Biochemical Characteristics of 130 Recent Isolates from *Haemophilus influenzae* Meningitis

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A total of 130 *Haemophilus* strains, comprising virtually all isolates from Danish and Norwegian cases of *Haemophilus* meningitis occurring in the period from October 1975 through September 1976, were examined by biochemical and serological means. All isolates were identified as *H. influenzae* and, except for one noncapsulated strain, possessed a capsule of serotype *b*. The vast majority of strains (93%) belonged to biotype I, which, in contrast to biotypes II and III, is rarely encountered as a commensal of the upper respiratory tract. This finding is a strong incentive for studies of possible additional virulence factors associated with biotype I organisms. The results are discussed in the light of North American reports, which have suggested changes in the etiology of *Haemophilus* meningitis.

Haemophilus influenzae serotype *b* is well established as one of the leading causes of bacterial meningitis. The unambiguous implication of this particular serotype of *H. influenzae* has been an important incentive to the development of a vaccine against *Haemophilus* meningitis. The clinical evaluation of a serotype *b* capsular polysaccharide vaccine has already been performed in large populations of children (19). However, during the last decade, a considerable number of North American cases of meningitis ascribed to *H. parainfluenzae* have been reported (1, 2, 6-9, 13, 16, 22). Some of these publications even seemed to suggest a major change in the etiology of *Haemophilus* meningitis. Thus, in a report from Rochester, Minn. (7), 7 out of 10 cases of *Haemophilus* meningitis observed during a 16-month period were ascribed to *H. parainfluenzae*. Likewise, Bachman (1) found 3 out of 11 cases from Baltimore, Md., to be caused by this organism.

The study reported here was undertaken to determine whether a similar change in the etiology of *Haemophilus* meningitis could be observed in Scandinavian countries. A recently developed identification system (11), which allows the subdivision of the *Haemophilus* species into biotypes, was applied in the study. Besides elucidating the above question, the examination of the biochemical characteristics of a large number of fresh isolates from *Haemophilus* meningitis would throw further light on our previous suggestion (11), that *H. influenzae* strains producing meningitis, apart from the serotype *b*

capsule, are distinct from strains colonizing the normal upper respiratory tract.

MATERIALS AND METHODS

Bacterial strains. Throughout the period from October 1975 to September 1976, all isolates from *Haemophilus* meningitis were collected from five Danish and five Norwegian clinical bacteriological laboratories. A total of 132 strains, comprising strains from virtually all cases of *Haemophilus* meningitis in the two countries during the 1-year period, were received as fresh cultures on agar plates or in transport medium. Two strains could not be recovered upon receipt.

Culture methods. Chocolate agar (10% heated defibrinated horse blood in blood agar base [Difco Laboratories, Detroit, Mich.]) was used for maintaining strains and for inoculum cultures for biochemical test substrates. Agar plates were incubated at 35°C in an atmosphere of air plus 10% carbon dioxide.

Identification procedures. The methods were essentially those described previously (11). The V-factor requirement was tested by observing satellite growth on autoclaved (120°C), 10% (vol/vol) citrate-stabilized calf blood agar cross-inoculated with a strain of *Staphylococcus aureus*. The X-factor requirement was determined by testing the ability of the strains to synthesize porphyrins from δ -aminolevulinic acid as described previously (10). Indole production was demonstrated in a 0.1% solution of L-tryptophan in 0.067 M phosphate buffer at pH 6.8 (Sørensen) (4). Urease activity was tested in the following substrate (14): KH_2PO_4 , 1 g; K_2HPO_4 , 1 g; NaCl, 5 g; and phenol red, 10 mg in 1 liter of distilled water (pH 7.0). After autoclaving, 10.4 ml of a filter-sterilized aqueous 20% urea solution was added. Ornithine decarboxylase activity was demonstrated in 1 ml of the medium of Møller (18). The substrates for the porphyrin, indole,

urease, and ornithine decarboxylase tests were inoculated by suspending one large loopful of bacteria from an agar culture in the respective substrates. The results of the tests were read after incubation for 4 h at 35°C. Production of acid from carbohydrates was determined in phenol red broth base (Difco) to which the respective sugars were added in filter-sterilized solutions to give a final concentration of 1% (wt/vol). Hemin and nicotinamide adenine dinucleotide were each added aseptically to a final concentration of 10 mg/liter (11). Results of the carbohydrate fermentation tests were read after incubation for 5 days.

The designation of the strains into species and biotypes followed the outlines reported previously (11).

Strains producing iridescent colonies on Levinthal agar after incubation for 18 h were typed according to Pittman (20), using antisera *a* through *f* (Hyland Laboratories, Inc., Costa Mesa, Calif.).

RESULTS

Patients. A total of 97 of the cases of meningitis from which the 130 *Haemophilus* strains were isolated appeared in Denmark, the remaining 33 cases being Norwegian. Seventy-eight percent of the patients were less than 3 years old, and 99% were less than 9 years old. Two patients who exceeded this age were 56 and 60 years old, respectively.

Characteristics of the bacterial isolates. All 130 strains were identified as *H. influenzae* on the basis of the criteria listed in Table 1. All strains fermented glucose, xylose, and ribose, whereas none of the strains fermented sucrose or lactose. All strains conformed with one of three of the five biotypes of *H. influenzae* described previously (11). A total of 93% of the strains belonged to biotype I (Table 1). The remaining nine strains were assigned to biotypes II and IV, respectively. One strain of biotype I, isolated from a 60-year-old patient, was noncapsulated. All other strains possessed a capsule of serotype *b*.

DISCUSSION

The age distribution of the 130 Danish and Norwegian patients from whom the *Haemophi-*

lus strains were isolated follows the classical pattern for *Haemophilus* meningitis originally described by Fothergill and Wright (5). Only two of our patients (56 and 60 years, respectively) exceeded the age of 9 years.

On the basis of several biochemical tests (Table 1), all 130 strains were identified as *H. influenzae*. All, except one noncapsulated strain, possessed a capsule of serotype *b*. The results of the biochemical tests revealed that all strains could be assigned to one of three of the five biotypes of *H. influenzae* described by Kilian (11). Of the 130 isolates, 121 (93%) belonged to biotype I. The remaining nine strains were assigned to biotypes II and IV. The significant predominance of biotype I strains confirms our previous observation (11) that in particular this biotype of *H. influenzae* is responsible for invasive diseases such as meningitis and epiglottitis. Furthermore, it strongly supports the previous suggestion by Kilian (11) that *H. influenzae* strains involved in these diseases, besides possessing a capsule of serotype *b*, are biochemically distinct from the *H. influenzae* that form part of the normal flora of the upper respiratory tract. The majority of such commensal strains belong to biotypes II and III (11). This observation is a strong incentive for studies of possible additional virulence factors associated with biotype I organisms.

Although the species *H. influenzae* thus appears to be heterogeneous in regard to pathogenicity and biochemical properties, the present study does support the old experience that this species is the one associated with meningitis. Several North American reports published from 1966 through 1975 seemed to indicate that *H. parainfluenzae* was also becoming a significant etiological agent in meningitis. Neither the age nor current health status of the patients involved in these reported cases differs from those of patients contracting *H. influenzae* serotype *b* meningitis.

H. parainfluenzae is found indigenously in the upper respiratory tract and is the predominant *Haemophilus* species found in the human

TABLE 1. Biochemical characteristics of 130 *Haemophilus* strains isolated from meningitis

Designation	No. of strains	V-factor requirement	Porphyrin test	Indole	Urease	Ornithine decarboxylase	Glucose, acid	Sucrose, acid	Lactose, acid	Xylose, acid	Ribose, acid
<i>H. influenzae</i> biotype I	121 (93.1) ^a	+	—	+	+	+	+	—	—	+	+
<i>H. influenzae</i> biotype II	6 (4.6)	+	—	+	+	—	+	—	—	+	+
<i>H. influenzae</i> biotype IV	3 (2.3)	+	—	—	+	+	+	—	—	+	+

^a Numbers within parentheses indicate percentages.

oral cavity. In saliva, bacteria of this species amount to a mean number of about 4×10^7 /ml and constitute about 3 to 10% of the bacterial deposits on the surfaces of teeth (12, 21). Like other oral bacteria, *H. parainfluenzae* is an occasional cause of bacterial endocarditis (15, 17). However, apart from this disease, in which the etiological agent is passively introduced into the blood stream, there has been no evidence in support of this organism having an invasive potential.

H. parainfluenzae is distinguished from *H. influenzae* by numerous biochemical characters, notably in being independent of the X-factor (hemin) and in being able to ferment sucrose but not xylose and ribose (11). In the reported cases of *H. parainfluenzae* meningitis, the responsible organisms were assigned to this species on the basis of their requirement for the V-factor but not the X-factor. The growth factor requirements were determined by the use of a disk method (1, 2, 7, 9, 22) or by testing for growth on a complex medium with and without blood (6, 8, 9, 13). Determined characters used to confirm the diagnosis included the inability to demonstrate agglutination with anticapsular typing sera *a* through *f* (6) and the testing for nitrate reductase, fermentation of maltose, lactose, and mannitol, and indole production (8). However, none of the characters revealed by these tests is indicative of the organisms belonging to *H. parainfluenzae*. In fact, a positive indole reaction, as recorded for one of the strains (8), is a good indication that the organism is not a strain of *H. parainfluenzae* (11). The problem and shortcomings of the disk and plate methods for the demonstration of the X-factor requirement have been discussed extensively in several publications (3, 10, 23). In a recently performed comparison between the results obtained by the use of the disk method and the porphyrin test (10), which demonstrates the actual enzymes involved in the biosynthesis of the X-factor, it was shown that, of 257 *Haemophilus* strains tested, 45 strains (18%) were misidentified by the use of the former method (M. Kilian and K. R. Eriksen, unpublished data). Therefore, it seems likely that some, if not all, of the published cases of meningitis assigned to *H. parainfluenzae* have been due to the isolates being incorrectly identified. Unfortunately, none of the isolates have been kept for further study (personal communication). As numerous *Haemophilus* species besides *H. influenzae* and *H. parainfluenzae* are recognized, it should be emphasized that a proper diagnosis of *Haemophilus* strains, in addition to the testing for growth factor requirements, requires the performance of several biochemical tests (11).

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