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Haemophilus influenzae b Infection in Rats: Effect of Splenectomy on Bloodstream and Meningeal Invasion After Intravenous and Intranasal Inoculations

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We investigated the effect of splenectomy on the susceptibility of rats to intravenous or intranasal inoculation of *Haemophilus influenzae*, type b. The 50% lethal dose for asplenic rats inoculated either by intravenous (i.v.) ($10^{4.7}$) or intranasal (i.n.) ($10^{4.6}$) injection was similar, but significantly lower than the 50% lethal dose value in sham-operated rats ($10^{8.6}$ i.v. and $10^{9.0}$ i.n.). Mean survival time was significantly longer for asplenic rats inoculated i.n. (49.3 h) compared to asplenic rats inoculated i.v. (24.4 h). Similarly, sham-operated rats inoculated i.n. survived significantly longer after i.n. challenge (mean survival time, 171.4 h) than after i.v. challenge (34.7 h). Bacteremia was detected in 100% of asplenic rats and in 80% of sham-operated rats. The geometric mean number of bacteria in the blood of asplenic rats ($10^{4.90}$ per ml) was significantly greater than in sham-operated rats ($10^{3.29}$ per ml). Meningitis was detected in 7 of 15 randomly sacrificed asplenic rats, whereas none of 15 sham-operated rats had evidence of meningeal invasion. Thus, the asplenic rat was more susceptible to experimentally induced *H. influenzae* bacteremia, meningitis, and fatal sepsis and offers a biologically relevant experimental model for investigating the role of the spleen in defense against infection with encapsulated bacteria.

Impaired function or absence of the spleen substantially increases human susceptibility to bacterial sepsis (3, 4). The majority of these infections are caused by encapsulated strains of *Streptococcus pneumoniae, Haemophilus influenzae* type b, or *Neisseria meningitidis* (3, 4, 8). Experimental studies with laboratory animals also have demonstrated the importance of the spleen in affecting clearance of *S. pneumoniae* (5, 10, 11, 12). In these studies, bacteria were inoculated by intravenous (i.v.) or intraperitoneal injections. Although these studies have contributed much useful data, these models do not simulate the pathogenesis of *S. pneumoniae* sepsis in asplenic humans. Recently, Dickerman et al. induced *S. pneumoniae* sepsis in asplenic mice via the respiratory tract, because the pathogenesis of this infection more closely resembled that occurring in humans (2).

Although *S. pneumoniae* account for approximately 75% of septic episodes among individuals with deficient splenic function, about 15% are caused by *H. influenzae* b (8). The present studies were performed to investigate the role of the spleen in host defense against *H. influenzae*. We also sought to gain insight into the role of the spleen as a determinant of bacterial invasion of the central nervous system, since an increased frequency of meningitis is a biologically relevant and clinically important consequence of *H. influenzae* b bacteremia among individuals with splenic deficiency. With this latter objective in mind, we elected to induce bacteremia by intranasal (i.n.) inoculation, since invasive *H. influenzae* b infection in humans is initiated via the respiratory tract.

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MATERIALS AND METHODS

Animals. Two-month-old albino Sprague-Dawley rats (strain COBS/CD) were purchased from Charles River Breeding Laboratories, Inc., and maintained under standard laboratory conditions.

Bacteria. *H. influenzae* b was a one-step streptomycin-resistant mutant derived from strain Eag. The source, method of storage, and characteristics of this strain have been described (1, 7), as have the procedures for its growth and quantitation (7).

Inoculation procedures. Ten days after surgery, animals were inoculated by i.n. injection with a technique similar to that previously described (7), except that before being given the bacterial suspension, the rats were lightly anesthetized with ether to facilitate instillation of the inoculum (50 μl) into the left nostril. i.v. inoculations were made into the tail vein; the rat

was lightly anesthetized with ether, and the desired number of bacteria, suspended in 500 μl of phosphate-buffered saline, was inoculated by using a tuberculin syringe and a 26-gauge needle.

Culture methodology. Blood cultures were obtained from the tail vein. The rat was placed in a restrainer, and the tail was warmed to dilate the blood vessels. The tip of the tail was incised by using a scalpel blade. One hundred microliters of undiluted blood and 100 μl of blood diluted 10^{-1} to 10^{-3} in chilled phosphate-buffered saline were cultured to estimate the number of bacteria per milliliter of blood. Cerebrospinal fluid was obtained for cell count and culture as was previously described (6).

Experimental procedure. To determine the effect of splenectomy on susceptibility to either i.v. or i.n. challenge with *H. influenzae* b, 168 rats were divided into two equal groups: 84 underwent splenectomy and 84 underwent a sham operation in which the peritoneal cavity was opened, the spleen was seen, and the peritoneum was closed. Forty-two asplenic and 42 sham-operated animals were then challenged i.v. or i.n. with varying numbers of *H. influenzae* b. After bacterial challenge, the animals were observed at 12-h intervals to determine the rate and number of deaths. The mean 50% lethal dose (LD_{50}) for each group of animals was determined by using the previously described method of Reed and Muench (9).

In a separate experiment, the effect of splenectomy on susceptibility to bacteremia and meningitis was investigated in 40 asplenic and 40 sham-operated rats inoculated i.n. with approximately 10^6 *H. influenzae* b. Blood cultures were obtained 4, 12, 18, 20, 24, 48, and 72 h after inoculation from randomly selected groups of five asplenic and five sham-operated rats. Also, at 24, 48, and 72 h, five asplenic and five sham-operated rats were randomly selected and sacrificed to determine the presence or absence of meningitis.

RESULTS

Effect of splenectomy on mortality, LD_{50}, and survival time after i.v. or i.n. inoculation with *H. influenzae* b. There was a significantly higher mortality in asplenic rats after either i.v. or i.n. inoculation of $>10^4$ *H. influenzae* b (Table 1). Overall, the case fatality rate in asplenic rats was significantly greater ($P < 0.0001$). Irrespective of the challenge route, the calculated mean LD_{50} was about 10,000 times less for asplenic than for sham-operated rats. However, survival time, defined as the interval from bacterial inoculation to time of recorded death, was significantly longer in rats challenged by the i.n. as compared to the i.v. route (Fig. 1). Mean survival time was 24.4 (standard deviation, ± 13.9) h after i.v. challenge of asplenic rats, compared to 49.3 (± 22.4) h in asplenic rats inoculated i.n. ($P < 0.01$). In sham-operated rats, i.v.-inoculated rats survived a mean of 34.7 (± 32) h as compared to 171.4 (± 46.8) h after i.n. challenge ($P < 0.001$).

Effect of splenectomy on susceptibility to bacterial invasion of the bloodstream and meninges. Asplenic rats developed bloodstream infection earlier and with significantly greater numbers of bacteria than did sham-operated rats (Fig. 2). Meningitis in asplenic rats, evidenced by positive cerebrospinal fluid cultures and increased cerebrospinal fluid granulocytes, was present in one of five rats 24 h after inoculation, two of five rats at 48 h, and four of five rats at 72 h. In contrast, none of the sham-operated rats developed meningitis.

DISCUSSION

These studies show the asplenic rats to be significantly more susceptible to experimentally induced *H. influenzae* b bacteremia, meningitis, and fatal sepsis than rats possessing a spleen. Although several studies have documented the enhanced susceptibility of asplenic laboratory animals to *S. pneumoniae* infection (5, 10, 12), altered susceptibility to other species of encapsulated bacteria has not been convincingly documented. In the present studies, splenectomy reduced the LD_{50} of *H. influenzae* b in the rat

TABLE 1. *Effect of splenectomy on mortality of rats inoculated by i.v. or i.n. injection with varying inocula of H. influenzae*

Variable	Intravenous		Intranasal		Significance[a]
	Asplenic	Sham-operated	Asplenic	Sham-operated	
No. of bacteria inoculated					
2×10^9	7/7[b]	6/7	7/7	2/7	<0.05
2×10^8	6/7	2/7	5/7	2/7	<0.05
2×10^7	7/7	0/7	5/7	1/7	<0.001
2×10^6	2/7	0/7	6/7	1/7	<0.05
2×10^5	7/7	1/7	4/7	0/7	<0.001
2×10^4	1/7	0/7	0/7	1/7	>0.1
LD_{50}	$10^{4.6}$	$10^{8.6}$	$10^{4.7}$	$10^{9.0}$	

[a] Compares mortality (from both i.v. and i.n. inocula) in asplenic versus sham-operated rats. Total, <0.0001.
[b] Total number dead/total number inoculated.

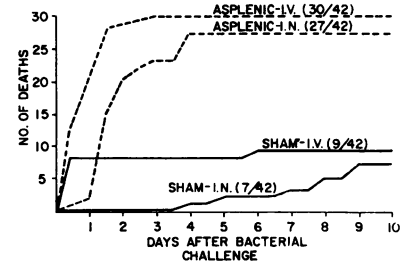

FIG. 1. *Number of deaths in asplenic and sham-operated rats after either i.v. or i.n. challenge. No deaths occurred after day 9.*

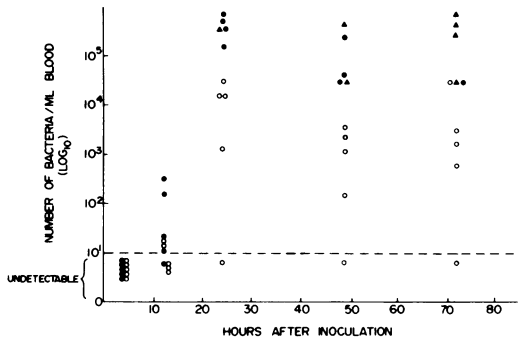

FIG. 2. *Magnitude of bacteremia in asplenic and sham-operated rats after intranasal inoculation of H. influenzae type b. ▲, Asplenic rats with meningitis; ●, asplenic rats without meningitis; ○, sham-operated rats without meningitis.*

by > $\log_{10^{4.0}}$ organisms, after either i.v. or i.n. inoculation. Thus, mortality rate was dependent upon the presence or absence of the spleen rather than the route of bacterial inoculation. Nonetheless, the time interval between inoculation and death was significantly longer in rats challenged by the i.n. compared to the i.v. route. This latter finding suggests some potential difficulties in interpreting experimental studies of the normal and asplenic host as they might relate to humans infected with encapsulated bacteria. Previous studies, such as those of Ellis and Smith (3), have emphasized the dual role of the spleen to act both as a biological filter and to enhance opsonization by synthesis of "early" antibody. However, these events were demonstrable only after i.v. administration of organisms or particulate antigen.

For the most part, natural infections involve spread of bacteria from epithelial tissues to the bloodstream. Under these circumstances, the evolution of bacteremia and the fate of intravascular bacteria are determined in part by host and microbial factors which influence the replication, removal, and bloodstream entry of bacteria from extravascular sites. The present studies indicate substantial differences in the time course of bacteremia after i.n. as opposed to i.v. challenge.

Our data may provide some insight into the increased susceptibility of the asplenic host to meningeal invasion. In humans with deficient or absent splenic function, bacterial meningitis due to encapsulated bacteria occurs with substantially increased frequency when compared to attack rates for the normal population (13). In the rat model of *H. influenzae* b meningitis, the magnitude of bacteremia appears to be a key determinant of meningeal invasion (6). In the present studies there were, on the average, 10 times more viable *H. influenzae* organisms in the blood of asplenic compared to sham-operated rats. Meningitis occurred in 47% of asplenic rats, but in none of the rats with intact spleens. Thus, the occurrence of meningitis appears to be directly related to the higher bacterial counts observed in the blood of the asplenic rats. However, it is also possible that the spleen plays some indirect role in facilitating removal of bacteria from the central nervous system, perhaps through production of opsonins or other factors which stimulate bacterial phagocytosis in the central nervous system.

In conclusion, although caution must be exercised in extrapolating these findings to infections in humans, i.n. inoculation of rats with *H. influenzae* appears to offer a biologically relevant experimental model for investigating the antibacterial defenses of the asplenic host to encapsulated bacteria.

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