

## Pathogenesis of Bloodstream Invasion with *Haemophilus influenzae* Type b

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Possible route(s) by which encapsulated bacteria invade the blood from the nasopharynx include (i) the direct invasion of submucosal blood vessels and (ii) clearance via lymphatics to regional nodes followed by bloodstream invasion. These possibilities were investigated in rats after intranasal inoculation with  $10^5$  *Haemophilus influenzae* type b. Within 24 h of inoculation, 10 of 42 rats with sterile blood cultures had similar numbers of *H. influenzae* b recovered from both cervical (local) and periliac (distant) lymph nodes, which suggested early bacteremic spread. When virtually continuous blood cultures were obtained for 30 min after inoculation with  $10^8$  *H. influenzae* b, early transient bacteremia was documented in four of eight rats. Also, we found no significant difference in bacteremia among rats whose cervical lymph nodes had been removed surgically compared with sham-operated rats. These findings favor the hypothesis of a rapid, perhaps direct invasion of pharyngeal blood vessels as an initial determinant of the systemic spread of *H. influenzae* b.

*Haemophilus influenzae* type b commonly colonizes upper respiratory mucosal surfaces and has a propensity for causing bacteremia and systemic disease in young children. The mucosal contact with *H. influenzae* b is felt to be the initial step in the pathogenesis of bacteremia with *H. influenzae* b, and the magnitude and duration of the bacteremia are major determinants of focal infections, such as meningitis (9). However, the events which occur between the initial mucosal contact and sustained bacteremia are unclear. Two distinct, but not mutually exclusive mechanisms for the development of bacteremia are as follows. (i) The organisms may enter directly into the bloodstream via mucosal or submucosal blood vessels; although subject to clearance by any of the reticuloendothelial organs, sufficient numbers may survive and replicate in one or more sites and result in sustained bacteremia. (ii) The organisms may be transported to regional lymph nodes, where some may survive, replicate, and result in bacteremia, entering the systemic circulation via the efferent lymphatics.

The young rat is a well-studied biologically relevant model for *H. influenzae* b meningitis, in which rats inoculated intranasally with *H. influenzae* b become colonized and bacteremic and subsequently develop meningitis (10). The present study utilized this model to examine the mechanism of bloodstream invasion after atraumatic intranasal inoculation, with emphasis on

the role of lymph nodes in the pathogenetic sequence.

### MATERIALS AND METHODS

#### Organism, growth media and preparation of inocula.

A one-step streptomycin-resistant mutant of *H. influenzae* b Eagan was used in all experiments (16). The organisms were grown to mid-logarithmic phase in brain heart infusion (BHI) broth supplemented with 10  $\mu$ g of hemin and 2  $\mu$ g of diphosphopyridine nucleotide per ml. The broth cultures were then centrifuged at  $5,000 \times g$  for 3 min, and the pellets were suspended in chilled 0.01 M phosphate-buffered saline containing 0.1% gelatin (PBS-G) and diluted for intranasal inoculation. Animal specimens (lymph nodes, pharyngeal washes, blood) were cultured on BHI agar containing 10% Levinthal base and 500  $\mu$ g of streptomycin per ml; other blood specimens were cultured in BHI broth containing hemin and diphosphopyridine nucleotide with or without 250  $\mu$ g of streptomycin per ml and 0.03% sodium polyanetholesulfonate (Sigma Chemical Co., St. Louis, Mo.)

**Animals and techniques.** Litters of 20-day-old rats (Sprague-Dawley, strain COBS/CD) were obtained from Charles River Laboratories, Wilmington, Maine. They were weaned at the time of inoculation and housed in separate filtered cages. Atraumatic intranasal inoculation (25- $\mu$ l volume) was performed in unprepared rats as previously described (11), and blood sampling was by heart puncture (only in sacrificed animals) or by tail vein puncture. Nearly continuous blood cultures were obtained in the search for early bacteremia by restraining rats in a towel (3) after intranasal inoculation and sampling blood after multiple tail vein punctures over 15 to 30 min. Blood

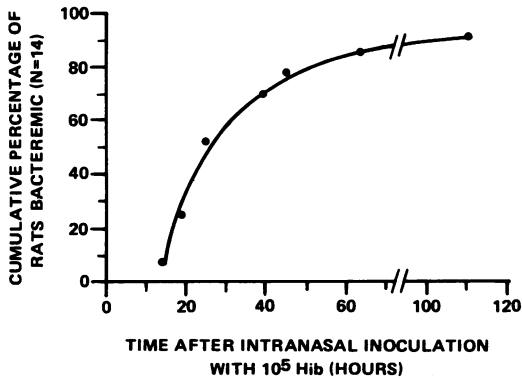


FIG. 1. Onset of sustained bacteremia after intranasal inoculation with  $10^5$  *H. influenzae* b (Hib).

obtained during consecutive 5-min intervals during the first 20 min was inoculated into supplemented BHI broth and cultured separately. Pharyngeal secretions were obtained by instilling 50  $\mu$ l of PBS-G in both nares of unprepared rats and collecting the secretions which pooled in the mouth.

**Lymph nodes: procurement and culture.** The locations and the drainage area for lymphatic vessels and lymph nodes of the white rat have been evaluated in detail (5, 8). Cervical lymph nodes provide primary drainage for the pharynx, the larynx, and the floor of the oral cavity, and the periiliac lymph nodes (drainage area: pelvis, genital organs, abdominal wall, rectum, and anus) were selected as accessible control lymph nodes having no anatomic continuity with the pharynx or gastrointestinal tract (excluding the rectum and anus). After the animals were sacrificed with ether anesthesia followed by exsanguination (to minimize blood passively residing in the lymph nodes), the lymph nodes were dissected, excised, ground in a tissue grinder containing PBS-G, and cultured on BHI agar as described above. In early experiments, touch preparations of cut sections of nodes were fixed in absolute ethanol and stained with hematoxylin and eosin to microscopically confirm by histological section the identification of these tissues as lymph nodes.

**Lymph node excision.** Fifteen-day-old rats were anesthetized with pentobarbital (0.5 mg) intraperitoneally and ether. One half of each litter had a bilateral excision of their cervical lymph nodes via a 2-cm transverse incision in the anterior neck; the other half of each litter was sham operated. All pups were returned to their mother, weaned, and intranasally inoculated at the age of 20 days.

**Statistical methods.** The Fisher exact test (two tailed) was used to analyze the data from the lymph node excision experiment.

## RESULTS

### Onset of bacteremia after intranasal inoculation.

After the intranasal inoculation of  $10^5$  organisms, sustained bacteremia occurred in most rats (Fig. 1). Although only one rat was bacteremic at 14 h after inoculation, 50% of the animals (7 of 14) were bacteremic by 25 h, and 93% of the

animals (13 of 14) were bacteremic at 110 h after inoculation.

**Recovery of viable *H. influenzae* b from lymph nodes.** If the pathogenesis of bacteremia with *H. influenzae* b involves the spread from pharynx to local lymph nodes to blood, then viable bacteria should be recovered from lymph nodes before the onset of sustained bacteremia, and lymph nodes not in anatomic continuity with the respiratory tract should not yield *H. influenzae* b. Table 1 shows that after the intranasal inoculation of  $10^5$  organisms, *H. influenzae* b was cultured from cervical lymph nodes in the absence of detectable bacteremia. However, *H. influenzae* b was also recovered in equal numbers (range, 5 to 400 organisms per lymph node) and with equal frequency from the periiliac lymph nodes, although they have no anatomic communication with the pharynx. (To avoid difficulty in the interpretation of positive lymph node cultures in the presence of bacteremia, we omitted three rats with detectable bacteremia from the data in Table 1). Thus, the bacteria reached lymph nodes distant from the pharynx before the onset of sustained bacteremia and did so as early as 5 min after inoculation. These observations suggested an early bacteremia as the most likely mechanism for organisms to reach these lymph nodes.

**Detection of early bacteremia.** In an initial attempt to document early bacteremia, we cultured large volumes of blood (1 ml) after intranasal inoculation to maximize the sensitivity of detecting circulating bacteria. Blood cultures were obtained from 44 rats 1 min to 3 h after the inoculation of  $10^5$  organisms (24 rats were cultured for 1 to 5 min after inoculation, 9 rats were cultured for 30 to 60 min after inoculation, and

TABLE 1. Recovery of viable *H. influenzae* b in local and distant lymph nodes after intranasal inoculation of  $10^5$  *H. influenzae* b

Time after intranasal inoculation	No. with positive cultures/no. tested in:			
	Pharynx	Blood <sup>a</sup>	Cervical lymph nodes <sup>b</sup>	Periiliac lymph nodes <sup>c</sup>
5-60 min	4/4	0/18	4/16	4/16
10-15 h	5/8	0/8	0/8	0/8
16-24 h	18/18	0/18 <sup>d</sup>	7/18	6/18

<sup>a</sup> Limit of detection, 10 organisms per ml (100  $\mu$ l of blood cultured).

<sup>b</sup> Lymph nodes which drain the naso- and oropharynx.

<sup>c</sup> Control lymph node having no continuity with the naso- and oropharynx.

<sup>d</sup> Three rats with positive blood cultures were excluded from the data shown to avoid the difficulty in the interpretation of positive lymph node cultures in the presence of bacteremia.

TABLE 2. Early bacteremia after intranasal inoculation of  $10^8$  *H. influenzae* b into rats

Rat no.	Bacteremia <sup>a</sup> at time after inoculation (min) of:				Any positive culture during first 30 min
	2-5	5-10	10-15	15-30	
1	-	-	-	-	No
2	+	+	-	-	Yes
3	-	-	-	-	No
4	-	+	+	-	Yes
5	-	+	-	-	Yes
6	+	-	-	+	Yes
7	-	-	-	-	No
8	-	-	-	-	No
Total					4/8

<sup>a</sup> Blood was obtained continuously via the tail vein and cultured in broth media (See the text). -, Denotes sterile blood culture; +, denotes blood culture which grew *H. influenzae* b.

11 rats were cultured 1 to 3 h after inoculation), but we were unable to document early bacteremia. However, when the intranasal inoculum was increased to  $10^8$  *H. influenzae* b and virtually continuous blood cultures were obtained throughout the first 15 to 30 min (see above), early bacteremia was detected in four of eight animals. The results of cultures of blood obtained at consecutive intervals after inoculation (i.e., 1 to 5 min, 5 to 10 min, 10 to 15 min, 15 to 30 min) in these four animals with early bacteremia showed bacteremia during certain time intervals and not during others (Table 2). Thus, we were able to document early bacteremia after intranasal inoculation with *H. influenzae* b.

**Effect of cervical lymph node excision upon onset of bacteremia.** If the cervical lymph nodes are critical in the pathogenesis of bacteremia, then their removal might be expected to alter the incidence of bacteremia or increase the time interval between inoculation and the onset of bacteremia. However, we found no statistically significant difference in either the incidence of bacteremia or the time interval between inoculation and the onset of bacteremia in the group whose cervical nodes had been excised compared with the sham-operated littermates (Table 3). Thus, we found no evidence of a critical role of the cervical lymph nodes in the pathogenesis of bacteremia with *H. influenzae* b.

### DISCUSSION

The 20-day-old rat is a useful model for the study of the early stages of *H. influenzae* b disease. It provides a consistent model for bacteremia: 93% of rats developed sustained bacteremia after intranasal inoculation with  $10^5$  organ-

isms. Furthermore, 20-day-old rats are much larger than 5-day-old rats, making surgery and repeated blood cultures possible. These older, weaned animals can be housed individually, thus avoiding the problem of the intralitter spread of *H. influenzae* b (4).

The simultaneous recovery of viable *H. influenzae* b from both local lymph nodes draining the pharynx and lymph nodes distant from the pharynx in the absence of detectable bacteremia indicates that these lymph nodes were most likely seeded via the bacteremic spread of organisms. Since the periiliac nodes drain only the distal gastrointestinal tract (rectum and anus), it is extremely unlikely that organisms recovered from these nodes shortly after inoculation could have entered via the gastrointestinal tract. Furthermore, we were unable to recover *H. influenzae* b from stools of rats whose nasopharynx was heavily colonized with *H. influenzae* b (L. R., unpublished data). It is unlikely that the recovery of organisms from these lymph nodes was due to blood containing *H. influenzae* b within these nodes. Since only a small amount of lymph node tissue was cultured (~1 mg), this explanation would require a bacteremia of at least  $10^3$  organisms per ml to account for even one organism in the lymph node. The number of animals in whose lymph nodes organisms were recovered may represent an underestimate as animal tissue inhibits the recovery of viable organisms (18). The rats subjected to the surgical excision of the lymph nodes draining the pharynx had neither a decreased incidence of bacteremia nor an increase in the time interval between inoculation and detectable bacteremia. Thus, these data offer no support for the hypothesis that lymphatics or lymph nodes draining the pharynx serve as the primary route or focus for sustained bacteremia. Furthermore, early bacteremia was documented in 50% of the animals

TABLE 3. Effect of prior cervical lymph node excision on development of bacteremia after intranasal challenge with *H. influenzae* b

Rat pretreatment	No. of rats (%) bacteremic <sup>a</sup> /no. of rats tested at (h):		
	24	48	72
Cervical lymph node excision	6/19 (32) <sup>b</sup>	12/19 (63)	15/19 (79)
Sham operation	3/18 (17)	10/18 (56)	13/18 (72)

<sup>a</sup> Limit of detection,  $10^2$  organisms per ml of blood ( $10 \mu$ l of blood cultured).

<sup>b</sup>  $P > 0.1$  comparing the two treatment groups at each time interval (Fisher exact test, two tailed).

by continuous blood cultures over the first 15 min after the inoculation of  $10^8$  organisms. The intermittent recovery of organisms during the first 30 min after inoculation implies either a transient (but recurrent) bacteremia or a low magnitude of bacteremia close to the limit of detection or both, resulting in the recovery of organisms in some samples and not in others on a chance basis. Thus, in this experimental model, *H. influenzae* b invades the blood shortly after inoculation, and this may provide an explanation for the dissemination of *H. influenzae* b.

Several studies performed during the 1930s provide data supporting the concept of an early bacteremia with a different encapsulated bacterium, *Streptococcus pneumoniae*. Within 1 h of the intratracheal inoculation of rabbits (2), the intranasal inoculation of mice (13), or the exposure of mice to *S. pneumoniae* in a spray chamber (17), organisms were recovered from the blood or spleen or both. In contrast to the implication of the above findings, it has been proposed that hematogenous invasion by encapsulated bacteria occurs after the penetration of the mucosal barrier and spreads via the local lymphatics to the local lymph nodes (1, 15). These nodes may confine the infection; alternatively, surviving bacteria may disseminate hematogenously after replication and spread within regional lymphatics and nodes. After the intranasal inoculation of rabbits with *S. pneumoniae*, Schultz et al. (14) recovered organisms in the cervical lymph of all animals within 3 h of inoculation but detected organisms in the blood of only one animal. These authors concluded that the organisms penetrated the draining lymphatics after being placed on the pharyngeal mucous membrane. However, blood samples were only obtained at 30-min intervals, whereas cervical lymph was collected and cultured continuously. Control lymph (from lymphatics in an area not contiguous with the pharynx) was not cultured, which would have ensured that organisms were only recovered from lymphatics in continuity with the inoculated mucosal surface. An equally plausible interpretation of these data would be that early bacteremia was not detected due to infrequent blood cultures, with clearance of organisms from the blood by lymph nodes and the escape of some of these organisms into the efferent lymph fluid.

The finding of early bacteremia after the intranasal inoculation of *H. influenzae* b raises a question regarding the mechanism of entry into the bloodstream. To reach the bloodstream, this nonmotile bacterium must pass through or between epithelial cells, penetrate the basement membrane and subepithelial tissue, and enter the endothelium of a blood vessel. A striking finding is that *H. influenzae* b inoculated intra-

nasally into rats induced a marked polymorphonuclear inflammatory response in the nasopharynx as early as 5 min after inoculation (12). Possible mechanisms for the entry of *H. influenzae* b into the circulation include the ingestion of *H. influenzae* b by polymorphonuclear leukocytes or macrophages, with survival (6) or adherence to macrophages, neutrophils, or erythrocytes and subsequent passive "piggyback" carriage into the systemic circulation. Alternatively, an intense inflammatory response may disrupt the mucosal barrier, allowing the entry of *H. influenzae* b into the capillaries. It is also conceivable that organisms are cleared to the local lymph nodes very early after inoculation, but escape this filter and arrive in the systemic circulation.

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