fluid within the right mastoid. No identifiable erosion or discontinuity along the petrous ridge of the right temporal bone was seen to suggest a communication with CSF. The brain appeared normal. An intrathecal dye study was not done.

Serum immunoglobulin measurements were as follows: IgG = 651 mg/dL, IgM = 76 mg/dL, IgA = 57 mg/dL, and IgE = 19 IU/mL, all in the normal range for age. Complement studies showed C<sub>3</sub> = 109 mg/dL, C<sub>4</sub> = 38 mg/dL, and CH<sub>50</sub> = 250 U, also in the normal range. Finally, the child was shown to mount a normal antibody response to tetanus booster (before = 0.17 IU/mL, 4 weeks after = 2.97 IU/mL). He has remained healthy over the 18 months since his hospitalization.

The bacterium isolated from the patient's blood was further characterized. An absolute requirement of both hematin and NAD for aerobic growth on brain-heart infusion (BHI) agar was demonstrated. The colonies were not iridescent on BHI agar and spontaneously agglutinated in PBS. The organism produced indole and was able to decarboxylate ornithine but failed to hydrolyze urea, placing it in *H. influenzae* biotype V according to the scheme of Kilian [25].

## Methods

**Bacterial strains and cultures.** Strain E1A is a spontaneous streptomycin-resistant mutant of *H. influenzae* type b (Eagan), originally isolated from a child with meningitis. It has been shown to establish bacteremia and meningitis in infant rats after intraperitoneal or intranasal inoculation [26]. Strain R906 [27], also identified as Goodgal, is a derivative of strain Rd, a capsule gene deletion mutant of a type d *H. influenzae* known to be avirulent in the

infant rat model [28]. Strain U11 is a spontaneous streptomycin-resistant mutant of nonencapsulated strain U1, also identified as Ramirez [26], and is unable to produce sustained bacteremia in infant rats. INT1 is the nontypeable *H. influenzae* isolated from the 30-month-old child with meningitis described herein. INT1a represents the same strain isolated from the blood of an infant rat 48 h after intraperitoneal inoculation. All bacterial strains were grown at 37°C in BHI broth (Difco) supplemented with NAD and hematin (sBHI) as described [29]. For plating, the broth was solidified with 2% agar (Difco).

**Estimation of capsular polysaccharide.** Bacteria were grown to midlog phase ( $A_{600} = 0.2$ ) or overnight and harvested by centrifugation at 12,000 g at 4°C for 15 min, and the supernatants were frozen at -70°C before ELISA. A 50- $\mu$ L aliquot of each supernatant and serial dilutions in PBS containing 1% gelatin (PBSG) were used to coat the wells of a 96-well microtiter plate (Cell Wells; Corning Glass Works, Corning, NY). The cells were resuspended in PBSG, the density adjusted to an  $A_{600}$  of 8.0, and the cell suspension sonified at 30 W while immersed in ice for 3 min. The sonicated suspension was centrifuged at 20,000 g for 20 min at 4°C, and serial dilutions in PBSG of the supernatant were used to coat the wells of another microtiter plate. Finally, the bacterial membrane pellet was resuspended in PBSG to the original density ( $A_{600}$  of 8.0), and serial PBSG dilutions were used to coat a third microtiter plate. All plates were stored at 4°C until used within 48 h of preparation.

Three antibody preparations were used: Difco anti-*H. influenzae* b (1:200 or 1:1000), Difco polyvalent antisera reactive to *H. influenzae* types a and c-f (1:200), and high-titer burro anti-*H. influenzae* strain E1A (1:5000). All dilutions were made in PBS containing 3% bovine serum albumin (PBS-BSA). Antibody capture was detected with peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-donkey IgG (Organon Teknika, Durham, NC), developed with diphenylamine, and quantitated by measuring the absorbance at 450 nm. Control wells, included on each plate, contained only PBS-BSA.

**Southern analysis of genomic DNA.** High-molecular-weight DNA was isolated from overnight bacterial cultures by the method of Berns and Thomas [30]. About 5  $\mu$ g of each sample was digested to completion with *Eco*RI (Life Technologies GIBCO BRL, Gaithersburg, MD) and separated by electrophoresis on a 0.7% agarose-TRIS acetate gel at 50 V. The DNA was then transferred to a nylon filter by the Southern method [31].

The plasmid pU038 was provided by E. R. Moxon (John Radcliffe Hospital, Oxford, UK) and contains a complete set of genes common to all encapsulated *H. influenzae*, known as the *cap* locus [32], as well as type b-specific genes. A digoxigenin-labeled pU038 probe was prepared (Genius System; Boehringer Mannheim, Indianapolis) and hybridized to the target filter under standard conditions, and the probe-positive bands were visualized by an immunochromiluminescent detection system (Lumi-Phos 530; Boehringer Mannheim).

**Experimental animals.** Outbred, pathogen-free, Sprague-Dawley COBS/CD rats were obtained from Charles River Laboratories (Wilmington, MA). Pregnant females and 25- to 30-day-old weanling males were housed individually at 21  $\pm$  2°C and 50% relative humidity on a 7 A.M. to 7 P.M. lighting schedule. Food and water were available ad libitum. After delivery, infant rats were caged with their mothers until 5 days of life, at which time all infants

were pooled, randomized, and returned to the adult females in litters of 9 or 10.

**Inoculation procedure.** Bacteria were grown in sBHI broth to a density of  $10^8$ /mL, harvested by centrifugation as above, resuspended in PBSG, and diluted in the same solution to the desired density. A volume of 0.1 mL was used for intraperitoneal inoculation of both weanling and infant rats; randomized litters received injections of one *H. influenzae* strain at a single inoculum.

**Determination of bacteremia and meningitis.** After inoculation, the 5-day-old rats were returned to their surrogate mothers and examined every 12 h. Animals that died were removed from the litter. At 48 h after inoculation, a 0.2-mL sample of blood was obtained from each infant rat by direct cardiac puncture using standard sterile technique. Serial dilutions were plated and incubated overnight at 37°C, and colony-forming units were counted to determine the level of bacteremia.

For weanling males, at 48–54 h the animals were anesthetized with 2% halothane and external jugular vein blood (0.1 mL) was obtained and added to 1 mL of sBHI broth. After overnight incubation, the turbid tubes were streaked to BHI plates, and NAD and hematin strips were used to verify the presence of *H. influenzae*. Cisternal CSF was obtained by the method of Moxon and Murphy [33]. In brief, a skin flap was reflected over the cisterna magna and the connective tissue scraped aside with a dental pick. The membrane was punctured with a 25-gauge needle, and as the V-shaped cavity filled with CSF, 50  $\mu$ L was withdrawn and added to 1 mL of sBHI broth. After overnight incubation, turbid cultures were processed as above.

**Transformation with high-molecular-weight INT1 DNA.** High-molecular-weight DNA was prepared from INT1 and strain U11 by the method of Hull et al. [34]. Strain R906 was rendered competent by the M-IV nongrowth media procedure of Herriott et al. [35]. Competent R906 ( $10^9$  cfu in 1 mL) were exposed for 30 min to 1–2  $\mu$ g of DNA from INT1 or U11 or no DNA. The cells were then pelleted at 4°C in a microcentrifuge, resuspended in 1 mL of warmed sBHI broth, and incubated for 1 h at 37°C. A 0.1-mL aliquot ( $\sim 10^8$  cfu) was used for intraperitoneal inoculation of weanling rats, which were then evaluated for development of bacteremia and meningitis at 48 h as described above.

## Results

**ELISA for capsular polysaccharide.** To eliminate the possibility of capsule production by INT1 below the detection level of latex agglutination, a sensitive type b capsular polysaccharide ELISA was done on culture supernatants, membrane fractions, and cell sonicate supernatants of INT1 in stationary growth phase (figure 1). The activity of all INT1 preparations was identical to the corresponding background values produced by strain R906, a capsule gene deletion mutant, and  $10^2$ - to  $10^5$ -fold less than similar preparations from the type b control strain, E1A. Identical differences were found when the bacterial strains were tested in the early log phase of growth (data not shown). INT1a, the same strain after intraperitoneal injection and recovery from an infant rat, was tested under the same conditions and shown to lack type b capsule (data not shown). Finally, direct agglutination and ELISA testing was done using

polyvalent *H. influenzae* antisera (types a, c–f; Difco), demonstrating the absence of production of other known capsular polysaccharides by INT1 (figure 1) or INT1a (data not shown).

**Southern blot analysis of capsule gene sequences.** A DNA probe containing the complete *cap* locus (pUO38) was used in Southern blot analysis of *Eco*RI genomic digests from INT1 and INT1a (figure 2). The hybridization pattern of INT1 revealed an 8.6-kb fragment that migrated together with a previously described band in strain Rd [36], as well as a unique 6.6-kb band. These fragments showing homology to *cap* gene sequences were unaltered by passage in an infant rat and distinct from the characteristic 20-, 10.2-, 9.0-, 4.4-, 2.7-, and 2.1-kb *Eco*RI restriction pattern seen in E1A, which contains the intact *cap* locus. It is unlikely that the hybridizing bands in INT1 were due to the  $\beta$ -lactamase gene (*bla*) of the vector, as no detectable hybridization was seen with pUC18/19 (data not shown).

**Bacteremia and mortality in infant rat model.** Forty-eight hours after intraperitoneal inoculation with  $10^5$  organisms, 6 of 9 infant rats inoculated with INT1 had detectable bacteremia ( $>5$  organisms/mL of blood), whereas 5 of 9 rats in the E1A litter died and the rest had high-grade bacteremia (table 1). When an inoculum of  $10^6$  organisms was used, 2 of 10 in each of the INT1 litters died, and an additional 6 demonstrated sustained bacteremia; mortality in the E1A litters was 100%. The level of bacteremia detected in the INT1-infected rats at 48 h ranged from  $5 \times 10^1$  to  $2 \times 10^7$  cfu/mL and was greater with increasing inoculum but fell short of the levels produced by strain E1A ( $2 \times 10^7$  to  $1 \times 10^8$  cfu/mL). Serous fluid obtained postmortem from the chest cavity of INT1-infected rats that died confirmed high bacterial burdens. Serving as a negative control, none of the infant rats inoculated with R906 at the higher inoculum developed detectable sustained bacteremia.

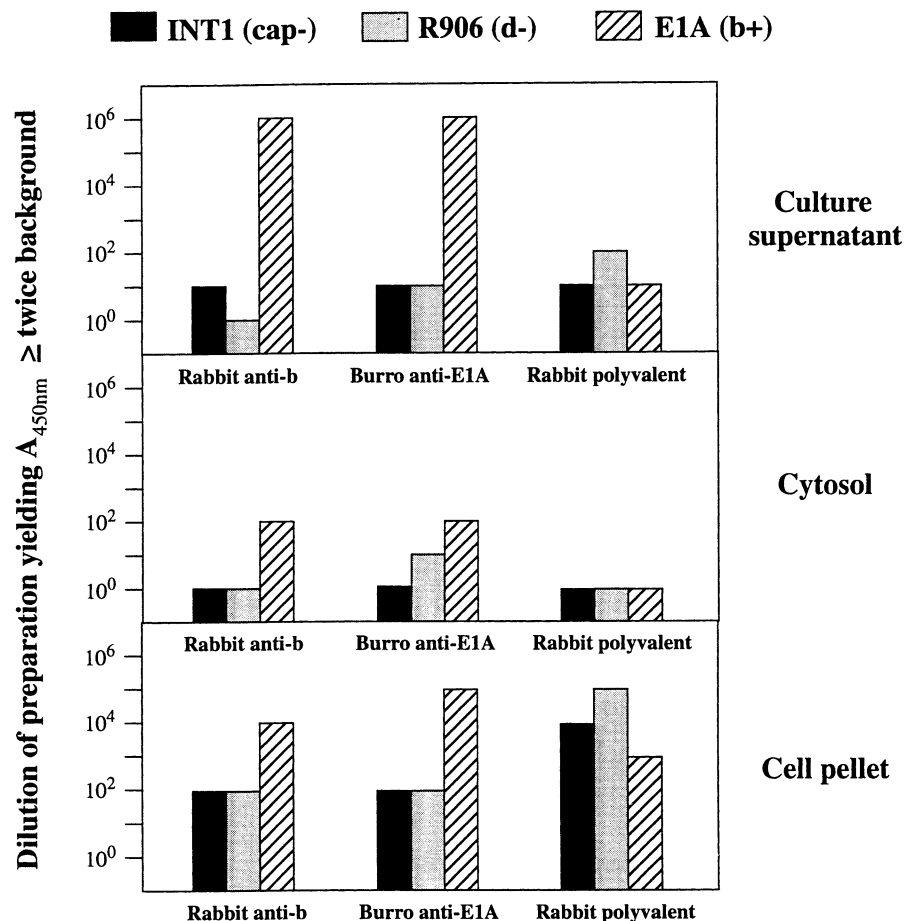
**Bacteremia and meningitis in weanling rat model.** Strain INT1 produced sustained bacteremia in 4 and meningitis in 3 of 6 weanling rats, compared with bacteremia in 6 and meningitis in 5 of 6 rats inoculated with E1A (positive control) (table 1). Strain R906 did not produce sustained bacteremia or meningitis in 8 weanling rats tested. Limits of detection in this weanling rat model were 10 cfu/mL for bacteremia and 20 cfu/mL for meningitis.

**Virulence of R906 transformed with INT1 DNA.** When competent R906 was exposed to high-molecular-weight INT1 DNA and transformants selected for in the weanling rat model, 5 of 6 rats developed sustained bacteremia, and 4 of these had concurrent meningitis (table 2). Control experiments demonstrated that the competence protocol itself did not render R906 virulent and transformation of R906 with U11 DNA did not result in transformants that cause sustained bacteremia or meningitis.

## Discussion

Bacteremia or meningitis with nontypeable *H. influenzae* are unusual infections in otherwise healthy children. Although the

**Figure 1.** Capsular antigen content of *H. influenzae* strains by ELISA. All preparations are from overnight cultures in supplemented brain-heart infusion broth. Rabbit anti-b = Difco polyclonal antisera vs. *H. influenzae* type b capsule, 1:200; burro anti-E1A = polyclonal antisera vs. *H. influenzae* strain E1A, 1:5000; rabbit polyvalent = Difco polyclonal antisera vs. *H. influenzae* types a, c–f capsules, 1:200.

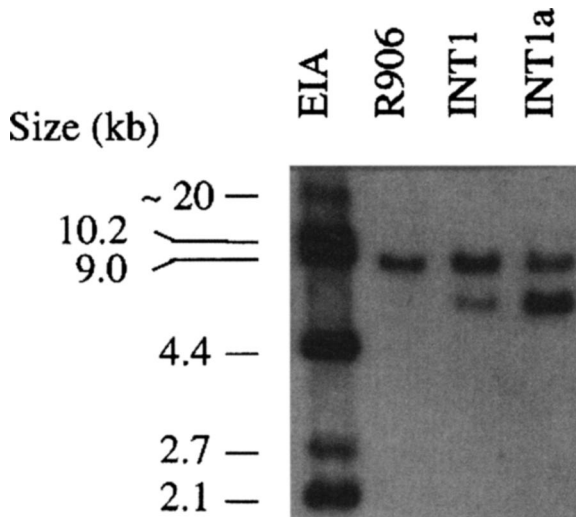


CSF pleocytosis in our patient may have represented a response to a parameningeal focus (e.g., mastoid), meningitis was suggested by clinical examination, and the sterile CSF culture was obtained only after intravenous antibiotics. After excluding an unrecognized immunodeficiency or anatomic abnormality in our patient, we sought to accurately define the capsulation status and animal virulence of the nontypeable *H. influenzae* isolate, INT1.

It is known that type b *H. influenzae* may spontaneously lose extracellular capsule expression at high frequency [36]. Catlin [37] described two such types of nonencapsulated variants, class I and class II, which were later shown [38] to produce small amounts of membrane-embedded capsular polysaccharide or even smaller amounts of intracellular capsular polysaccharide, respectively. However, ELISA testing of cytoplasmic contents, membrane fractions (cell pellet), and culture supernatants for type b capsule antigen showed that strain INT1 lacks immunologically detectable capsule. And whereas animal passage can effectively select for a small number of encapsulated organisms from a predominantly nonencapsulated culture [39], identical negative ELISA results for capsule antigen with strain INT1a demonstrated that was not the operative mechanism.

The 17-kb capsule gene locus (*cap*) of *Haemophilus* is divided into a serotype-specific central DNA region flanked by DNA common to all serotypes [40]. In the majority of type b strains, there is extensive duplication of these *cap* genes, with each cluster lying between direct repeats of an insertion sequence element, IS1016 [41]. An appreciable number of nontypeable isolates show partial hybridization with *cap* locus sequences, indicating that they may have evolved from encapsulated ancestors [42]. Southern blot analysis demonstrates that INT1 lacks the majority of hybridizing bands associated with an intact capsulation locus but does possess two bands with homology to the pUO38 *cap* gene probe. These findings suggest that INT1 has evolved from an encapsulated ancestor, although acquisition of partial *cap* or IS1016 sequences through in vivo transformation cannot be excluded.

The rat has proved to be a valuable animal model for the study of invasive disease due to *H. influenzae* and was chosen to examine the comparative virulence of the nonencapsulated isolate INT1. When inoculated intraperitoneally [1] or intranasally [43], E1A and other type b strains readily produce sustained bacteremia and meningitis in 5-day-old rats. Untypeable strains (U11, Rd, R906, KW20) have been used as negative



**Figure 2.** Southern blot hybridization of *EcoRI*-cut chromosomal DNA from *H. influenzae* strains probed with pUO38 to detect type b capsule gene sequences. E1A = type b<sup>+</sup> positive control, R906 type d<sup>-</sup> negative control; INT1 = nontypeable strain isolated from 2-year-old child with meningitis; INT1a = same strain isolated from infant rat 48 h after intraperitoneal inoculation.

controls in such studies, and, even with intraperitoneal inocula as large as 10<sup>8</sup> cfu, produce either very low-grade bacteremia that is cleared within several hours or no detectable bacteremia at all [1, 26, 44]. INT1a produced sustained bacteremia in infant rats at intraperitoneal inocula of 10<sup>5</sup> cfu, mortality in infant rats at inocula of 10<sup>6</sup> cfu, and sustained bacteremia and

**Table 1.** Virulence of *H. influenzae* strains after intraperitoneal inoculation of Sprague-Dawley rats.

Model, strain	Inoculum (cfu)	No. with bacteremia/total*	Mortality (infants) or no. developing meningitis/total (weanlings)
<b>Infants</b>			
E1A	10 <sup>5</sup>	9/9	4/9
	10 <sup>6</sup>	10/10, 10/10	10/10, 10/10
INT1	10 <sup>5</sup>	6/9	0/9
	10 <sup>6</sup>	8/10, 8/10	2/10, 2/10
R906	10 <sup>6</sup>	0/10, 0/10	0/10, 0/10
<b>Weanlings</b>			
E1A	10 <sup>6</sup>	6/6	5/6
INT1	10 <sup>6</sup>	4/6	3/6
R906	10 <sup>6</sup>	0/8	0/8

NOTE. Infants: 5 days old; weanlings: 4 weeks old. Capsule phenotype: E1A = b<sup>+</sup>, INT1 = cap<sup>-</sup>, R906 = d<sup>-</sup>. Cultures were done and cumulative mortality reported 48 h after inoculation. No mortality was observed at 48 h in weanling rats. Minimum bacterial density detectable in culture of blood of infant rats = 5 cfu/mL, blood of weanling rats = 10 cfu/mL, cerebrospinal fluid of weanling rats = 20 cfu/mL.

\* When 2 sets of data are given, 2 litters were tested.

**Table 2.** Virulence of *H. influenzae* strain R906 after transformation with high-molecular-weight DNA from nonencapsulated strain INT1 (intraperitoneal inoculation of weanling Sprague-Dawley rats).

Strain	Transforming DNA	Inoculum (cfu)	No. with bacteremia/total	No. with meningitis/total
E1A	—	10 <sup>6</sup>	4/4	4/4
INT1	—	10 <sup>8</sup>	5/6	4/6
U11	—	10 <sup>8</sup>	0/10	0/10
R906	—	10 <sup>8</sup>	0/10	0/10
R906*	None	10 <sup>8</sup>	0/10	0/10
R906*	U11	10 <sup>8</sup>	0/10	0/10
R906*	INT1	10 <sup>8</sup>	6/10	4/10

NOTE. Capsule phenotype: E1A = b<sup>+</sup>; INT1, U11 = cap<sup>-</sup>; R906 = d<sup>-</sup>. Cultures were done 48 h after inoculation. Minimum bacterial density detectable in culture of blood = 10 cfu/mL, cerebrospinal fluid = 20 cfu/mL.

\* After induction of competence in M-IV media.

meningitis in weanling rats at inocula of 10<sup>6</sup> cfu. To our knowledge, this is the first demonstration that a nonencapsulated *H. influenzae* (INT1) can produce sustained bacteremia, meningitis, or fatalities in an immunocompetent animal model.

*H. influenzae* may be rendered competent to take up purified DNA, and transformants can be shown to express acquired genes such as those for antibiotic resistance [32, 35] or capsule production [36, 39]. Competence media formulations induce the expression of six novel outer membrane polypeptides (“competence proteins”), which appear to be responsible for DNA uptake [45]. As a preliminary test to demonstrate that INT1 possesses a specific genetic determinant(s) related to bloodstream invasion and resistance to immune clearance, high-molecular-weight DNA isolated from INT1 was used to transform the nonvirulent, nonencapsulated *H. influenzae* strain R906. Intraperitoneal inoculation of the transformation mixture resulted in bacteremia and meningitis in weanling rats. Neither the competence protocol itself nor the introduction of DNA from a control unencapsulated *H. influenzae* strain (U11) produced virulent R906 derivatives in this model system.

The minimal implication of our findings is that certain non-encapsulated *H. influenzae* possess one or more virulence factors capable of mediating bloodstream invasion and resistance to host immunologic defenses. Given that DNA from INT1 exhibits partial hybridization to *cap* locus gene sequences, it is possible that these noncapsular virulence factors were present in an encapsulated ancestor of INT1 and may perhaps be widespread among current invasive isolates independent of capsulation status. Epidemiologic data support the existence of important noncapsular virulence factors even among type b strains, because invasive Hib disease appears limited to a small number of clonal populations [46] and clonal variation is observed in their predilection to cause meningitis [47]. An extreme hypothesis would hold that type b capsular polysaccharide is and has been merely a phenotypic marker for independently acting virulence genes in close linkage disequi-

librium with the *cap* locus. It is noteworthy that isogenic capsule-deficient mutants of Hib demonstrate enhanced adherence to and invasion of cultured human epithelial cells [48]. The ability of type b capsular polysaccharide to promote intravascular survival, however, appears indisputable [1, 4], and it is in this regard that the demonstrated virulence of the nonencapsulated strain INT1 is novel.

The prevalence of INT1 and other strains of similar phenotypic potential is unknown. INT1 belongs to *H. influenzae* biotype V, distinguishing it from several genetically distinct nonencapsulated type IV clones recently associated with obstetric and neonatal infections [21, 46]. Biotype V strains have been isolated most frequently from acute otitis media or as respiratory tract commensals [25, 49]. However, a small percentage of type b strains belong to biotype V, and at least one clinical series noted an unusually high recovery rate of nontypeable biotype V strains from blood and spinal fluid specimens [50].

Molecular cloning studies and further *in vivo* testing have begun in our laboratory in an attempt to identify the precise nature of the virulence factor(s) that allow INT1 to produce invasive disease.

#### Acknowledgments

We thank Marguerite Cervin, Carla Clausen, James C. Hurley, and Delilah Stevens for their assistance.

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