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Sporadic Invasion of Cultured Epithelial Cells by *Haemophilus influenzae* Type b

PAUL D. R. JOHNSON,[1]* FRANCES OPPEDISANO,[1] VICKI BENNETT-WOOD,[2] G. LYN GILBERT,[1]† AND ROY M. ROBINS-BROWNE[1]

Department of Microbiology and Infectious Diseases, Royal Children's Hospital,[1] and Department of Microbiology, University of Melbourne,[2] Parkville, Victoria 3052, Australia

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While working with an in vitro invasion assay, we observed that *Haemophilus influenzae* type b occasionally exhibits highly invasive behavior. The phenomenon is not inhibited by colchicine or cytochalasin but is dependent on the presence of supplemental CO_2. We propose that sporadic invasiveness may correlate with the unknown events that precede *Haemophilus influenzae* type b bacteremia.

Haemophilus influenzae type b (Hib) penetrates the nasopharyngeal mucosa of its host by an unknown mechanism. While working with an in vitro invasion assay, we unexpectedly observed that Hib occasionally displays high-level invasion of cultured epithelial cells. The aim of this report is to characterize this phenomenon.

Clinical isolates of Hib were obtained as previously described (2, 4, 5, 10). Hib strain Eagan (1) and the following mutants derived from it, Eagan-AH1-2 (20) and Eagan pES3ΔX-3 and Eagan *galE galK* (15), were kindly supplied by E. R. Moxon, Oxford, United Kingdom. All three mutants carry mutations within the *lic* loci which are involved in the phase variation of Hib lipopolysaccharide (LPS) (14, 20). Hib C54 Cap^+ P^+ (19) is a constitutively fimbriated strain of Hib kindly supplied by S. Falkow, Stanford, Calif. Nontypeable isolates of *H. influenzae* were obtained from routine clinical specimens.

Invasion assays were performed as previously described (17) with the addition of 10% Levinthal base. In brief, 3.0×10^7 Hib organisms from an overnight broth culture were incubated with cell monolayers (usually HEp-2) in 24-well tissue culture trays for 3 h. The monolayers were then washed and exposed to 100 μg of gentamicin per ml for a further 90 min, after which cells were lysed with digitonin and numbers of surviving bacteria were estimated by colony counts. Adhesion was assessed by the same method except that gentamicin was omitted. Because gentamicin remains in the extracellular supernatant, invading bacteria could be identified by their phenotypic resistance to gentamicin.

In general, Hib demonstrated a very weak propensity to invade in this assay. By contrast, a decapsulate mutant of Hib was consistently 20 to 100 times more invasive than its wild-type parent and a clinical nontypeable *H. influenzae* isolate (RCH no. NTHI 26.0) was consistently over 1,000 times more invasive than Hib (Table 1).

However, when the same Hib isolate was assayed for invasion in multiple replicates, occasional assays yielded counts much higher than expected. Differences between replicates amounting to as much as 10,000-fold were sometimes observed. When the data were displayed graphically, a right-skewed distribution emerged whenever a clinical isolate of Hib was tested for invasion in a sufficient number of replicates (Fig. 1 and 2). This phenomenon, termed sporadic high-level invasion, was observed with Hib and HEp-2, HeLa, and Madin-Darby canine kidney (MDCK) cell lines. No comparable effect was observed when Hib was tested for adhesion (Fig. 1).

Single colonies from assays exhibiting high-level invasion were characterized as Hib and repassaged through further assays for a total of six cycles. The majority yielded the same low counts as the original strain, but sporadic high-level invasion continued to be observed as before. Hib strain Eagan, three *lic* mutants of this strain, and a constitutively fimbriated strain of Hib also displayed the phenomenon, as did a decapsulate mutant of Hib. Sporadic invasion continued to be observed when the assay was performed with cytochalasins B and D and colchicine in the range of 0.5 to 50 μg/ml and when the incubation temperature was varied between 37 and 39.5°C.

However, sporadic high-level invasion was not observed when invasion assays were performed without supplemental CO_2. In one experiment, the mean count for 264 replicate invasion assays performed in the absence of supplemental CO_2 was 83 CFU per assay, with results for all assays lying within 2 standard deviations of this mean. In contrast, 10% of the results for 264 replicate assays in the second arm of the experiment performed with 5% supplemental CO_2 were between 3 and 35 standard deviations greater than the mean observed in the first arm ($P = 0.003$; Mann-Whitney-Wilcoxon test) (Fig. 2). When the progressive pH reduction that normally occurs when assays are performed with supplemental CO_2 was mimicked in assays performed in air by progressive titration with dilute hydrochloric acid, sporadic high-level invasion was not restored.

In vitro assays employing cultured epithelial cells, despite their artificial nature, have provided important insights into the behavior of pathogenic bacteria (7, 8, 12, 17, 18).

We observed sporadic high-level invasion with several clinical isolates of Hib, a decapsulate mutant of Hib, and one isolate of *H. influenzae* type f obtained from cerebrospinal fluid. Sporadic high-level invasion was also displayed by *lic* mutants of Hib, suggesting that the phenomenon is not related to known mechanisms of LPS phase variation. We have not observed this phenomenon with a variety of other pathogens.

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, Royal Children's Hospital, Parkville, Victoria 3052, Australia. Phone: 61-3 9345 5735. Fax: 61-3 9345 5764. Electronic mail address: pdrjoh@netspace.net.au.

† Present address: Department of Clinical Microbiology, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Westmead, N.S.W. 2145, Australia.

TABLE 1. Adhesion and invasion by *H. influenzae* isolates

Capsular type	Source	No. of isolates	% Recovered bacteria (mean ± SD)[a]		Adhesion/invasion ratio[b]
			Adhesion	Invasion	
b	Child with epiglottitis	9	13.1 ± 5.0	0.0006 ± 0.0005	0.0046
b	Child with meningitis	11	12.5 ± 5.1	0.0007 ± 0.0001	0.0056
b	Throat swab	8	13.1 ± 4.8	0.00051 ± 0.0004	0.0040
b	Blood or CSF[c]	15	11.9 ± 5.6	0.0011 ± 0.002	0.0092
Decapsulate mutant of Hib RCH no. 70C−	Laboratory	1	31.8 ± 10.1	0.027 ± 0.0086	0.0849
NTHI[d]	Nonsterile sites	18	6,102 ± 72,165	22.6 ± 50.3	0.37

[a] Results are expressed as percentages of bacteria originally added per well.
[b] The ratio was determined by the following formula: percent invasion/percent adhesion × 100.
[c] CSF, cerebrospinal fluid.
[d] NTHI, nontypeable *H. influenzae*.

Colchicine and cytochalasin, agents which inhibit eukaryotic cell microtubular and cytoskeletal function, respectively, did not appear to inhibit the phenomenon. Recently, it has been reported that cytochalasin D and colchicine also fail to prevent eukaryotic cell entry by *H. influenzae* biogroup aegyptius (16).

Sporadic high-level invasion was not seen in the absence of supplemental CO_2. The permissive effect of CO_2 appeared to be independent of pH, and it cannot be attributed to an effect on growth rate (Fig. 3). The concentration of CO_2 in the human upper respiratory tract is approximately 5%, compared with an atmospheric concentration of 0.03%. We propose that the sensing by Hib of an increase in CO_2 concentration may lead to phenotypic changes, such as increased invasiveness, that enhance colonization and persistence. Regulation of virulence by carbon dioxide has also been described for several other species of bacteria (6, 11, 13).

There are at least five chromosomal loci in *H. influenzae* that contain multiple repeated tetramers, misreading of which may randomly modulate expression of downstream genes (9). In this way, a replicating clonal group of bacteria, all experiencing the same initial conditions, can express different phenotypes simultaneously. While high-level invasion occurs too infrequently to be explained by this type of mechanism alone and is not selectable, it is conceivable that several regulatory systems could interact to produce an occasional invasive phenotype. Hib organisms that become internalized may then pass beyond the mucosal barrier and, protected by their antiphagocytic capsules, establish bacteremia.

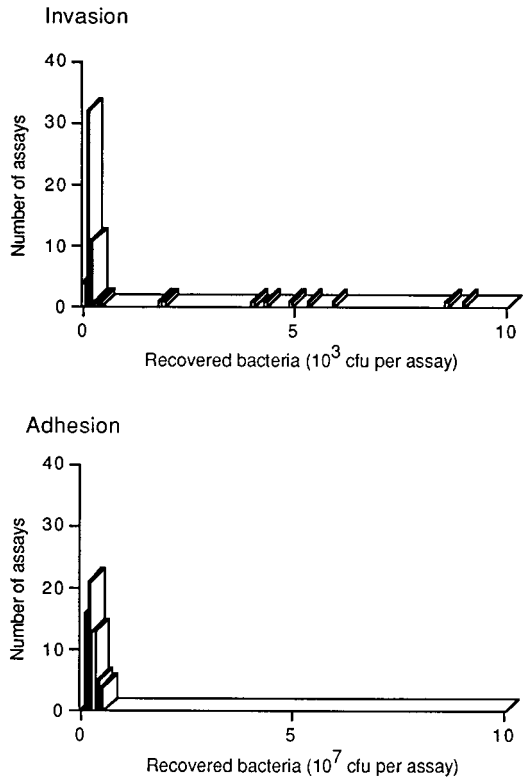

FIG. 1. Sporadic high-level invasion does not reflect variations in adhesion. The upper panel shows a frequency distribution of results from individual invasion assays, obtained with 23 different clinical Hib isolates. The lower panel shows results obtained with the same isolates assayed for adhesion in the same experiments.

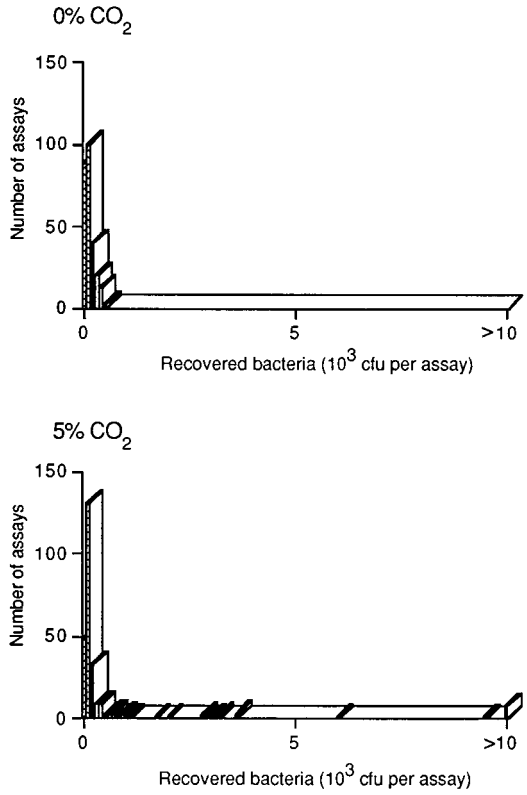

FIG. 2. Effect of supplemental CO_2 on sporadic high-level invasion. The panels show the frequency distribution of results from 528 replicate invasion assays performed with a single clinical isolate of Hib (RCH no. 70). One half of the replicates were incubated without CO_2 (upper panel), and the remainder were incubated with 5% supplemental CO_2 (lower panel).

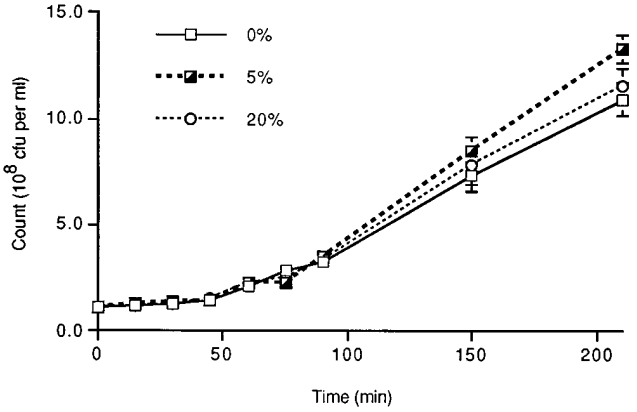

FIG. 3. Growth curves of Hib RCH no. 70 in atmospheres containing three different concentrations of supplemental CO_2, 0, 5, and 20% (means ± standard deviations for four observations at each time point).

Alternatively, sporadic high-level invasion may not be under direct genetic control. For example, single isolates of methicillin-resistant *Staphylococcus aureus* give rise to subpopulations with varied susceptibilities to methicillin. At least 10 chromosomal genes and a wide variety of environmental factors appear to control this phenomenon (3).

We have demonstrated that in the presence of physiological levels of CO_2, Hib sporadically exhibits a highly invasive phenotype in vitro. Further study of the phenomenon could advance our understanding of how Hib initiates invasive disease.

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