Production of *Haemophilus influenzae* b Meningitis in Infant Rats by Intraperitoneal Inoculation

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Sprague-Dawley rats have a marked age-related susceptibility to *Haemophilus influenzae* type b that does not correlate with serum bactericidal activity. Eighty percent of 5-day-old animals that survive to 48 h after an intraperitoneal inoculation of a mean lethal dose of bacteria have histologically documented meningitis. Animals surviving the inoculations as infants manifest cerebral dysfunction as adults. This model should facilitate experimental study of bacterial meningitis.

Bacterial meningitis continues to be a health problem of infants and children. Since the introduction of antibiotics, the childhood mortality from this disease has remained at approximately 5 to 10%, while neurological sequelae, such as deafness, seizures, and mental retardation, affect 30 to 50% of the survivors (15).

Approximately 65% of the children with bacterial meningitis are between 3 months and 6 years of age (6, 20, 21). Recent epidemiological surveys of meningitis suggest that *Haemophilus influenzae* b, the most common etiologic agent in childhood, has an annual attack rate of approximately 1 in every 2,000 children in this age group (J. V. Bennett and J. B. Robbins, personal communication). As this affects the developing central nervous system (CNS) of young children, it follows that bacterial meningitis is one of the most common causes of postnatally acquired cerebral dysfunction.

The bacteria that most commonly cause childhood meningitis are generally susceptible to available antibiotics. Thus, it seems likely that further reductions in the prevalence, morbidity, and mortality will be realized only through prevention, early diagnosis, and new modes of management, which maintain normal brain metabolism and integrity.

An animal model in which the age-dependent susceptibility and the pathological lesions are similar to those observed in human disease and one in which a significant percentage of survivors demonstrate cerebral dysfunction would allow evaluation of pathophysiology, such as the basis of host susceptibility, mode of bacterial invasion, and the biochemical and pathological mechanisms involved in the genesis of neurological sequelae. With such a model, eventual outcome could be interpreted in terms of these parameters and their relationship to antibiotic therapy.

We have examined the susceptibility of infant rats, in the absence of CNS trauma, to *H. influenzae* b inoculated intraperitoneally. Infant rats were chosen because of the known age-related susceptibility of young children to meningitis and because of the large foundation of neurochemical and behavioral data available on this animal. Further, our results indicate that rats have marked age-related susceptibility to this bacterium, develop meningitis with classic histological changes, and, if they survive, demonstrate cerebral dysfunction.

MATERIALS AND METHODS

Bacteriological methods. *H. influenzae* b (Eagan) was isolated from a child with meningitis. The strain was originally isolated on chocolate agar, but had been passed repeatedly on brain heart infusion (BHI) agar supplemented with heme and oxidized nicotinamide adenine dinucleotide (NAD$^+$). A streptomycin-resistant mutant (E-1) was selected on supplemented BHI agar containing streptomycin at 500 μg/ml. Grace Leidy, Babies Hospital, New York, provided strain U-1, originally identified in her laboratory as strain Ramirez which does not produce detectable capsular antigen. A high-level, streptomycin-resistant

mutant, U-11, was developed with identical methods used to produce E-1. These *H. influenzae* strains were inoculated into brain heart broth containing a source of heme (supernatant fluid of horse blood diluted 1:3,000 with distilled water, centrifuged, and the supernatant fluid sterilized by filtration through a 0.22-μm pore size membrane filter [Millipore Corp.]) and NAD$^+$ (Sigma Chemical Corp., St. Louis, Mo.) at 0.1 μg/ml. They were grown at 37 C to a density of 10^{10}/ml (an absorbance of 0.5 in Lumetron model #401 using the 490-nm filter), harvested by centrifugation at 5,000 × g at 4 C, resuspended in skim milk, and frozen in portions at −70 C. To assay viable bacteria in the organs of infected rats, the organs were removed under aseptic conditions, weighed and homogenized (10 ml/g wet weight) in phosphate-buffered saline (PBS) with a standard clearance Potter-Elvehjem homogenizer in an ice bath, diluted in PBS, plated on a supplemented BHI agar plate containing strep-tomycin at 500 μg/ml, and incubated overnight at 37 C.

Inoculation of animals. *H. influenzae* b (E-1) and the unencapsulated avirulent strain U-11 were grown in supplemented BHI broth to a density of 10^8 organisms/ml, harvested by centrifugation at 5,000 × g at 4 C, resuspended in a phosphate buffered saline containing 0.1% gelatin (PBS-G), diluted in the same media to the desired density, and inoculated inter-peritoneally in a volume of 0.1 ml between 9 a.m. and 1 p.m.

Animals used. Rats used in this study were derived from the outbred strain COBS/CD, originally ob-tained from Charles River Breeding Laboratories. These animals were bred in the Children's Hospital Medical Center animal quarters, maintained at 70 ± 5 F at 50% relative humidity on a 7 a.m. to 7 p.m. light schedule in a pathogen-free environment. Sero-typing of *H. influenzae* and the bactericidal assay of rat sera was performed as previously described (2).

Operant conditioning. The acquisition of a contin-uous reinforcement schedule was observed in a Lehigh Valley model #113-04 automated operant conditioning apparatus. We allowed animals, which had spontane-ously survived a mean lethal dose (LD$_{50}$) inoculation at 5 days of age, ad lib access to water from 21 days (weaning) to 35 days of age. At that time, access to water was restricted to 1 h per day (9 a.m. to noon). At 38 days of age, the animals were placed in the apparatus, and the acquisition was observed. This test system requires a force of 5 × g to depress the standard rodent lever (operanda) for a reinforcement of 20 μliters of water. The animals received water ad lib for 1 h after exposure in the apparatus.

Histologic methods. In a separate experimental series, 26 rats were given intraperitoneal inoculations of *H. influenzae* type b or untypable *H. influenzae* or PBS-G in identical volumes. The animals were decap-itated 48 h after inoculation. Tissue samples were collected from each rat, fixed in 10% buffered neutral Formalin, imbedded in paraffin, cut 6 μm thick, and stained with hematoxylin and eosin (H & E), Giemsa, Goodpasture's, and Brown & Brenn stains (for bac-teria). Portions of lung, liver, spleen, kidney, heart, trachea, mandibular lymph node, urinary bladder, pancreas, small intestine, large intestine, stomach, salivary gland, adrenal, femorotibial joint, bone mar-row, middle ear, and five coronal sections of the decalcified skull were examined with a microscope. The tissues were examined by one of us (D.R.A.) without knowledge of the inoculum. We defined meningitis as the presence of inflammatory cells within the meninges or in the subarachnoid space.

Deoxyribonucleic acid (DNA) quantitation. DNA was measured by the Zamenhof (25) modifica-tion of the method of Kissane and Robins (14).

RESULTS

Recovery of H. influenzae from rat tissues. Before attempting to assay the viable bacterial count in the organs of infected animals, it was important to determine if there were substances in infant rat tissues or homogenization condi-tions which would affect the viability of *H. influenzae*. Accordingly, in reconstruction ex-periments, dilutions of *H. influenzae* were added to weighed organs derived from 5-day-old rats. Organs were then homogenized in an ice bath with a standard clearance Potter-Elevh-jem Teflon homogenizer in PBS-G at 10 ml/g (wet weight).

Table 1 depicts the recovery of the two strains of *H. influenzae* studied from homogenates of

TABLE 1. *Percent recovery of Haemophilus influenzae from organ homogenates of 5-day-old rats*

Organ	Recovery of *H. influenzae* (%)					
	b(E-1)Sm[a]				U-11 Sm	
	10^2/ml[b]	10^4/ml	10^6/ml	10^8/ml	10^2/ml	10^4/ml
Brain	97.6				93.4	
Lung	95.8				68.7	90.4
Kidney	109.0				82.3	89.0
Spleen	75.7	69.8	79.0	50.5	103.8	
Liver	31.3	90.0			14.5	88.4
Blood	88.3	85	82	78.3	69.5	98.9

[a] Strain of *H. influenzae*.
[b] Number of organisms added (final concentration).

the various organs of the 5-day-old rat. With the exception of the liver and spleen, 10^2 *H. influenzae* b (E-1) organisms per ml of homogenate (10^3/g) are recovered at an acceptable rate, approximately 90%. Organisms from liver homogenate are recovered satisfactorily at a density of 10^4/ml. Although percentage recovery of b (E-1) organisms from spleen is greatest at lower organism densities, the absolute recovery at higher densities is sufficient to allow evaluation of in vivo microorganism duplication. Strain U-11 was more easily recovered at lower organism densities from most tissue homogenates than was strain b (E-1).

Age-dependent lethal effect of H. influenza in infant rats. A series of pilot experiments was conducted to determine if intraperitoneal inoculation of *H. influenzae* would produce meningitis and if this effect was restricted to type b organisms. This proved to be the case in 40 infant rats, in which one-half received type b organisms and the remaining untypable. Therefore, further studies were undertaken to examine the quantitative relationships of inoculum to death, the kinetics of infection, and the effects of the inoculation. Figure 1 relates age to the minimal lethal dose (MLD) of *H. influenzae* b (E-1). As can be seen, there was an 8-log increase in MLD in the first 3 weeks of life, the time from birth to weaning. Animals inoculated

FIG. 1. *Relationship between age and minimal number of bacteria which effect death within 96 h after intraperitoneal inoculation of H. influenzae b. Data was obtained from experiments involving 390 animals.*

with greater than 10^8 organisms usually died within 2 h after injection. Postmortem examination of these animals revealed only diffuse petechiae. Animals which expired when inoculated with less than an MLD of type b organisms died 18 to 72 h after inoculation. Inoculation of 10^8 U-11 organisms was not lethal. This age-dependent susceptibility was not related to the presence or absence of serum bactericidal antibodies, as is the case with humans (5). In experiments involving 202 rats, we found noninfected animals of 1 to 97 days had no significant serum bactericidal activity. Those of 175–199 days had mean titers of two. Survivors of *H. influenzae* b inoculation developed serum bactericidal activities with a mean reciprocal titer of 400 (Table 2). Figure 2 depicts the temporal course of the magnitude of bacteremia in 5-day-old infant rats after the intraperitoneal inoculation of 10^3 *H. influenzae* b (E-1), approximately an LD_{50}. The earliest time that bacteremia was detectable was 4 min after inoculation. The concentration of organisms in blood at 12 h, 2 × 10^4 ml, and at 24 h, 7 × 10^5/ml, reflected bacterial replication. The presence of bacteremia corresponded to the clinical situation in which a positive blood culture occurred most often in *H. influenzae* meningitis (79% in reference 22).

Infant rats with *H. influenzae* b infection were observed to have poor weight gain, tremors, and hair ruffling. Prior to death, animals stopped nursing, lay on their side, and were unresponsive to painful stimuli. The peak incidence of deaths of infant rats given an LD_{50} was 24 to 72 h, with only occasional deaths occurring after that time.

Animals inoculated with 10^4 U-11 (avirulent) organisms had a transient bacteremia from 30 min to 4 h after inoculation. The maximum of 165 organisms/ml of blood was reached at 4 h, while no U-11 organisms were isolated at 8 h. U-11 organisms were found in the lung, kidney, and spleen 60 min after inoculation with an organism density of 10^3 to 5 × 10^3/g. But these organisms decreased to the detection limits at 12 h after inoculation and were not detectable 24 h after inoculation.

Figure 3 depicts the temporal course of *H. influenzae* b (E-1) concentration in lung, spleen, and brain relative to the concentration present in these organs was several log units greater than that present in the blood of these organs. Thus, the observed bacterial density must represent sequestration of microorganisms. The sum of the concentration of the organisms in the organs examined is greater than the inoculum size. This also indicates bacterial multiplication.
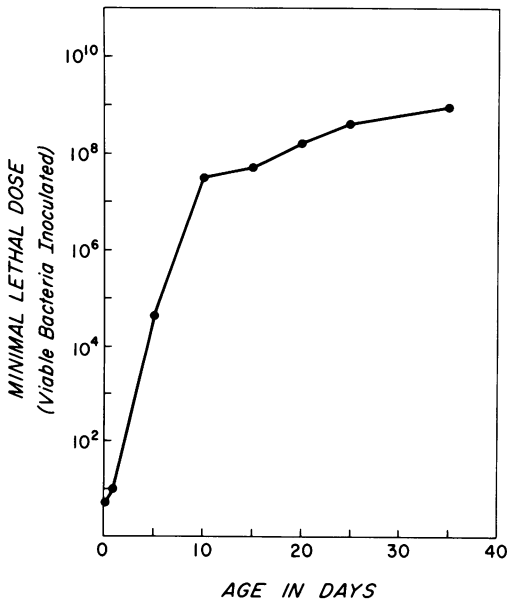
TABLE 2. *Relationship between animal status and bactericidal titer*

Rat	Animal age (days)	No. of animals	No. of samples	H. influenzae b inoculation[a]	Mean reciprocal bactericidal titer
Normal	1–20	162	27	None	0
	25–97	15	15	None	0
Survivors of infection	140	6	6	IP at 14 days of age	400
	21	3	3	IN at 5 days of age	300
	140	4	4	IN at 5 days of age	512

[a] IP, Intraperitoneal; IN, intranasal.
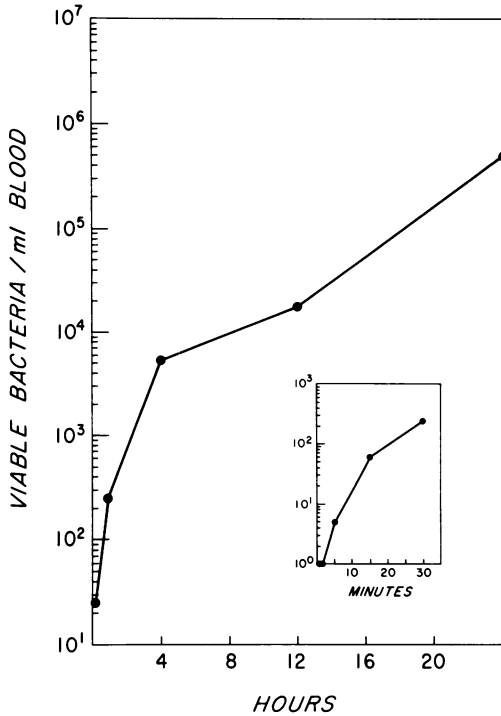
FIG. 2. *Kinetics of bacteremia in 5-day-old rats after the intraperitoneal inoculation of 10^3 H. influenzae b (E-1). One-tenth of a milliliter of blood was collected from the jugular vein and plated on supplemented BHI agar. Each point represents the mean of eight to ten animals.*

FIG. 3. *Recovery of total H. influenzae b (E-1) from several organs of infant rats after intraperitoneal inoculation of 10^3 organisms.*

Histopathological examination: central nervous system. Eighty percent of all 5-day-old rats surviving to 48 h after the inoculation of a LD_{50} of *H. influenzae* type b had dural, subarachnoid, or leptomeningeal accumulations of polymorphonuclear leukocytes.

In the separate experiments designed to evaluate the distribution of histologic lesions, 15 of the 16 rats given *H. influenzae* type b had widespread purulent and fibrinous pachymeningitis and leptomeningitis. The thickness of the purulent meningeal exudate ranged from small periarterial accumulations of in-
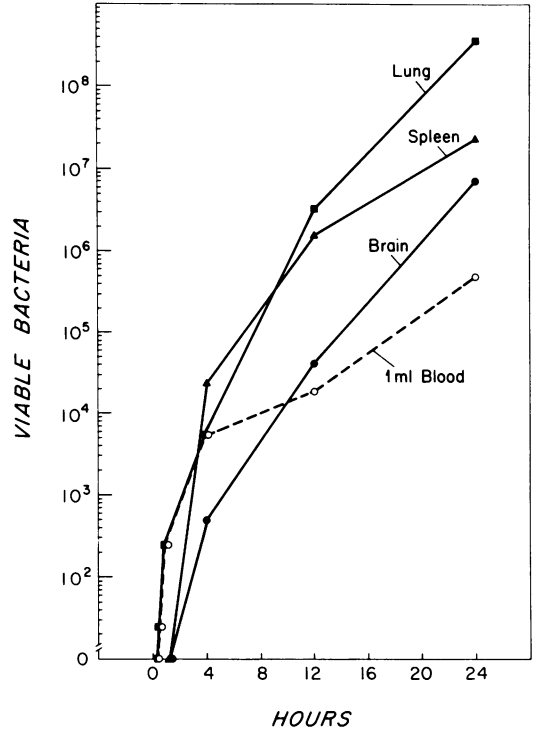
flammatory cells to cell layers filling the space between the pial surface and the inner surface of the skull. Polymorphonuclear leukocytes predominated in the cellular reaction, but there were also some macrophages, occasional lymphocytes, and rare plasma cells present. Fibrin strands were usually among the inflammatory cells. The meningitis was always severe in the dura mater around the dorsal longitudinal sinus (Fig. 4 and 5) and lateral sinuses (Fig. 6). The dorsal longitudinal sinus had more inflammatory infiltration in the frontal regions and near the confluence of sinuses. Leptomeningitis (Fig. 7) was always less severe than pachymeningitis and occurred most often in the dorsal longitudinal fissure, basilar frontal regions, and around
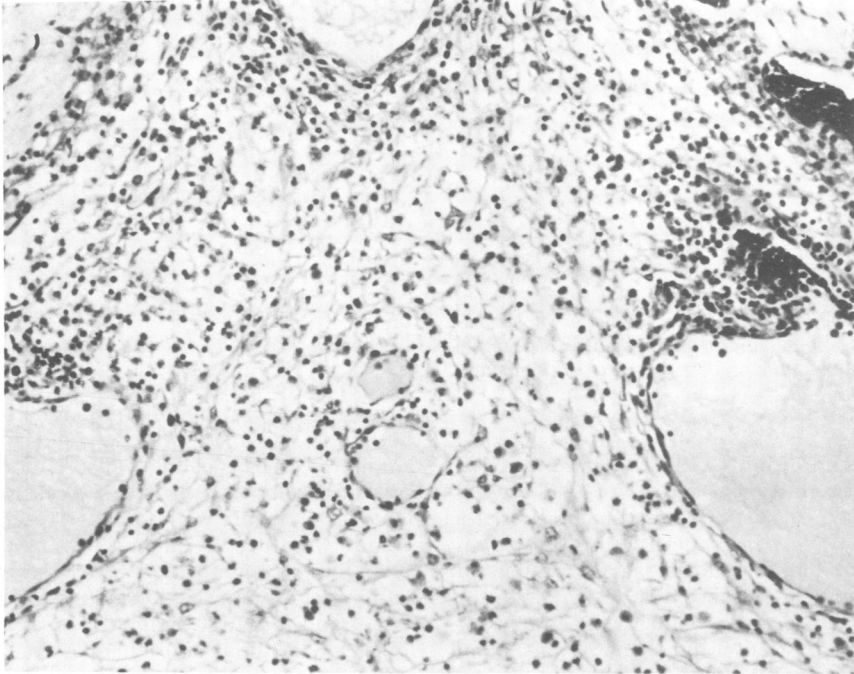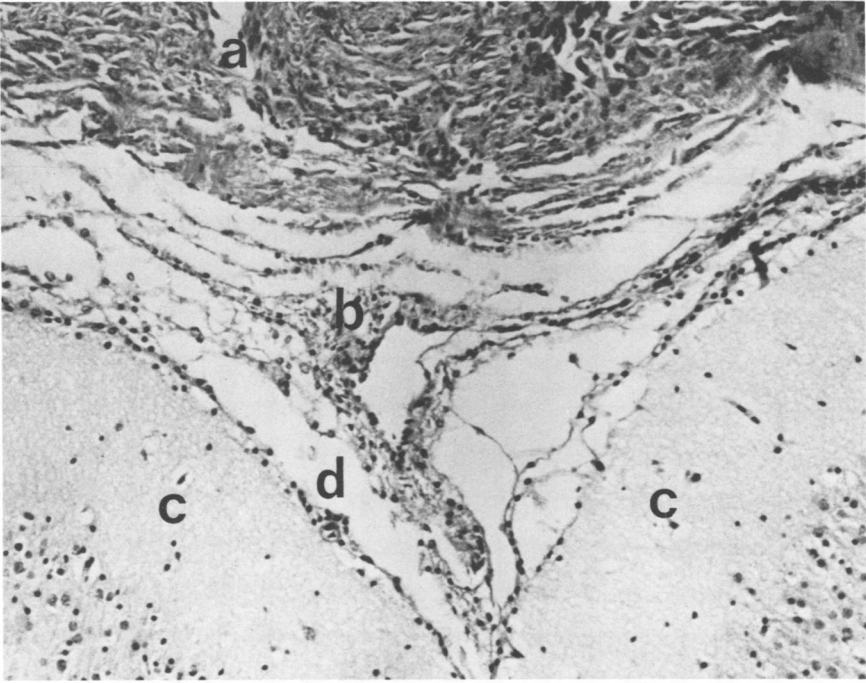
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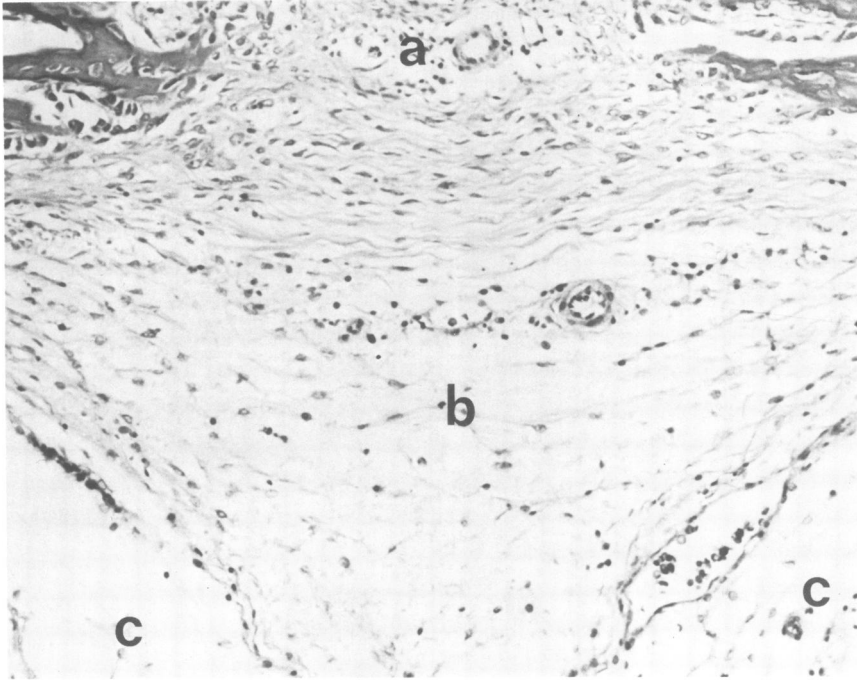
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FIG. 4. *Frontal dorsal longitudinal sinus in a 7-day-old rat showing a loose reticular meshwork.* $\times 100$, *H & E stain. A, Saggital suture indicated by the dura mater (a, b) and the cerebral cortex (c) in a normal rat. B, Two days postinoculation. Reticular meshwork is filled with polymorphonuclear leukocytes.*
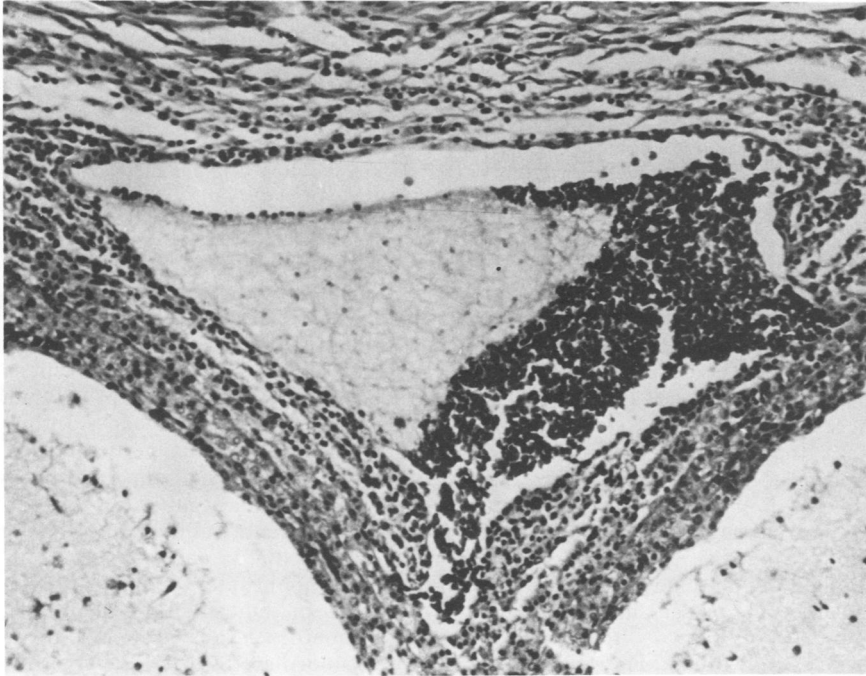
FIG. 5 *Parietal dorsal longitudinal sinus in a 7-day-old rat.* ×*100, H & E stain. A, Dorsal saggital suture indicated by dura mater (a) and sinus (b), cerebral cortex (c), and subarachnoid space (d). B, Forty-eight hours postinoculation, the same area is heavily infiltrated by inflammatory cells.*
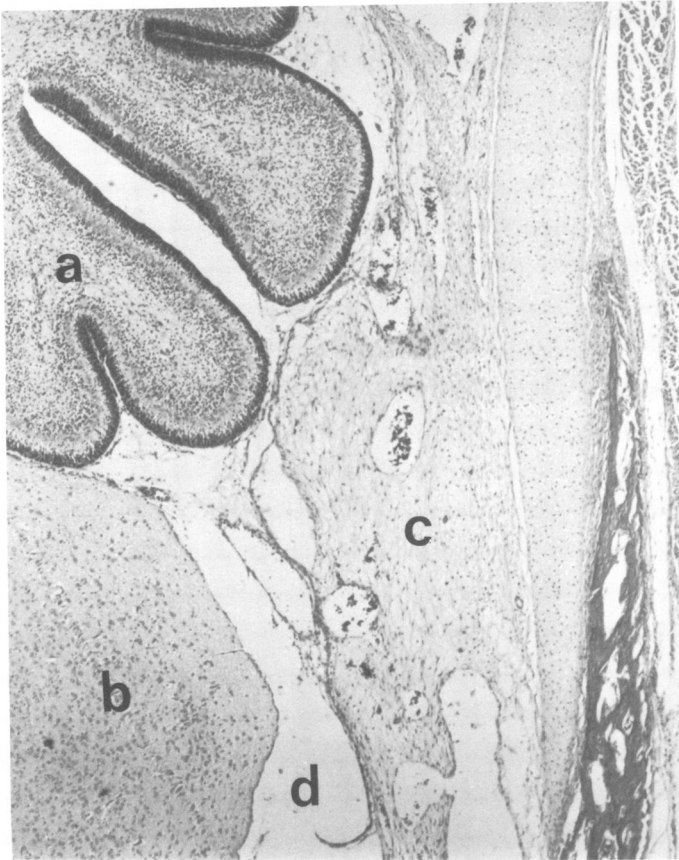
Fig. 6. *Lateral sinus adjacent to the cerebello-medullary angle.* ×25, *H & E stain. A, Normal rat: cerebellum (a), medulla (b), dura surrounding the lateral sinus (c), and the subarachnoid space (d). B, Same area 48 h postinoculation showing the subarachnoid space filled with, and the dura infiltrated by, inflammatory cells.*

lateral aspects of the pons. Giemsa-stained pleomorphic bacilli, consistent with *H. influenzae,* were present in all intracranial inflammatory foci. They could not be stained with the other staining methods. Occasional polymorphonuclear leukocytes were found in the superficial layers of the cerebral cortex, and a few neurons of the molecular layer had nuclear fragmentation. No other lesions were found in frontal, parietal, or occipital cortex, deep cerebral gray matter, cerebral white matter, ventricles, choroid plexus, ependyma, midbrain, pons, cerebellum, or medulla. There were no microscopic lesions present in the blood vessels of the meninges of brain.

Other organ systems. Fourteen of sixteen rats with meningitis had mild to moderate purulent otitis interna (Fig. 8) which was always less severe than the meningitis. In appropriate sections, columns of polymorphonuclear leukocytes were present in the cochlear aque-

duct connecting the subarachnoid space with the endolymphatic space of the internal ear. Of the rats with meningitis, 15 had mild purulent synovitis of femorotibial joints and eleven had small atrial subepicardial or ventricular subendocardial accumulations of polymorphonuclear leukocytes and macrophages. Inflammatory foci were located in the peripancreatic mesentery or on the surface of the gastrointestinal tract. Seven rats had mild acute necrosis of the proximal renal tubules; one case occurred in a U-11 inoculated animal. Seven had mild purulent panophthalmitis. There were no histologic lesions in the remaining organs. Histologic inflammatory lesions were not demonstrable in rats inoculated with PBS-G or untypable *H. influenzae.*

Behavior of survivors. Figure 9 depicts the rate of acquisition of a continuous reinforcement schedule of 40 animals inoculated with 10^3 organisms at 5 days of age. Survivors of type b

infections learned the operant schedule at a slower rate than litter mates inoculated with strain U-11. In addition, the final mean response rate per session in survivors inoculated with type b organisms was significantly lower (274 versus 666) than that of animals inoculated with U-11 (T = 4.725; $P < 0.001$). This decreased response rate did not depend upon animal size or force required to depress the operanda. The biochemical basis of this functional cerebral deficiency has not been defined as yet. However, the total brain DNA content in adult animals who spontaneously survived *H. influenzae* b inoculation at 5 days of age was 70% of that found in U-11 inoculated animals (Table 3). This difference was significant at $P < 0.005$.

DISCUSSION

Bacteria which cause meningitis could colonize in the upper respiratory tract and reach the central nervous system, either by contiguous spread or by invasion of the blood stream and subsequent localization. Previous models developed for the study of meningitis employed adult animals, and most introduced the bacteria directly into the central nervous system, e.g., the lumbar subarachnoid space (5), the cisterna magna (3), or the brain (8). These methods did not permit study of the initial stages in the pathogenesis of meningitis, often produced local rather than diffuse inflammation, and had the unwanted side effect of direct trauma to the brain or spinal cord. Weed et al. (23) produced bacterial meningitis in animals without direct nervous system inoculation by altering cerebrospinal fluid dynamics in the presence of bacteremia. This model has been that contemporarily used (15–17) but requires larger animals. It involves a nonphysiological manipulation to induce meningitis, namely the withdrawal of cerebrospinal fluid.
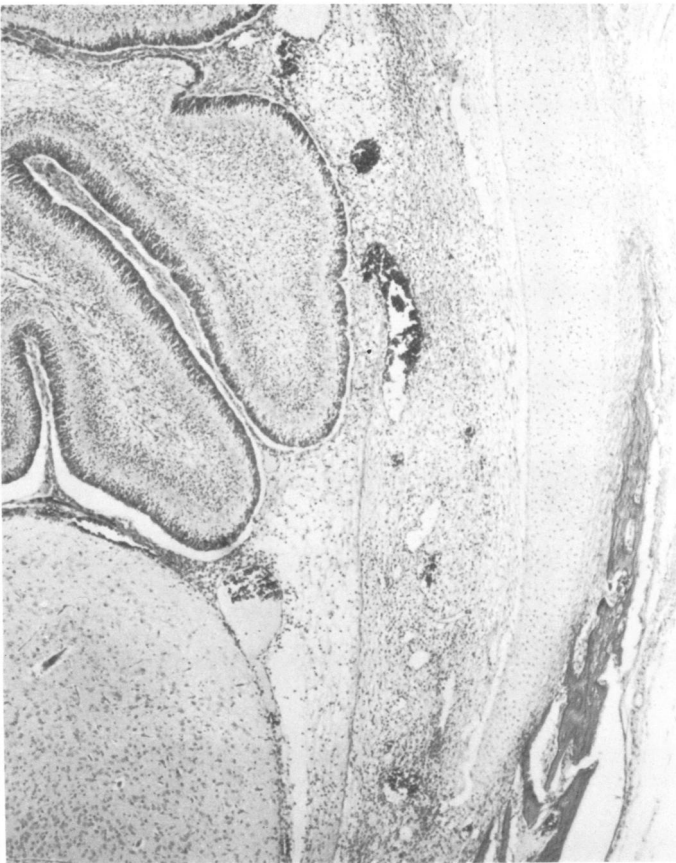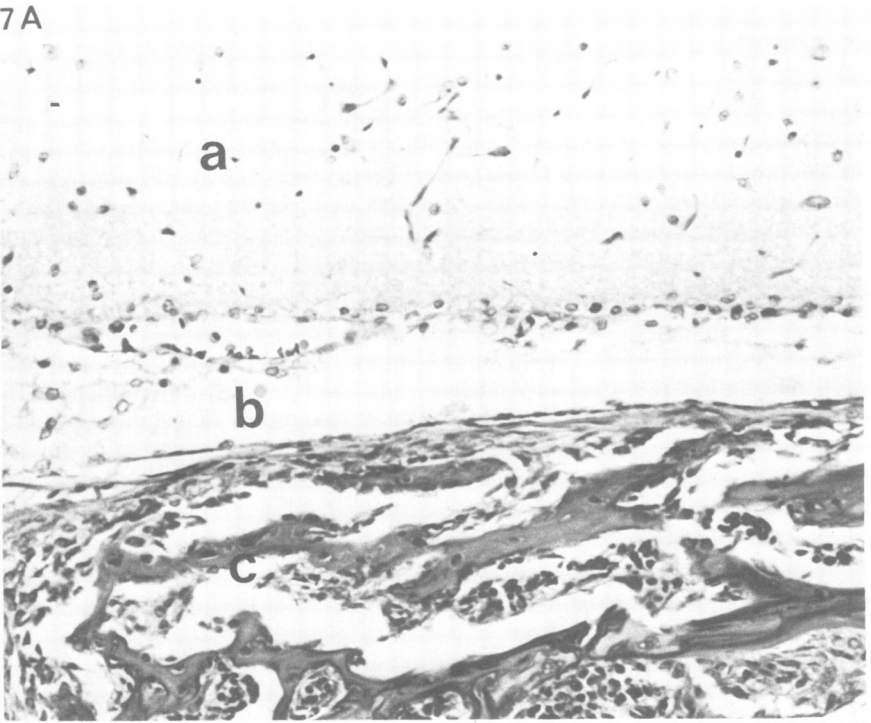
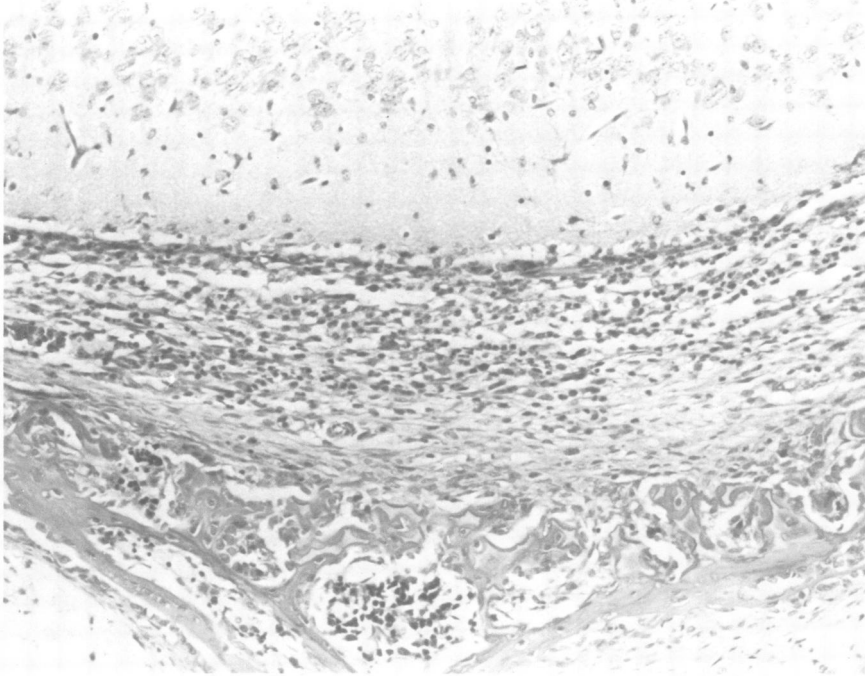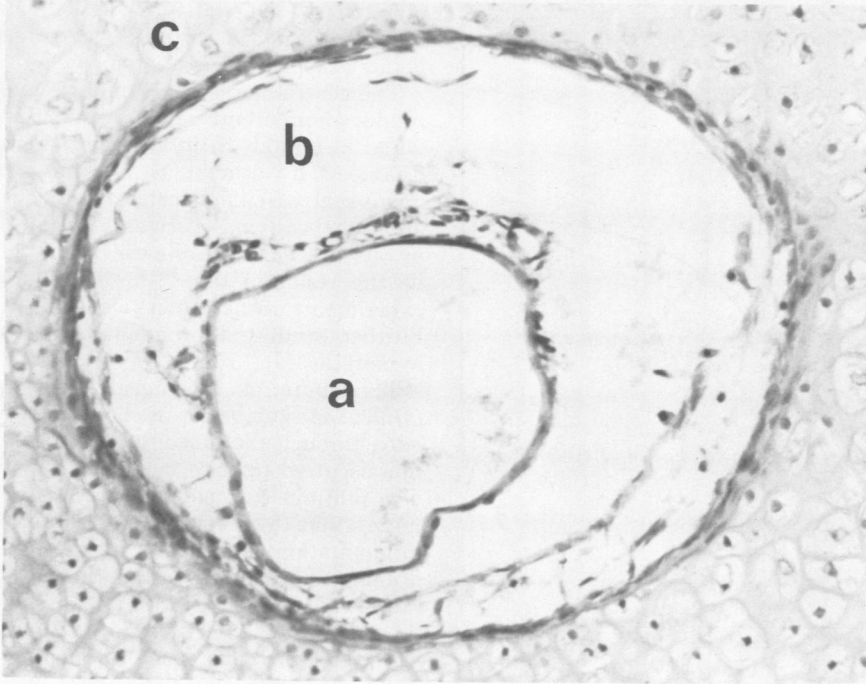Harter and Petersdorf (11) suggested that the

6 B

FIG. 6. *Continued.*

FIG. 7. *Inferior temporal subarachnoid space in a 7-day-old rat.* ×100, *H & E stain. A, Normal animal: temporal lobe (a), subarachnoid space (b), and temporal bone (c). B, Animal inoculated 48 h prior to sacrifice with the subarachnoid space and dura infiltrated with inflammatory cells.*
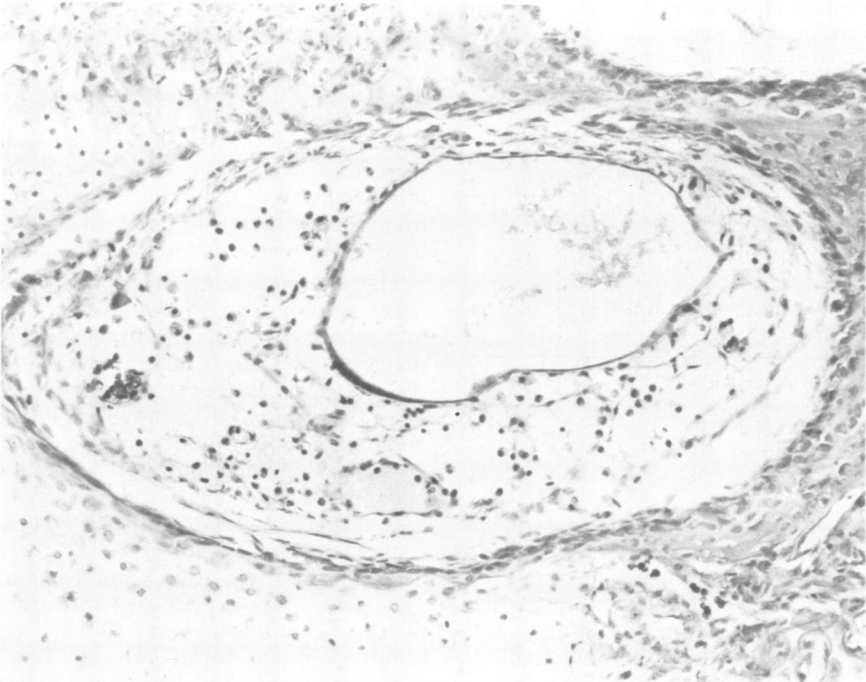
Fig. 8. *Horizontal semicircular canal in a 7-day-old rat. ×100, H & E stain. A, Control animal: canal (a), endolymyphatic space (b), cartilaginous petrous bone (c). B, Section prepared from a meningitic animal showing polymorphonuclear leukocytes and macrophages in the endolymphatic space.*
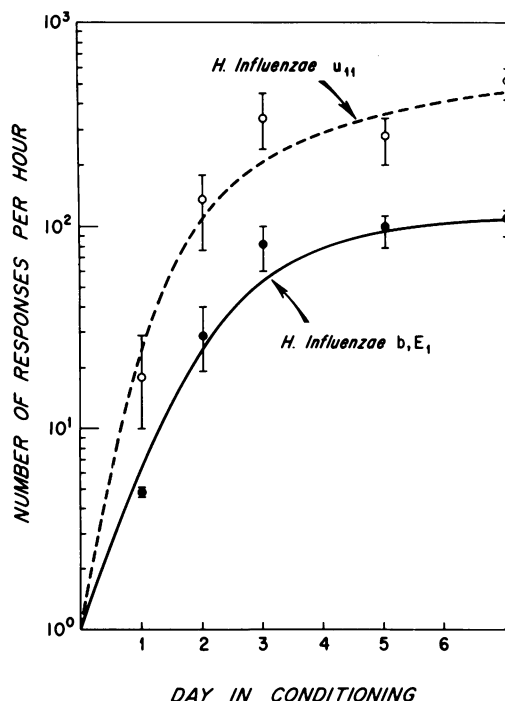
FIG. 9. *Acquisition of a continuous reinforcement schedule in 20 animals inoculated intraperitoneally with 10^3 H. influenza b (E-1) and 20 inoculated with 10^4 of the untypable strain at 5 days of age. Conditioning was begun at 38 days of age. Symbols: circles, means; brackets, standard deviations.*

TABLE 3. *Total brain DNA content in H. influenzae-inoculated rats*[a]

Animal inoculation	Amt of DNA/brain (μg)[b]		
	Mean	σ	SEM[c]
H. influenzae (U-11)	1494.41 (10)	288.49	91.23
H. influenzae b (E1)	1042.09 (9)	276.37	92.12

[a] Adult animals who spontaneously survived inoculation at 5 days.

[b] t = 3.49; 0.05 < P > 0.01.

[c] SEM, Standard error of the mean.

following criteria characterize the ideal model for the study of bacterial meningitis: (i) portal of entry and route of dissemination should be equivalent to that in man, (ii) microorganism in question should be pathogenic for man as well as animal, (iii) course should be predictable to allow evaluation of therapy, (iv) the lesions must be morphologically similar to those in man, (v) the illness must be reproducible within the limits of biological variation, and (vi) tech-

nique must be relatively simple. Our model fulfilled the above criteria with respect to all points except the portal of entry.

The central nervous system histologic lesions produced in infant rats were similar to that found in human *H. influenzae* b meningitis as described by others (1, 2). The absence of meningeal vasculitis, cortical necrosis, gliosis, and subependymal perivascular inflammation in rats proved a notable exception. This could be the result of the 48-h time course in the experiments rather than a species difference. Further studies are under way to test this hypothesis.

The route of meningeal invasion by *H. influenzae* type b in this model was, undoubtedly, through the blood, as detectable bacteremia occurred prior to meningeal inflammation. The findings that pachymeningitis was always more severe than leptomeningitis, and that dural inflammation was most extensive around the draining sinuses, suggested that bacteria initially invaded the intracranial structure at those sites. Whether the *H. influenzae* arrived in the subarachnoid space via meningeal vessel walls and flowed with the cerebrospinal fluid to the draining sinuses, or whether they colonized the dorsal longitudinal sinus and spread to the leptomeninges is currently being investigated.

As the otitis interna was always less severe than the meningitis, and meningitis could occur in the absence of otitis interna, the latter could be an extension of the meningeal infection. The mild focal fibrinous peritonitis undoubtedly resulted from intraperitoneal inoculation but seemed of little biological significance. The mild synovitis and panophthalmitis may result from simple filtration of blood-borne bacteria by the anterior uveal and synovial membranes.

There are other aspects of this model which deserve emphasis. Adjuvants were not needed to promote the infection. In most other models previously described, an adjuvant or some unphysiological maneuver was necessary to consistently obtain the meningitis. For example, Hamburger et al. (9) injected microorganisms in an agar suspension and used animals that had been previously splenectomized. Weed et al. (23) altered cerebrospinal fluid dynamics (by a cisterna magna puncture) during the course of bacteremia to consistently obtain meningitis.

In older literature on experimental meningitis, the inoculum size was frequently referred to as an overnight broth culture. This did not allow any estimation as to the number of viable organisms that were actually inoculated. The more contemporary literature has observed that a minimum of 10^3 pneumococci had to be

injected into the cisterna magna of mongrel dogs in order to observe a 75% incidence of meningitis (16). This contrasted to our model, where the distal (intraperitoneal) inoculation of 10^3 *H. influenzae* b into infant rats consistently produced histologically documented meningitis.

Other investigators have observed *Haemophilus* meningitis in animals other than man. Felton and Wegeforth (4) were able to produce *Haemophilus* meningitis in cats by the intrathecal administration, in large volume, of an overnight culture of *H. influenzae* b. Wollstein (24) produced meningitis in two animal species, mice and guinea pigs, by direct intraperitoneal inoculation of cerebrospinal fluid from children with *H. influenzae* b meningitis. There was no postmortem description of a meningitis process. However, its existence was implied on the basis of positive piarachnoid cultures. She also noted minimal peritonitis, as well as the ability to reisolate *H. influenzae* from "many other organs." Kasahara (12) produced *Haemophilus* b meningitis in adult rabbits after the "intraspinal" inoculation of an unspecified amount of the microorganism. This data suggested to us that *H. influenzae* b did possess potential virulence for many laboratory animal species. Although there was not detailed bacteriological characterization, Kennedy et al. (13) isolated a *Haemophilus*-like organism from calves dying of an illness which histologically was pyogenic meningitis. He also noted that this *Haemophilus*-like organism was lethal when inoculated intraperitoneally in mice of unspecified age, and that there was recovery of the organism from brain and peritoneal cavity at autopsy. Thus it appears there were naturally occurring *Haemophilus*-like species which can produce meningitis in animals without overt extraneous intervention.

The infant rat model differed significantly from that recently reported utilizing infant rabbits (19). In the rabbit model, the age-dependent susceptibility seemed to correlate with the presence in serum of antibodies which were bactericidal for *H. influenzae* b. We found a more marked age-dependent susceptibility in infant rats (on the basis of minimal lethal dose), but the relationship did not depend on detectable bactericidal antibodies.

Currently, our model appears to be the best available for the study of bacterial meningitis, and it provides a unique opportunity to delineate in biochemical and anatomical terms the genesis of cerebral dysfunction caused by this infection. As an example, the lower brain DNA content might be correlated with the histological finding of neuronal necrosis. Studies are in progress to integrate histological, biochemical, and behavioral consequences of *H. influenzae* meningitis in infant rats. It seems likely that the results of future studies with models such as ours will allow pathophysiological description of other types of bacterial and possibly viral meningitis; and that these models may allow insight into the mechanism of certain noninfectious causes of cerebral dysfunction.

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