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Haemophilus influenzae bacteremia and meningitis resulting from survival of a single organism

(streptomycin/central nervous system/nasopharyngeal colonization/pathogenesis of central nervous system infection/encapsulated bacteria)

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ABSTRACT **Infant rats were infected intranasally with mixtures of streptomycin-sensitive and streptomycin-resistant strains of *Haemophilus influenzae* type b and cultures of nasopharyngeal washings, blood, and cerebrospinal fluid were obtained. If the infecting organisms cooperated with each other during the establishment of infection, nasopharyngeal, blood, and cerebrospinal fluid cultures should have contained mixtures of the variants. If each organism acted independently, then with small infecting inocula all the organisms in nasopharynx, blood, or cerebrospinal fluid should be descended from a single bacterium. Cultures should then contain only one of the variants.**

Single variant nasopharyngeal cultures were obtained from 8 out of 19 (42%) rats when the intranasal inoculum was <100 organisms. As the inoculum was increased, single variant cultures were less frequently observed. When the inoculum was $\geq 10^5$ organisms, nasopharyngeal cultures were always mixtures. Single variant blood cultures were obtained in 46 of 67 (68.7%) episodes of bacteremia when rats were inoculated intranasally with 10^8 organisms. Single variants were isolated from the cerebrospinal fluid of 13 of 19 (68.4%) rats with meningitis whose blood contained both streptomycin-sensitive and streptomycin-resistant variants. When the blood contained a single variant, this same variant was cultured from the cerebrospinal fluid on 39 of 40 (97.5%) occasions. These studies demonstrated that invasive *H. influenzae* b infections of infant rats resulted from independent action, as opposed to cooperative interaction of intranasally inoculated organisms. The results also suggested that the meninges were invaded by the hematogenous route.

Intranasal inoculation of infant rats with *Haemophilus influenzae* type b results in bacteremia and meningitis and has proved to be a useful experimental model of the human disease (1–3). These studies provided evidence that the pathogenesis of meningitis involved three sequential events: nasopharyngeal colonization, bacteremia, and central nervous system invasion, although direct invasion of the meninges by contiguous spread from the nasopharynx was not precluded. The studies also revealed that an inoculum of at least 1000 organisms was a prerequisite for the induction of bacteremia and meningitis. This observation might be explained as follows. First, it is possible that every organism in the initial inoculum behaves in a manner that is uninfluenced by the other bacteria present (independent action) (4–6). Each organism has a low probability of successfully overcoming the host defenses; however, the probability is not zero. As the dose of organisms is increased, it becomes progressively more likely that one of them will establish a local or distant focus of infection. Under these circumstances, the bacteria recovered from blood or cerebrospinal fluid (CSF), for example, would be descended from a few organisms, perhaps even a single organism of the original inoculum (4–7). Alternatively, organisms *en masse* may possess properties that are denied to single bacteria (cooperative action). When the inoculum attains a critical magnitude, infection ensues (8, 9). Under these circumstances, bacteria cultured from the blood or CSF would be descendents of many different organisms of the original inoculum.

The following study was designed to test the theory of independent action compared to that of cooperative action in the pathogenesis of *H. influenzae* type b, bacteremia, and meningitis. The results also provided data relevant to the relationship between nasopharyngeal colonization, bacteremia, and invasion of the central nervous system.

MATERIALS AND METHODS

The Eagan strain of *H. influenzae* type b and its one-step streptomycin-resistant mutant, the media used for its growth, the methods of intranasal inoculation, the technique of blood and CSF sampling, and the quantitation of bacteria in blood and CSF were identical to those used previously (1–3). Natural litters of 5-day-old rats (Sprague–Dawley, strain COBS/CD) were obtained from Charles River Laboratories, Wilmington, MA. Inocula containing an approximately equal number of the streptomycin-sensitive (SM^S) and streptomycin-resistant (SM^R) variants were prepared as follows. Each variant was grown overnight on solid medium at 37°. A few colonies of each variant were suspended in 15 ml of liquid broth in separate erlenmeyer flasks and incubated with vigorous shaking. When the cultures reached mid-logarithmic phase (absorbance 0.1 in Lumetron model no. 401 with the 490 nm filter), the broth cultures were centrifuged at $5000 \times g$ at 0° and the pellets were resuspended in chilled phosphate-buffered saline. Equal volumes of the suspensions in phosphate-buffered saline containing the Sm^S and Sm^R variants were combined and agitated on a Vortex. Serial 10-fold dilutions were performed to estimate the viable counts. Dilutions yielding 40–200 colonies were plated onto media with and without 400 μg of streptomycin per ml. This permitted estimation of both the total count of viable bacteria and the proportion of Sm^S and Sm^R variants. As an additional check on the proportions of each variant, the number of Sm^R variants was estimated by replica plating (10) onto streptomycin-containing medium. The proportions obtained by these independent estimates were averaged for each experiment. Overall, the mean proportion of Sm^R variants was 52%, with a standard error of 1.7%. Preliminary experiments established that the virulence of the Sm^S and Sm^R variants was similar. Twenty rats were inoculated intranasally with either the Sm^S or the Sm^R variant. Bacteremia and meningitis, respectively, occurred in 25% and 23% of Sm^S inoculated rats and 18% and 13% of rats inoculated with the Sm^R variant.

In all of the experiments, 5-day-old rats were inoculated in-

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Abbreviation: CSF, cerebrospinal fluid.

Table 1. Nasopharyngeal cultures

Intranasal organisms	Positive/total (%)	Cultures scored: Indeterminate	Pure Sm^S	Pure Sm^R	Mixed (%)
3–9	5/27 (18.5)	2	3	0	0 (0)
26–90	28/54 (51.9)	12	1	4	11 (39.3)
$1.2–9 \times 10^2$	24/27 (88.9)	4	2	2	16 (66.7)
$1.2–9 \times 10^3$	18/19 (94.7)	2	0	2	14 (77.8)
$1.2–9 \times 10^4$	9/10 (90.0)	1	1	0	7 (77.8)
1.7×10^5	10/10 (100.0)	0	0	0	10 (100.0)

Results of nasopharyngeal cultures 24 hr after intranasal inoculation of different numbers of an equal mixture of streptomycin-sensitive (Sm^S) and streptomycin-resistant (Sm^R) *H. influenzae* type b into 5-day-old rats.

tranasally with 10 μl of suspension in phosphate-buffered saline, containing the desired number of an equal mixture of the Sm^S and Sm^R variants.

Nasopharyngeal cultures were performed by instilling 20 μl of sterile phosphate-buffered saline into the right nostril and culturing 10 μl of fluid recovered from the left nostril.

Nasopharyngeal, blood, and cerebrospinal fluid cultures were initially plated on antibiotic-free media. Colonies of *H. influenzae* were scored as Sm^S or Sm^R by replica plating onto plates containing streptomycin. All cultures containing both Sm^S and Sm^R variants (whatever the proportion of each) were considered to be mixed. All cultures that contained eight or more colonies of the same variant were considered to be pure. This was reasoned as follows: The probability (P) of obtaining a pure culture of only one variant from a 50% mixture of Sm^S and Sm^R organisms is $2(0.5)^n$, where n is the number of colonies scored. If $n = 8$, $P = 1/128$, which is <0.01. Cultures consisting of less than eight colonies of one variant were considered indeterminate.

RESULTS

Nasopharyngeal cultures

Table 1 shows the results of nasopharyngeal cultures. When less than 100 bacteria were inoculated, the cultures often yielded pure cultures of either the Sm^S or Sm^R variant. As the dose increased, so did the number of mixed cultures. With an inoculum of $\geq 10^5$ bacteria, all nasopharyngeal cultures were mixed.

Bacteremia

Fig. 1 shows the results of blood cultures. When the dose was 10^4–10^5 bacteria, 58 of 60 (96.7%) rats had pure Sm^S or Sm^R

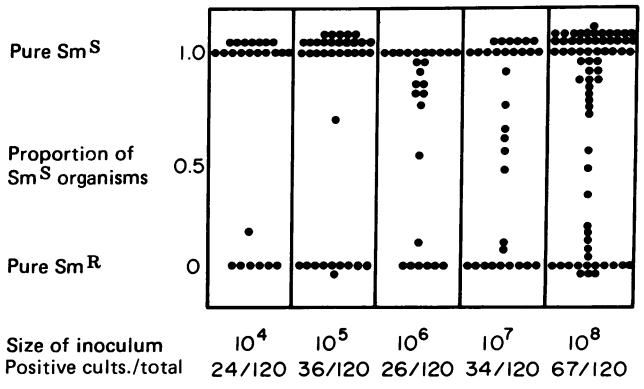

FIG. 1. Proportion of Sm^S and Sm^R variants of *H. influenzae* type b recovered from blood cultures. Groups of 40 rats each received inocula of 10^4–10^8 intranasal organisms. Cultures were obtained 2, 3, and 5 days after inoculation.

Table 2. Proportion of Sm^S and Sm^R variants in blood

Cultures scored	Time after inoculation: 5 min	30 min	3 hr	6 hr	10 hr	20 hr	54 hr
Indeterminate	0	0	1	5	0	1	0
Pure Sm^S	0	0	0	0	2	9	9
Pure Sm^R	0	0	0	0	1	1	6
Mixed	3	2	0	1	4	4	5

Numbers of blood cultures containing one or both variants at different times after the intranasal inoculation of 10^8 *H. influenzae* type b consisting of equal numbers of Sm^S and Sm^R variants are shown.

blood cultures. As the size of the inocula increased, the proportion of cultures containing only one of the variants decreased, but even with an inoculum of 10^8 organisms, 46 of 67 (68.7%) rats had single variant blood cultures.

Because blood cultures were performed on only 10 μl of blood, it was possible that some cultures scored as pure were in fact taken from rats with mixtures of Sm^R and Sm^S variants, but with one predominating. We, therefore, performed the following experiment. Three rats with intense ($>10^4$/ml) Sm^S bacteremia were exsanguinated and the blood (1–1.5 ml for each rat) was added to 10-ml aliquots of broth, incubated overnight, and finally plated onto media containing streptomycin. No Sm^R colonies grew; therefore, there were less than 1 in 10,000 Sm^R variants present.

Observations up to this stage were on rats with sustained, intense bacteremia detected 2–5 days after inoculation. However, previously published studies had shown that bacteremia could be detected within minutes of intranasal inoculation (3). Therefore, serial blood cultures were performed (Table 2). For 6 hr after inoculation, all of six blood cultures scored were mixed, compared to 13 of 41 (31.7%) blood cultures obtained 10–54 hr after inoculation. The incidence of mixed cultures less than 6 hr after inoculation was significantly different from that observed more than 6 hr after inoculation ($\chi^2 = 7.50$; $P < 0.01$).

Meningitis

Growth of *H. influenzae* in CSF occurred only in rats with associated bacteremia (Table 3). The CSF cultures contained the same variant as that found in the blood in 39 of 40 rats with single variant blood cultures. Among 19 rats with mixed bacteremia (demonstrable in two or more consecutive blood cultures obtained at intervals of at least 24 hr), 13 CSF cultures contained only a single variant and 6 contained both Sm^S and Sm^R variants.

DISCUSSION

The hypothesis that all but one bacterium of an infecting inoculum may be eliminated has been advanced by others (4–9). Evidence supporting this concept was obtained by analysis of dose-mortality curves and by studying experimental salmon-

Table 3. Comparison of blood and CSF cultures

CSF culture	Blood culture: Sm^S	Sm^R	Mixed	Totals
Sm^S	25	0	7	32
Sm^R	0	14	6	20
Mixed	1	0	6	7
Totals	26	14	19	59

Number of rats are given with blood and CSF cultures that contained Sm^S or Sm^R or mixtures of Sm^S and Sm^R variants of *H. influenzae* type b.

ellosis (7). The present investigation provides evidence supporting the applicability of independent bacterial action in the pathogenesis of *H. influenzae* type b bacteremia and meningitis. The results also furnished evidence that CNS invasion occurred by the hematogenous route.

Independent bacterial action was demonstrated at all three stages of the pathogenesis of the experimental infection. When small numbers of organisms (<100) were inoculated intranasally, 42% of nasopharyngeal cultures grew only one variant. As the inoculum was increased, more rats became colonized; the proportion of mixed cultures increased so that with inocula of $\geq 10^5$ bacteria, all rats were colonized with a mixture of Sm^S and Sm^R organisms. Inocula of $\geq 10^4$–10^8 bacteria resulted in bacteremia, the majority of which comprised pure cultures of either the Sm^S or Sm^R variant. Elimination of one variant did not occur during penetration of the nasopharynx, since early blood cultures (obtained up to 6 hr after inoculation) were mixed. It was observed that early bacteremia often spontaneously resolved and did not recur. However, when persistent bacteremia did supervene, it was most commonly with a single variant. Thus, it would seem likely that many organisms initially penetrated the nasopharynx and entered the bloodstream. Subsequently, a focus was established either paranasally or distant from the nasopharynx which resulted in sustained bacteremia (11). The only reasonable explanation for the data is that organisms isolated from the blood of rats with sustained bacteremia were often the progeny of a single bacterium. Elimination of all but one organism was also demonstrated during invasion of the central nervous system. Rats with mixed Sm^S and Sm^R bacteremia developed meningitis in which 13 of 19 (68%) CSF cultures grew only the Sm^S or the Sm^R variant.

These conclusions depended upon secure evidence that the variants were of approximately equal virulence. In fact, there were 147 single variant blood cultures (100 Sm^S and 47 Sm^R) and 52 single variant CSF cultures (32 Sm^S and 20 Sm^R). Thus, although there was evidence for a small difference in the virulence of the variants, the difference could not account for the preponderance of pure cultures of both variants.

Another possible source of error was that cultures scored by us as pure were in fact mixtures of variants. Thus, if relatively small numbers of Sm^S colonies were sampled (<200), small numbers (<1%) of Sm^R variants might not have been detected. In three rats with Sm^S bacteremia, we were able to show that less than 1 in 10,000 of the organisms were Sm^R variants. However, when plates yielded growth of Sm^R organisms only, we could not be certain that every single colony had transferred to streptomycin plates. Because of this source of error, it is possible that a few of the cultures with low colony counts that were scored by us as pure were in fact taken from rats with mixed infections. However, it seems most improbable that our results can be completely explained in this way. Considering the nasopharyngeal cultures scored as Sm^R, the actual numbers of colonies counted were 11, 14, 17, 20, 70, 102, and >300. Of 27 Sm^R blood cultures, only 3 were scored on the basis of less than 20 colonies, and 24 were scored by counting more than 100 colonies. There were 20 pure Sm^R cultures of CSF; 7 contained 20–100 and 9 contained >100 colonies. We, therefore, are of the opinion that very few cultures were incorrectly scored as pure.

These experiments also provided clear evidence that the meninges were invaded by the hematogenous route. Nasopharyngeal cultures from rats given $>10^4$ intranasal bacteria were mixed, yet only one rat out of forty had CSF infection with a variant not found in the bloodstream. If bacteria reached the central nervous system by contiguous spread from the nasopharynx (12) (perhaps via olfactory neurons), many CSF cultures should have yielded either one or both of the variants when they were absent from the blood but present in the nasopharynx.

It may be worthwhile to consider the possible relevance of these observations. In a longitudinal study, 38.5% of children acquired *H. influenzae* type b (at least one positive nasopharyngeal culture) by 5 years (13). However, *H. influenzae* meningitis has always been relatively uncommon; the present incidence of 8,000–10,000 cases annually (14) in the U.S. population represents an attack rate of about 1 per 1000 children. The vast majority of these children do not suffer from any obvious immune defect either before or after their bout of meningitis. The theory of independent action would explain these observations as follows. The number of *H. influenzae* b inhaled as a consequence of exposure of a child to a carrier is unknown. The actual number is not critical since the theory predicts that one organism may be sufficient to colonize the nasopharynx. Once colonization and bacterial replication have occurred, there is the possibility of tissue invasion, each organism having a low, but finite probability of penetrating the nasopharyngeal mucosa and establishing a contiguous or distant focus from which sustained bacteremia can be maintained. Bacteremia in human beings with *H. influenzae* b is, in fact, not uncommon (15), but many of these individuals do not develop meningitis. The theory of independent action predicts that any organism in the bloodstream has some finite probability of establishing meningitis. The incidence of meningitis will be a function of the number of children with bacteremia as well as the magnitude and duration of bacteremia. Since the U.S. population contains about 6,300,000 children below the age of 2, a number of them, relatively small but absolutely large, will develop *H. influenzae* b meningitis as a consequence of natural exposure to small numbers of organisms. There need be nothing wrong with these children; they are simply the victims of random selection.

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