STUDIES ON HAEMOPHILUS INFLUENZAE*

II. A Comparative Study of the Virulence of Smooth, Rough, and Respiratory Strains of Haemophilus influenzae as Determined by Infection of Mice with Mucin Suspensions of the Organism

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Following the lead of Nungester, Wolf, and Jourdonais (1) and Miller (2) who had succeeded in producing infections in animals normally resistant to various organisms by suspending the organisms in mucin, Fothergill, Dingle, and Chandler (3) carried out a series of experiments which led to the following conclusions: (*a*) the suspension of *Haemophilus influenzae* in mucin enhances the power of this organism to infect mice, (*b*) *in vivo* multiplication of the organism occurs with subsequent death of the animal, (*c*) repeated mouse passage of a strain of *H. influenzae* appears to increase its "mucin virulence," and (*d*) by means of this method protection tests and passive immunization experiments can be carried out. It was suggested that this method could also be used to determine the comparative virulence of smooth and rough strains of *H. influenzae.* The present communication describes the results of such a comparative study.

Methods and Materials

Smooth Strains.—A total of seventeen smooth strains was used in this study. With the single exception of strain 26, which was obtained from a blood culture, all the smooth strains were Pittman type b of meningeal origin. Since most of

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them had been kept in whole, defibrinated, rabbit blood for periods of time varying from 6 months to 2 years, it was felt necessary to check on the "smoothness" of each strain by studying the colony morphology and by utilizing the precipitin reaction to determine the presence or absence of specific precipitinogen (soluble specific substance) at the time of each experiment. This procedure was also followed in the case of freshly isolated cultures (66, 82, 83). In tabulating the results, which are shown in Table II, only those smooth strains which were smooth, opaque, and fluorescent on plating and which gave strongly positive precipitin reactions (1–32) against type b antiserum were included. The virulence of all strains was determined in mice. The results of these determinations will be discussed later.

Rough Strains.—Of the twenty-two rough strains included in this study, one (No. 19) was an old, degraded, respiratory strain, twelve were "converted" rough strains derived from smooth meningeal strains by the method described in a previous paper (4), and nine (64, 72, 73, 74, 76, 77, 78, 79, 81) were respiratory strains freshly isolated from the nasopharynx of patients with various types of upper respiratory infections. As in the case of the smooth strains, each rough strain was checked for its degree of "roughness" by plating at the beginning of the experiment. As a further check, a precipitin test was done with each strain against a potent type b[1] antiserum both before and after the determination of its virulence in mice. In no instance was any soluble specific substance demonstrable in any of the converted rough or respiratory strains either before or after this single mouse passage.

The results of the precipitin reactions of the smooth, converted rough, and respiratory strains are shown in Table I. All the smooth strains included in the series produced on Levinthal agar plates completely smooth, glistening, opaque, fluorescent colonies. The converted rough strains on the same medium produced colonies which varied in roughness from slight surface wrinkling with slight serration of the edges to complete loss of colony contour. The colonies of all the rough strains, however, were uniformly bluish, translucent, and non-fluorescent. In colony appearance the respiratory strains were without exception essentially smooth in surface contour with regular edges, but like the converted rough strains were bluish, translucent, and non-fluorescent.

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[1] At the beginning of this study sera of other smooth types were not available. Since then, however, we have received additional smooth cultures (a, b, c, d, e, and f) from Dr. Pittman, to whom we wish to express our gratitude. Rabbits are now being immunized with these strains and although the sera are not quite up to titre, preliminary precipitin tests with these sera against the respiratory strains have given uniformly negative results. Since in addition, these respiratory strains have produced only transparent, non-iridescent colonies on Levinthal agar plates, we feel fairly confident that they may be classified as rough, and non-precipitinogen-forming.

TABLE I

Precipitin Reactions of Smooth, Converted Rough, and Respiratory Strains against Type b Antiserum

Strain No.	Dilution of antigen					
	Undiluted	1-2	1-4	1-8	1-16	1-32
Smooth						
1	++	++	+	+	+	+
3	++	++	+	+	+	+
26	++	++	+	+	+	+
38	++	++	+	+	+	+
40	++	+	+	+	+	+
44	++	+	+	+	+	±
62	++	++	+	+	+	+
66	++	+	+	+	+	−
82	++	++	++	+	+	+
83	++	++	++	+	+	+
F	++	++	+	+	+	+
Converted Rough						
1	−	−	−	−	−	−
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	−	−	−	−	−	−
5	−	−	−	−	−	−
14	−	−	−	−	−	−
15	−	−	−	−	−	−
19	−	−	−	−	−	−
26	−	−	−	−	−	−
36	−	−	−	−	−	−
38	±	−	−	−	−	−
40	−	−	−	−	−	−
44	−	−	−	−	−	−
Respiratory						
64	−	−	−	−	−	−
72	−	−	−	−	−	−
73	−	−	−	−	−	−
74	−	−	−	−	−	−
76	−	−	−	−	−	−
77	−	−	−	−	−	−
78	−	−	−	−	−	−
79	−	−	−	−	−	−
81	−	−	−	−	−	−

Mucin suspensions were prepared according to the technique described in the first paper of this series (3). The organisms were grown on peptic digest blood agar plates heavily inoculated from a 6 to 8 hour broth culture for 15 to 18 hours at 37°C. The confluent growth from each plate was scraped up in 1 to 2 cc. of sterile infusion broth and the yield from 4 to 5 plates was pooled giving a final total volume of 5 to 6 cc. of suspension for each organism. In the case of smooth strains a homogeneous, dense suspension resulted with little tendency to settle out. Suspensions of rough organisms equally dense and homogeneous originally, however, rapidly settled out leaving an almost clear supernatant over a dense flocculum. In all instances broth was used in making suspensions and dilutions. In preparing mucin suspensions for mouse inoculations the original broth suspension was diluted 1–4 with mucin. From this mixture further dilutions in mucin were carried on decimally. A separate sterile pipette was used for making and mixing each dilution. A similar series of dilutions was prepared in sterile infusion broth for inoculation of control mice and for plate counts. In order to obtain as accurate results as possible with this method of counting, both pour plates and streak plates were prepared of the higher dilutions; 1 cc. amounts being used in the pour plates and 0.1 cc. and 0.2 cc. amounts on the streak plates. Thus for any given dilution a triplicate count was obtained, the average of which was used in determining the minimal lethal dose for a given strain. Mice were inoculated intraperitoneally with 1 cc. amounts of either the mucin or the broth suspension. In the case of the smooth strains, six mice were inoculated with each dilution, four receiving the suspension in mucin, and two the control suspension in broth. In the case of the rough and respiratory strains, except in a single instance, eight mice were inoculated with each dilution, four receiving the suspension in mucin, and four the control suspension in broth.

Albino Swiss mice of inbred stock were used in all experiments. In the early virulence determinations only four to five mice were autopsied and cultured for a given strain. Around the middle of the experimental period, however, mouse typhoid began to appear in the mouse colony. From that time on all inoculated mice that succumbed were autopsied and cultures were made of the peritoneal exudate and heart's blood. For any given strain, the virulence determination was repeated if more than one or two mice showed evidence of typhoid infection in addition to the experimental infection, but in no instance was the occasional death due to typhoid infection included in the calculation of the M.L.D.

RESULTS

The results of the virulence determinations of smooth, converted rough, and respiratory strains of *H. influenzae* are shown in Table II. With regard to the smooth strains, it is to be noted that whereas the mucin virulence of individual strains varied widely with respect to the dilution of the suspension (1–4000 to 1–4,000,000) this variation was

TABLE II

Comparative Mucin Virulence of Smooth, Converted Rough, and Respiratory Strains of Haemophilus influenzae in Mice

Smooth Strains

Dilution of suspension	Strain 1		Strain 3		Strain 26		Strain 38		Strain 40		Strain 44		Strain 62		Strain 66		Strain 82		Strain 83		Strain F	
	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B
1–4	4/0	1/1	4/0	2/0	4/0	0/2	4/0	2/0	4/0	1/1	4/0	2/0	4/0	2/0	4/0	1/1	4/0	2/0	4/0	1/1	4/0	1/1
1–40	4/0	0/2	4/0		4/0		2/2	1/1	4/0	0/2	2/2	1/1	3/1	1/1	3/1		3/1		4/0		4/0	
1–400	4/0	1/1	4/0		4/0		2/2	1/1	3/1	1/1	3/1		4/0		4/0		4/0		3/1		4/0	
1–4,000	4/0		4/0		3/1		3/1		2/2		2/2		3/1		4/0				2/2		4/0	
1–40,000	1/3		3/1		1/3								3/1		1/3				1/3		3/1	
1–400,000	0/4		1/3										0/4									
1–4,000,000	1/3		1/3										1/3									
1–40,000,000																						
M.L.D.	300,000		400,000		500,000		950,000		850,000		30,000		60,000		180,000		1,600,000		300,000		350,000	

Converted Rough Strains

Dilution of suspension	Strain 1		Strain 2		Strain 3		Strain 4		Strain 5		Strain 14		Strain 15		Strain 19		Strain 26		Strain 36		Strain 38		Strain 40		Strain 44	
	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B
1–4	4/0	2/0	4/0	3/1	4/0	2/2	0/4	0/4	3/1	3/1	4/0	3/1	3/1	1/3	4/0	0/4	2/2	1/3	2/2	0/4	2/2	1/3	3/1	2/2	3/1	1/3
1–40	3/1		3/1	1/3	4/0	1/3			2/2		4/0	1/3		1/3			4/0		2/2		4/0		1/3	2/2	3/1	
1–400	2/2		3/1		1/3						2/2						2/2				2/2					
1–4,000											1/3															
1–40,000																										
1–400,000																										
M.L.D.	30,000,000		90,000,000		500,000,000		Infinity		500,000,000		2,000,000		2 billion		120,000,000		12,000,000		500,000,000		5,200,000		7.1 billion		5,000,000	

Respiratory Strains

Dilution of suspension	Strain 64		Strain 72		Strain 73		Strain 74		Strain 76		Strain 77		Strain 78		Strain 79		Strain 81	
	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B	M	B
1–4	4/0	2/2	4/0	4/0	2/2	1/3	4/0	1/3	4/0	2/2	3/1	0/4	4/0	0/4	4/0	2/2	3/1	1/3
1–40	1/3		3/1		2/2	1/3	2/2		4/0		3/1		2/2		1/3		3/1	
1–400			3/1						2/2		1/3		2/2		1/3			
1–4,000													1/3					
1–40,000																		
1–400,000																		
M.L.D.	3 billion		8,500,000		6,000,000		17,000,000		1,300,000		30,000,000		10,200,000		1.6 billion		40,000,000	

M = mucin suspension. B = broth suspension. Numerator = number of mice dying. Denominator = number of mice surviving.
M.L.D. = number of organisms killing 50 per cent or more of injected mice.

apparent rather than real. By taking into account the number of organisms originally present in a given suspension and computing the M.L.D. from that for each strain, the corrected value was obtained. Thus, some constancy in mucin virulence, as shown by M.L.D. values, was exhibited by the smooth strains, the figures ranging from 30,000 to 1,600,000 organisms.

No such constancy was seen in the M.L.D. values for the converted rough and respiratory strains, although much more regularity was observed in the dilution range resulting in death, the majority of mice having succumbed in a dilution of from 1–40 to 1–400. For the converted rough strains the M.L.D. values vary from 2 million to 7 billion;

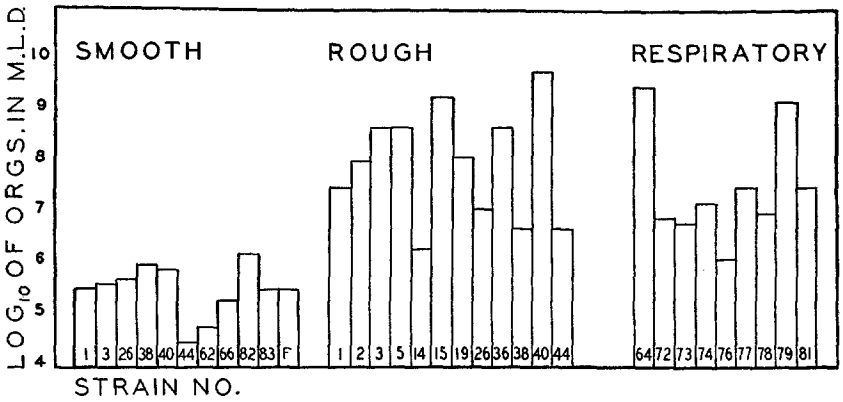

FIG. 1. The virulence of smooth, rough (artificially dissociated), and respiratory strains of *H. influenzae*.

for the respiratory strains the range was from 1.3 million to 1.6 billion organisms. Notwithstanding this wide range in virulence among the converted rough and respiratory strains themselves, the fact that they differed significantly in virulence as a group from the smooth strains is shown by the magnitude of the comparative M.L.D. values. This is better illustrated by Fig. 1, in which the logarithmic values for the M.L.D. figures are plotted side by side for the three series. By referring to Fig. 1 it will be noted that the smooth strains were considerably more virulent for mice than either the converted rough or respiratory strains. The respiratory strains did not differ significantly in virulence from the converted rough strains.

As stated previously, only those smooth strains which were entirely smooth and which formed significant amounts of soluble specific substance (type b) were included in the above group. A number of smooth strains[2] which had undergone partial dissociation spontaneously, however, were titrated in mice to determine their virulence. The results of one of these determinations are shown in Table III.

This particular strain, No. 2, was isolated from the cerebrospinal fluid of a patient with *H. influenzae* meningitis over 3 years ago. Originally a completely smooth strain producing an abundance of type b specific substance, at the time of mouse virulence titration it no longer produced any precipitinogen, although on Levinthal agar

TABLE III

Mucin Virulence of a Partially Dissociated Smooth Strain

Dilution of suspension	Strain 2	
	M	B
1–4	4/0	2/0
1–40	2/2	
1–400		
1–4,000		
1–40,000		
1–400,000		
1–4,000,000		
M.L.D.—120,000,000		

plates it produced colonies only moderately rough in appearance. Titration of its virulence in mice established the fact that it had dissociated to the extent of having an M.L.D. in the rough rather than in the smooth range. This was found to hold true for all the other partially dissociated, smooth strains studied. In some instances the only evidence of dissociation was the appearance of rough colonies

[2] The results of some of these titrations were reported in the first paper of this series to illustrate variations in virulence among smooth strains themselves. On further examination, however, it was found that those strains which presumably were less virulent for mice were actually not entirely smooth according to all the criteria enumerated herein. Therefore they have not been included in the smooth series represented in Table II. They are mentioned at this point because they really belong in the partially dissociated group.

among the smooth ones with the precipitinogen formation by the culture apparently little diminished; in other instances, as in the example given above, the colony appearance of the strain was essentially unchanged but the organism had lost the ability to produce soluble specific substance either partially or completely. In all instances, however, these strains were found to have M.L.D.'s in the rough range.

DISCUSSION

The above results indicate that it is possible by means of the mucin method of infecting mice to compare the virulence of smooth, converted rough, and respiratory strains of *H. influenzae*. By expressing virulence in terms of minimal lethal doses, it has been possible to demonstrate the fact that smooth strains are considerably more virulent for mice than either converted rough or respiratory strains and that the last two groups are essentially equal in virulence. Moreover, it has been found that the numerical difference between the M.L.D. range of the smooth group and the M.L.D. range of the rough group as a whole is well within the limits of statistical validity, notwithstanding the rather wide variation in individual M.L.D. values occurring within the two rough groups themselves.

It is well to point out at this juncture that allowance has been made for the errors inherent in the plate count method of estimating numbers of bacteria in a given suspension. Little reliance can be placed on estimates by this method in regard to absolute numbers of viable organisms present. In this study, however, relative rather than absolute values were sought. Since the conditions under which the suspensions were prepared were kept as constant as possible, and since virulence titrations checked remarkably well, it is felt that M.L.D. values of the order of magnitude obtained are truly significant in a comparative study of this sort. In this connection a possible explanation may be given for the somewhat lower M.L.D. values obtained in the respiratory group as compared with the converted rough group. With suspensions of approximately the same density and death points falling in the same range, plate counts of the number of organisms in the former group were almost invariably slightly lower than those in the latter group. Since the freshly isolated respiratory strains grew poorly on culture media until after several transplants, it is felt that

inhibition of growth was responsible for the lowering of the plate count among these strains. This view is supported by the fact that no such inhibition occurred with respiratory strains which had been kept on artificial media for some time.

With regard to the relationship between virulence and serological as well as morphological characteristics, it might be of interest to try to correlate the present findings with the results of other investigators as well as with the results previously reported (4). At that time it was stated that strains of *H. influenzae* isolated from pathogenic sources, particularly from the cerebrospinal fluid of patients with *H. influenzae* meningitis, were of the smooth or S variety (originally described by Pittman (5)), and that almost invariably they formed a single homogeneous, serological group (Pittman type B). When smooth strains were dissociated into rough forms, however, it was found that the latter made up a serologically heterogeneous group, which heterogeneity was not essentially different from that found in the ordinary respiratory strains of this organism. Completely dissociated rough strains produced bluish, non-iridescent colonies and no longer formed specific precipitinogen. Likewise, in our experience, respiratory strains, with the exception of those isolated from the nasopharynx of patients with *H. influenzae* meningitis, produced bluish non-iridescent colonies and did not elaborate specific soluble substance. In examining large numbers of respiratory strains, both Pittman (6) and, more recently, Platt (7) have found a few which were of the smooth, iridescent variety culturally and which serologically were capable of being typed by means of the precipitin reaction.[3] In general, however, their investigations, as well as those of other workers in this field, have emphasized the fact that smooth strains of *H. influenzae* from pathogenic sources are essentially homogeneous serologically, whereas dissociated strains and respiratory strains are serologically heterogeneous. The present study which has brought out more clearly the association of smoothness with virulence and, conversely, roughness (natural and artificially induced) with avirulence, therefore tends to confirm previous findings in this field. It is, of course, possible that an occasional virulent respiratory strain

[3] Most of these strains have fallen into the Pittman type a and e groups.

would have been found in this study if a much larger series of strains had been examined.

It should perhaps be emphasized once more that so far in our experience only those smooth strains which have had all the attributes of smoothness, *i.e.*, made up entirely of smooth, opaque, fluorescent colonies and producing specific soluble substance in a high titre, have fallen in the virulent range. Those which have dissociated in one or more of these respects have so far been found to be remarkably diminished in virulence. Preliminary virulence titrations of the strains a to f,[4] indicate that most of them tend to belong in the less virulent group. The most probable explanation for this observation is that some of these strains have undergone partial dissociation since by cultural and serological methods some of them have been found to be deficient in one or more smooth characteristics.

It is felt that the method described in the preceding pages should be of value in determining the virulence of strains of *H. influenzae* of known and unknown origin. Except in a comparative study of this nature, however, a more accurate method than the one here employed for estimating the number of bacteria in a given suspension would be a desideratum.

This study also casts some doubt on the possibility that *H. influenzae* plays even a secondary rôle in the pathogenesis of human epidemic influenza. In any future studies on this question it will be essential to determine the virulence and other properties of the organism before it can reasonably be assigned specific pathogenic importance.

SUMMARY

1. A comparative study of the mucin virulence of smooth, converted rough, and respiratory strains of *H. influenzae* was carried out in mice.

2. The smooth strains were found to be significantly more virulent than either the converted rough or respiratory strains.

3. The converted rough and respiratory strains were found to be approximately equal in virulence.

4. A diminution in virulence was observed in those smooth strains which had dissociated spontaneously either partially or completely.

[4] Kindly furnished us by Dr. Pittman.

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