STUDIES ON RESPIRATORY DISEASES

XXIX. THE INFLUENCE OF ANTI-SERUM AND OF ANIMAL PASSAGE UPON THE VIRULENCE AND ELECTROPHORESIS OF PNEUMOCOCCI[1]

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I. INTRODUCTORY

In earlier publications of this series, Falk, Gussin and Jacobson (1925, Falk, Jacobson and Gussin (1925) and Falk and Jacobson (1925, 1926, 1926a) discussed at length the parallel relations between the virulence and the electrophoretic potentials of pneumococci. It was found that the potentials are different for the several types of pneumococci and that the sequence of decreasing potentials (types III, I, II, IV) is identical with the sequence of decreasing virulence for white mice. It was found further that variant strains (Blake) of type I pneumococci which differ in their virulence for mice also differ, in a parallel manner, in their electrophoretic potentials. These differences were demonstrated upon single cell subcultures as well as upon the original strains.

Falk and Jacobson also found for pneumococci, as had already been found for other bacteria, that the electrophoretic potential difference (P.D.) is a function of the pH of the menstruum and that the relative agglutinability—specific (serum) and non-specific (acid-alkali)—is an inverse function of the P.D. The correlation between P.D. and inagglutinability was further evidenced by the inversion of the relative suspension stability of

[1] This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

the *A. B. C.* strains, tested with agglutinating serum, after they had been washed sufficiently to invert the relative magnitudes of the potentials.

Many investigators have emphasized in recent years the significance of "smooth" and "rough" variations in bacteria. It has generally been found that strains which give "smooth" colonies on suitable media are (relatively) highly virulent and that strains which give "rough" colonies are slightly or not at all virulent when tested in appropriate animals. Under specified conditions, "smooth" strains can be converted to the "rough" varieties (*vide* Reimann, 1925; Amoss, 1925) and "rough" to the "smooth" (Jordan, 1926).

The normal and variant strains of pneumococci upon which we have already reported were all strains which gave "smooth" colonies on pepton, serum and blood agar media. Even the strain *C*, which is ordinarily without virulence for mice, gives "smooth" colonies. If a mouse be killed with a very large dose of these organisms, only virulent organisms of the smooth A variety are recovered.

The experiments reported in this paper were undertaken to determine whether "rough" colony varieties of these pneumococci could be produced, and whether changes in virulence would be associated with alterations in P.D. We also undertook a series of experiments to determine changes in virulence and P.D. upon successive passage through white mice of cultures of significantly different virulence and P.D.

II. PRODUCTION OF "ROUGH" STRAINS[2]

The cultures and methods used in these experiments were described in detail in the earlier publications which have been cited. Culture "*A*" is a virulent, type I pneumococcus; cultures "*B*" and "*C*" are variant strains of lesser virulence which were obtained by Professor F. G. Blake from "*A*" by growth in the presence of specific anti-serum and which he kindly placed ar our disposal. The P.D., agglutination and other charac-

[2] We are indebted to Miss Becky Bradley for technical assistance in the course of these experiments.

teristics of these organisms have been described at length by Falk and Jacobson (1925, 1926, 1926a).

Cultures *A*, *B* and *C* were grown in Blake's broth to which had been added 10 per cent of specific antiserum (New York State Department of Health, type I serum, free from preservative). The cultures were incubated at 37°C. and transferred at twenty-four-hour intervals. Control cultures were carried similarly in Blake's broth.

After the fourth transfer, the cultures were plated on sheep's blood agar plates each day and the colonies which developed after sixteen to twenty-four hours' incubation at 37°C. were examined. Definitely "rough" colonies were predominant after the 12th transfers in the broth containing anti-serum. Subcultures were taken from isolated "rough" colonies to blood agar slants. The *A* cultures were carried for 23 transfers; and *B* and *C* strains for 12. Subcultures taken from the 12th and 23rd transfers were used for virulence and other experiments. The notation used to describe our cultures may be illustrated as follows:

A orig. B orig. C orig.	Subcultures taken from stock cultures which had been kept on blood agar slants.
A_{12}, A_{23}, B_{12}, C_{12},	Subcultures taken from smooth colonies obtained by plating out after the 12th or 23rd transfers in Blake's broth + anti-serum.
$A_{12}R$, $A_{12}VR$, $A_{23}R$, etc. $B_{12}R$, $B_{12}VR$, etc. $C_{12}R$, $C_{12}VR$, etc.	Subcultures taken from "rough" colonies obtained by plating out 12th or 23rd transfers in Blake's broth + anti-serum.

Measurements of P.D., virulence for white mice, agglutination and precipitation tests were made by the methods described by Falk, Gussin and Jacobson (1925). Virulence is expressed in M.L.D. (cc.); P.D., in μ/sec. Typical results are illustrated in tables 1 to 4.

From table 1 it appears that smooth colony strains obtained from the 23rd transfer gave virulence and P.D. measurements typical of the original strain. The rough strain ($A_{23}R$) showed

entire lack of virulence for white mice, reduced P.D. and a tendency to spontaneous clumping and sedimentation. After 23 transfers in broth containing anti-serum, both *S* and *R* varieties were still co-existing in the culture. Indeed, in another series of experiments, in which virulence and P.D. were being modified by treatment with pepton in several concentrations, direct observations in the electrophoresis apparatus showed that

TABLE 1

Virulence and P. D. of S and R Strains of Pneumococci.

CULTURE	COLONY FORM	M.L.D.	P.D.
		cc.	*μ/second*
A original	S	10^{-7}	7.1
A_{23} (1)	S	10^{-7}–10^{-8}	6.4
A_{23} (1-XX)	S	10^{-7}–10^{-8}	6.7
A_{23} (3)	S	10^{-7}–10^{-8}	6.0
$A_{23}R$	R	—*	3.2†

* No deaths with 0.5 cc. of undiluted culture or with smaller quantities.
† Culture showed spontaneous agglutination and sedimentation.

TABLE 2

Virulence and P. D. of S and R strains of pneumococci

CULTURE	COLONY FORM	M.L.D.	P.D.
		cc.	*μ/second*
A original	S	10^{-7}	7.1
A_{12} (1)	S	10^{-7}	5.5
A_{12} (3)	S	10^{-7}	7.9
A_{23} (1)	S	10^{-7}	6.5
A_{23} (3)	S	10^{-7}	6.9
$A_{12}R$	R	—*	5.4
$A_{23}R$	R	—*	2.6

* No deaths with 0.5 cc. of undiluted culture or with smaller quantities.

two varieties of cocci were present—some which moved at high and some at low cataphoretic velocities.

From the data in table 2, it is clear that the findings are entirely similar to those presented in table 1.

In table 3, measurements on the original strains (*A*, *B* and *C*) show the characteristic differences in virulence and P.D. The A_{12} and A_{23} strains, although still showing smooth colonies,

show reduced virulence and reduced P.D. The $B_{12}R_1$ strain was rough but showed no appreciable modification in either virulence or P.D. by comparison with the *B* original strain.

TABLE 3

Virulence and P. D. of S and R strains of pneumococci

CULTURE	COLONY FORM	M.L.D.	P.D.
		cc.	*μ/second*
A original	S	10^{-7}	8.5
B original	S	10^{-3}	5.0
C original	S	—*	2.9
A_{12}	S	—*	5.0†
A_{23}	S	—*	2.5†
$B_{12}R_1$	R	10^{-3}	5.1
$C_{12}VR_1$	R	0.5	2.5

* No deaths with 0.5 cc. of undiluted culture or with smaller quantities.
† Cultures showed spontaneous agglutination and sedimentation.

TABLE 4

P. D., spontaneous agglutination and serum precipitation with S and R strains of pneumococci

CULTURE	COLONY FORM	P.D.	SPONTANEOUS AGGLUTINATION	SERUM PRECIPITATION	
				1 hour	24 hours
		μ/second			
A original (1)	S	7.1	−	++++	++++
A original (2)	S	7.9	−	++++	++++
B original	S	4.0	+	−	++
C original	S	1.9	+	−	++
$A_{12}VR$	R	1.4	+	−	++
$A_{23}VR$	R	1.7	+	−	++
$B_{12}VR$	R	2.9	+++	−	++
$B_{12}VR_1$	R	2.2	+++	−	++
$B_{12}VR_2$	R	2.2	+++	−	++
$C_{12}R_1$	R	1.7	+++	−	++
$C_{12}R_2$	R	1.9	+++	−	++

The $C_{12}VR_1$ strain, although rough in colony form, shows no significant change in either virulence or P.D.

From the data in table 4 it appears that the rough strains of *A* and *C* showed marked reductions in P.D. The reductions for the rough *B* strains were not so great. The tendency of a

culture to show spontaneous agglutination and sedimentation is seen to be an approximate reciprocal of the magnitude of the P.D.

It is significant to note in table 4 that the capacity of the supernatant fluid (obtained by centrifugation of cultures in Blake's broth) to give a precipitin reaction with type I anti-pneumococcus serum within 1 hour (or within the day) is restricted to the *A* original cultures. Neither the variant strains *B* and *C* (Blake) nor the rough varieties produced by 12 or 23 transfers in broth containing anti-serum showed a prompt precipitin reaction. These strains showed a slight but definite precipitin reaction after they were held in the ice box for some twenty-four hours.

III. EFFECTS OF ANIMAL PASSAGE

In earlier publications we pointed out, as Blake and Trask first reported, that when a mouse is killed with the *A* strain of pneumococcus (type I, original) the organisms that are recovered from the heart blood or peritoneal exudate are indistinguishable from the organisms which had been injected. We also confirmed the finding that an occasional mouse injected with a large dose of the *C* strain dies and that from the peritoneal exudate or heart blood only *A* type organisms (of high virulence, high P.D., and low agglutinability) are recovered. Preliminary experiments with the *B* strain, which is intermediate between the *A* and *C* strains in virulence, P.D. and other characteristics, had given variable results. The experiments reported here were undertaken to determine more specifically the behavior of the *A*, *B* and *C* strains upon serial passage through white mice.

Blood agar slant cultures of the original *A*, *B* and *C* strains were covered with a little sterile Blake's broth, incubated over night at 37°C. and the cultures in the broth injected intraperitoneally into healthy white mice. With the *A* strain 0.5 cc. quantities were injected; with the *B* strain 0.75 cc.; and with the *C* strain—which in 0.5 cc. quantity is normally without virulence for mice—1 cc. quantities were injected. The mice died regularly eighteen to thirty-six hours later. A little of the

heart blood was streaked on blood agar plates directly after the animal's death and cultures of pneumococci were recovered from discrete colonies. These were tested with agglutinating serum and with bile and were used subsequently for further injections into mice. A blood agar slant culture of each recovered strain was preserved in the ice chest.

The blood agar slant cultures of the original strains (*A* original, *B* original, *C* original) and of the strains recovered from serial passages through mice (A_1, A_2, etc., B_1, B_2, etc., C_1, C_2, etc.) were used for virulence, P.D. and agglutination tests by the methods which have been described.

TABLE 5

Virulence and P. D. of A, B and C strains of pneumococci after four passages through mice

CULTURE	M.L.D.	P.D.
	cc.	*μ/second*
A original	10^{-7}	7.1
A_4	10^{-7}–10^{-8}	6.8
B original	10^{-2}	5.4
B_4	10^{-3}	3.9
C original	—*	4.1
C_4	10^{-4}†	5.7

* No deaths with 0.5 cc. quantities of undiluted culture.

† Not tested beyond 0.0001 cc. quantity.

The results of the first series of experiments, made after 4 animal passages, are presented in table 5.

The data in table 5 show that both the virulence and P.D. of the *A* and *B* strains were essentially unaltered by 4 passages through mice. The *C* strain had been markedly increased in virulence and perhaps significantly increased in P.D.

The experiment was repeated after 8 animal passages. The results are presented in table 6.

It is evident from table 6 that animal passage has not significantly modified the virulence, P.D. or agglutination reactions of strains *A* and *B*. Strain *C* showed an increase in virulence, an increase in P.D. and a reduction in agglutinability so that, with respect to these characteristics, it is indistinguishable from

strain *A*. It would appear, therefore, that the direct or inverse parallelisms between virulence, P.D., and agglutinability which were discussed in earlier publications are confirmed by measure-

TABLE 6

Virulence and P. D. of A, B and C strains of pneumococci after eight passages through mice

CULTURE	P.D.	M.L.D.	AGGLUTINATION BY TYPE I SERUM
	μ/second	*cc.*	
A original	6.1	10^{-3}	1:40
A_1	6.5		1:40
A_2	6.5		1:40
A_3	6.7		1:40
A_4	8.1		1:40
A_5	4.5		1:40
A_6	5.2		1:40
A_7	6.1		1:40
A_8	6.7	10^{-3}	1:40
B original	5.6	10^{-2}–10^{-3}	1:80
B_1	4.5		1:80
B_2	4.9		1:80
B_3	4.4		1:80
B_4	4.4		1:80
B_5	4.1		1:80
B_6	3.7		1:80
B_7	3.8		1:80
B_8	4.0	10^{-2}	1:80
C original	4.1	—*	1:1280
C_1	4.2		1:160
C_2	5.3		1:30
C_3	5.8		1:20
C_4	5.3		1:20
C_5	5.6		1:20
C_6	(lost)		1:20
C_7	7.2		1:20
C_8	6.0	10^{-3}	1:20

* No deaths with 0.5 cc. quantities of undiluted culture.

ments on cultures subjected to animal passage. The relative constancy of the *B* strain and its failure to revert to the *A* type is a further indication of its comparative stability. This would

appear to confirm the conclusion from the study of single cell strains that the *B* variant is not a mixture of *A* and *C*.

Inasmuch as all three strains have never given detectable "rough" colonies in this set of experiments, it is apparent that strains of pneumococci of different virulence are not necessarily separable into homologous *R* and *S* categories.

After the completion of the series of animal passages which have been described, we undertook two more, similar experiments with the *B* strain. In each experiment, the *B* strain was passed successively through eight mice and the cultures were tested for virulence and P.D. as in the first experiment. The results showed again the greater variability of this strain in virulence and in P.D. as compared with strains *A* and *C*. But they also showed unequivocally in each experiment that after eight passages the characteristics of the *B* strain had not changed significantly towards those of the *A* or the *C* strains.

We have repeatedly observed when conducting electrophoresis measurements that each culture of the *B* strain consists of organisms which, as individuals, give electrophoretic mobilities intermediate between the mobilities of *A* and *C* organisms. The *B* strain is not, from this point of view, a mixture of cocci of the *A* and *C* varieties.

IV. SUMMARY AND CONCLUSIONS

1. The *B* and *C* strains of pneumococci used in these experiments are derivatives of the *A* strain (type I). They were obtained by Professor F. G. Blake by growth in the presence of specific antiserum. The sequence of decreasing virulence for white mice, decreasing P.D. and increasing agglutinability is: *A*, *B*, *C*.

2. Of the colonies of *A*, *B* and *C* strains which form on pepton, serum or blood agar plates, only "smooth" varieties have been observed.

3. Strains which give "rough" colonies on blood agar plates were produced from *A*, *B* and *C* cultures by growth in broth to which specific anti-serum had been added.

4. After 12 or 23 transfers in serum broth, none of the cul-

tures had been completely converted to the *R* varieties. Organisms of both the *S* and *R* varieties could be recovered.

5. Some of the *S* varieties which were recovered after 23 transfers in broth containing anti-serum showed the virulence and P.D. which are characteristic of the original cultures.

6. Some of the *S* varieties recovered showed reduced virulence and P.D.

7. The "rough" varieties recovered after 12 transfers of *B* and *C* strains in broth + anti-serum showed the same virulence and P.D. as the original *B* and *C* cultures.

8. It would appear that strains of pneumococci which differ significantly in virulence are not necessarily separable into homologous *S* and *R* categories.

9. The original *A* strain gives a prompt precipitation reaction with specific anti-serum. The original *B* and *C* strains and the *R* derivatives of *A*, *B* and *C* strains give only a slight, delayed reaction after twenty-four hours.

10. The *A* and *B* strains show no significant changes in virulence, P.D. or agglutination after 4 and 8 passages through mice.

11. Passage of the *C* strain through mice results in a reversion of its characteristics to those of the *A* strain.

12. In all the cases studied, alterations in the virulence of pneumococci for white mice were accompanied by parallel alterations in P.D. and by reciprocal alterations in agglutinability.

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