STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

I. The Capacity of Strains of Pneumococcus Type III to Grow at 41°C. and Their Virulence for Rabbits

By JOHN F. ENDERS, Ph.D., and MORRIS F. SHAFFER,* Ph.D.

(From the Department of Bacteriology and Immunology, The Harvard Medical School, Boston)

(Received for publication, April 16, 1936)

Although the well known change from smooth to rough types, with the accompanying loss of antigenic elements, presents an explanation for many instances of loss of virulence by bacteria, it fails to account adequately for differences in the degree of virulence observed among various strains of a species all of which possess the characteristics associated with the smooth form. A notable example of this latter type of variation is found among strains of Pneumococcus Type III of human origin when injected into rabbits.

Tillett (1) demonstrated that the majority of strains isolated by him from cases of Type III pneumonia were, although virulent for mice, avirulent for rabbits in the sense that upon injection, even in large numbers, they produced a transient bacteremia which in nearly all cases terminated in recovery. He was able to obtain one strain which killed rabbits in moderate dosage on isolation and which, upon passage in these animals, attained an M.L.D. of 0.0001 cc. when introduced intravenously, but was unable to observe any biological property of the rabbit-virulent strain by which it could be distinguished from the avirulent cultures. Similar differences in the virulence of Pneumococcus Type III strains have been noted by others (Levy-Bruhl (2), Watson and Cooper (3)) but no entirely satisfactory explanation of this behavior has been presented.

One of the two principal objectives, then, of these experiments and

* Fellow in Medicine of the National Research Council.

those recorded in the papers which follow has been to reveal attributes of the organisms from several smooth strains of Pneumococcus Type III which might account for the differences in virulence. Since the studies of previous workers (*e.g.* Singer and Adler (4), Tillett (5, 6, 7)) on the mechanism of the natural immunity of the rabbit against this microbe do not completely elucidate the means whereby it is removed from the blood stream and destroyed in the body, a second aim of our work has been to investigate further the defensive factors of not only the rabbit but also the mouse and (to a limited extent) of man against infection with this type of pneumococcus.

In the present paper it has been shown that marked differences exist in the ability of various strains of Pneumococcus III not only to remain viable, but to multiply at 41°C.— a temperature rapidly attained or exceeded and maintained for varying periods of time by the body of rabbits, following infection. The virulence of these strains for this animal has also been determined with the purpose of revealing a possible correlation between this property and the capacity to proliferate at this temperature.

Materials and Methods

Strains Employed.—13 strains of Pneumococcus Type III have been studied. Of these, strain SV,[1] originally isolated by Tillett from a case of lobar pneumonia, is highly virulent for both rabbits and mice. Intradermal injection of 0.001 cc. of a 16 hour blood broth culture into a normal rabbit will bring about the death of the animal. Strain CH was also derived from a case of lobar pneumonia and is the stock strain maintained for several years in this laboratory where it is passed each week through a mouse as routine. Intraperitoneal inoculation of 0.5 cc. of 10^{-6} dilution of a 16 hour blood broth culture of CH or SV regularly kills mice in about 48 hours. The remaining 11 strains were isolated from human cases of lobar pneumonia, meningitis, and otitis media within a period of 18 months. 2 of these, Nos. 298 and 312, were studied within a week following their isolation. Stock cultures of these strains are grown in normal defibrinated rabbit's blood under a vaseline seal and kept in the ice box. Transfers are carried out at intervals of 4 to 6 weeks.

Strain SV is passed through a rabbit at intervals of 3 to 4 weeks. The remaining strains were not passed through rabbits prior to the beginning of this work, except in the cases of strains IE and CSp which had been inoculated into rabbits once and recovered in pure culture about 1 year previously. All strains were

[1] Sent to us through the courtesy of Dr. K. Goodner of The Rockefeller Institute.

characterized by the possession of capsules, and produced large mucoid colonies on blood agar. They agglutinated to titer in anti-Type III pneumococcus horse serum and showed no tendency to flocculate in 1/10 dilution of an anti-Type VIII pneumococcus horse serum which agglutinated the homologous type organisms in a titer of 1/80.

The 2 strains of R variants derived respectively from the smooth strains CH and SV were obtained after 8 transfers in 10 per cent anti-Type III pneumococcus rabbit serum infusion broth. These fulfilled all the criteria for the rough form. 0.7 cc. of a 15 hour blood broth culture of each failed to kill mice subsequent to intraperitoneal injection.

Method Used in Determining Growth Curves.—16 hour blood broth cultures of the various strains were diluted in sterile infusion broth. Freshly drawn defibrinated rabbit blood was inoculated with 1 per cent of its volume of a 1/100 dilution of the culture; 2 to 3 cc. of rabbit blood were usually employed. In each case duplicate cultures were made: one was kept at 37°C. in the incubator and the other placed in a water bath at 41°C. ± 0.1°. At intervals 0.1 cc. samples were removed and dilutions prepared in broth. Measured portions of these were placed in Petri dishes containing 0.5 cc. of defibrinated horse blood and melted infusion agar added. After 48 hours incubation, the colonies were counted. Defibrinated rabbit blood was used as the medium since it would most closely approximate the nutritive environment in the host.

Method of Determining the Virulence for Rabbits.—0.1 cc. of blood broth culture was inoculated into 10 cc. of 0.1 per cent dextrose 0.5 per cent rabbit serum infusion broth. After 7½ hours incubation various amounts of this culture were injected intravenously into normal rabbits. When quantities larger than 1 cc. were tested, the culture was centrifugalized at 2800 R.P.M. for ½ hour and the deposit taken up in 1 cc. of sterile broth which was injected. The number of viable bacteria per 1 cc. of inoculum was determined in each case by the plating method. The temperatures of the animals were recorded each morning and evening.

Variation in the Capacity of 13 Strains of Pneumococcus Type III to Grow at 41°C.

In Table I are presented data which represent the numbers (expressed as the logarithms) of viable organisms per cubic centimeter of defibrinated rabbit blood (incubation at 41°C.), at various intervals following inoculation with each of 13 smooth strains of Pneumococcus Type III and the R variants derived from two of these smooth forms. Figures are also included which denote the numbers of viable organisms present after 24 hours incubation in similar cultures maintained at 37°C. Counts at the latter temperature for earlier intervals were obtained, but are not recorded except in the case of strain SV (whose

TABLE I

Growth of Pneumococcus Type III Strains in Defibrinated Rabbit Blood at 41°C.

Strain	Period of incubation						
	0 hrs.	2½ hrs.	5 hrs.	7½ hrs.	9 hrs.	12 hrs.	24 hrs.
SV (Tillett)	4.21 4.49*	5.39 5.47*	7.10 7.10*	7.85 7.96*	8.73 8.26*	n.d. n.d.	7.05 9.38*
CSp	4.60	4.39	6.14	8.03	n.d.	n.d.	7.98
B	4.48	4.40	6.07	7.18	n.d.	n.d.	8.35 9.46*
IE	5.02	4.17	4.08	4.08	n.d.	4.38	5.55
Tirrell	5.97	4.50	4.30	4.39	n.d.	4.91	5.33
CH	3.80	5.11	6.35	6.04	5.28	n.d.	0.00 9.78*
669	4.19	2.48	<2.00	n.d.	n.d.	n.d.	0.00 9.82*
681	4.05	3.54	4.11	3.85	<3.00	n.d.	0.00 9.38*
A305	4.43	3.84	3.86	3.75	n.d.	n.d.	0.00 9.47*
3843	4.20	3.60	3.04	<2.00	n.d.	n.d.	0.00 9.48*
3838	4.39	4.20	4.11	3.69	n.d.	n.d.	0.00 8.96*
298	4.04	2.00	2.00	<2.00	n.d.	n.d.	0.00 9.12*
312	4.40	3.03	2.31	<2.00	n.d.	n.d.	0.00 9.35*
SV (rough form)	4.46	4.34	4.35	5.09	5.79	n.d.	6.57
CH (rough form)	5.48	4.15	3.15	2.30	2.00	n.d.	0.00

Results given as $\log_{10}$ of number of viable pneumococci per cc. of blood.
n.d. = not done.
*Indicates values for duplicate cultures at 37°C.

37°C. values are given as an example) since they showed that the growth rates of all were quite comparable to that of strain SV at this temperature.

From these results it is apparent that individual strains of this type of pneumococcus exhibit marked diversity in their capacity to multiply

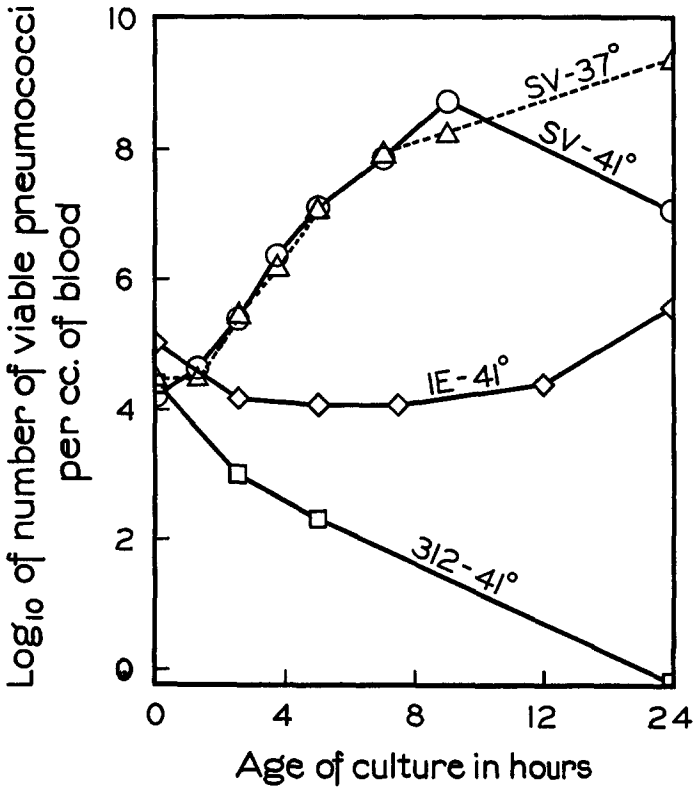

FIG. 1. Growth curves at 41°C. of Pneumococcus Type III strains typical of each of the three groups, as well as the SV growth curve at 37°C. O——O strain SV (group 1); ◇——◇ strain IE (group 2); □——□ strain 312 (group 3); △------△ strain SV (37°).

at 41°C. On the basis of such differences it is possible to divide the strains which have been examined into three groups:

1. Those which grow at approximately the same rate and attain almost the same maximal numbers at 41°C. as at 37°C.

2. Those which increase more slowly at 41°C. than at 37°C. or which remain practically stationary at the higher temperature.

3. Those which show little or no evidence of increase at 41°C. and which after a variable interval at this temperature decrease in numbers. This diminution in most instances may progress until no viable organisms remain.

In Text-fig. 1 are presented in the form of growth curves the data for 3 strains each representative of one of these groups. For purposes of comparison the curve for strain SV at 37°C. is included, since this exemplifies the rate of multiplication of all the other strains at this temperature.

It is clear from the figures included in Table I that of the 13 strains studied, 3 clearly fall into the first group, 2 in the second, and 7 in the third. Strain CH, although difficult to classify has been more or less arbitrarily assigned to group 2 since it grew at 41°C. during the early hours following inoculation, but soon the numbers declined and the culture was found to be sterile at 24 hours.

That the growth property at 41°C. of a given strain depends upon stable metabolic processes is indicated by the fact that this remains qualitatively uninfluenced by the S→R change. Thus, it will be seen that the R form derived from strain SV retains the ability to grow at 41°C., whereas that from strain CH, although not exactly reproducing the growth curve obtained with the S form, nevertheless, like the latter, is inhibited at this temperature.

Virulence of the Strains for Rabbits

Our observations on the inability of the majority of our strains to grow well at 41°C. suggested a possible relationship between this behavior and the avirulence for the rabbit of most strains of Pneumococcus Type III reported by other workers, since the temperature of this animal has been found to rise to 41°C. or higher 3 to 5 hours after intravenous infection. Fluctuations occur thereafter but body temperature of 40.3–41.3°C. may be maintained for considerable periods of time. Conversely it seemed probable that the strains which demonstrated their capacity to survive and multiply at this temperature

might prove virulent, particularly since strain SV which was known to be highly virulent for rabbits had been found to grow well at 41°C. Accordingly, a series of rabbits were injected intravenously with the organisms from 5 cc. of a young (7½ hour) culture at 37°C. of each of 11 strains, the virulence of which was not definitely known. Strains SV and CH were not tested at this time since their relative virulence had been determined in previous experiments.

This preliminary test showed that among the 11 strains, 3 only (IE, CSp, B), were capable of killing rabbits following the injection of 5 cc. of culture. Additional animals were then inoculated with smaller quantities of cultures of these 3 strains. The rabbits injected with 0.1 cc. of the cultures of strains IE and CSp succumbed, while strain B proved to be definitely less virulent since this quantity failed to kill either animal following generalized invasion of the body. Again, of two rabbits each receiving 1 cc. of B culture, only one died and this animal was inoculated with a culture obtained from the heart's blood of the rabbit which had succumbed to 5 cc. These findings, with additional experimental details, are recorded in Table II. It will be seen that all the 7 strains which failed to withstand a temperature of 41°C. (group 3) were not fatal to rabbits in the amounts of culture injected. In contrast both strains (B and CSp) in group 1 were able to bring about death of the animals, although the degree of virulence is obviously not identical. In group 2, of 3 strains only strain IE possessed to a marked degree the property of invading the blood stream and overwhelming the animal. The Tirrell strain possessed little or no virulence, although its growth curve was quite similar to that of strain IE. Certain characteristics exhibited by strain CH which will be described in subsequent communications probably account for its lack of virulence and that of the Tirrell strain. It should be emphasized in respect to the quantities of culture which in these experiments have led to the death of rabbits that although apparently large, they nevertheless indicate a considerable degree of virulence since it has been found by practically all workers (*cf.* Tillett) that the virulence of Pneumococcus Type III for rabbits, without repeated passage through these animals, in no instance approaches that of organisms such as Pneumococcus Type I.

TABLE II

Virulence for Rabbits of Various Strains of Pneumococcus Type III

Rabbit No.	Weight	Strain Pneumococcus III	Group on basis of growth at 41°C.	No. of organisms in volume of culture injected	Result, death or survival of rabbit	Remarks
	gm.					
1-9	1500	SV	1	0.1 cc. culture	D < 48 hrs.	This dose does not represent the M.L.D. for this strain
7-0	2010	CSp	1	$2.4 \times 10^8 \Rightarrow 1.0$ cc.	D < 40 hrs.	2 other animals inoculated. 1.0 cc. and 0.1 cc. of culture CSp passed once through a rabbit; both died
4-69	1050	CSp	1	$2.4 \times 10^7 \Rightarrow 0.1$ cc.	D < 47 hrs.	
2-5	1450	CSp	1	$3.8 \times 10^5 \Rightarrow 0.01$ cc.	S	
8-9	1480	B	1	5 cc. culture	D 96 hrs.	
6-5	2020	B	1	$2.6 \times 10^8 \Rightarrow 1.0$ cc.	S	
2-80	1840	B	1	$2.3 \times 10^8 \Rightarrow 1.0$ cc.	D 144 hrs.	Pericarditis—large numbers of organisms in the blood; culture passed once through a rabbit
2-1	1730	B	1	$2.3 \times 10^7 \Rightarrow 0.1$ cc.	S	Culture passed once through a rabbit
4-74	1020	B	1	$2.6 \times 10^7 \Rightarrow 0.1$ cc.	D 192 hrs.	Infection of right eye. Infection of knee joint with pneumococcus—No organisms in heart's blood
3-69	About 1500	IE	2	$4.5 \times 10^9 \Rightarrow 5$ cc.	D < 96 hrs.	
2-3	2000	IE	2	$4.5 \times 10^8 \Rightarrow 10$ cc.	D 95 hrs.	
3-77	1650	IE	2	$4.5 \times 10^7 \Rightarrow 0.1$ cc.	D < 96 hrs.	

2-4	1610	Tirrell	2	8 $\times 10^8 \Rightarrow$ 5 cc.	S	
4-75	2150	Tirrell	2	1.8 $\times 10^9 \Rightarrow$ 5 cc.	D < 96 hrs.	Culture of heart's blood showed only 4 $\times 10^4$ organisms per cc. Few organisms previously found in blood. Doubt as to death from pneumococcus infection
9-2	2500	CH	2	10 cc. culture	S	5 hr. culture used for inoculum
6-69	1710	669	3	3.5 $\times 10^9 \Rightarrow$ 5 cc.	S	
4-61	1860	681	3	9.0 $\times 10^8 \Rightarrow$ 5 cc.	S	
1-5	1240	A305	3	5 $\times 10^9 \Rightarrow$ 5 cc.	S	
1-7	1450	3843	3	2.5 $\times 10^9 \Rightarrow$ 5 cc.	S	
1-4	1660	3838	3	4 $\times 10^9 \Rightarrow$ 5 cc.	S	
4-67	1620	298	3	1.8 $\times 10^9 \Rightarrow$ 5 cc.	S	
4-76	1750	312	3	7.0 $\times 10^9 \Rightarrow$ 5 cc.	S	

DISCUSSION

In assessing the significance of these results, we may assert with considerable confidence that those strains which are unable to increase at 41°C., though endowed with the complete antigenic equipment of the smooth virulent organism contained in the capsule, cannot, under the conditions of temperature existing in the body of the rabbit during infection, multiply sufficiently to bring about the death of the animal. We have investigated too few strains to give the results any statistical validity, but those we have obtained suggest that the majority belong to this group. The fact that most Pneumococcus Type III strains are not virulent for rabbits is, therefore, entirely in agreement with our findings.

Conversely, evidence for believing that a correlation exists between the ability to withstand 41°C. and virulence is the fact that only among such "thermo-resistant" strains were found those possessing this property of rabbit virulence. It is clear, however, that an additional factor or factors must be involved in determining whether thermo-resistant strains will produce an infection which terminates fatally, since in this class we have found, according to the criteria arbitrarily selected, avirulent, slightly virulent, and virulent varieties. The results of an analysis of one of these factors will be found in the following papers.

The conception that the high normal body temperature of certain species of animals plays a defensive rôle in immunity to infection by microorganisms has been in the minds of workers since the experiments of Pasteur demonstrated that the chicken, normally refractory to anthrax, became susceptible after the temperature had been lowered by immersion in cold water. Nearly as old is the hypothesis that fever itself is an immunological response. Various methods have been employed in attempts to demonstrate experimentally the validity of these hypotheses. They have consisted in depressing or raising the normal body temperature by physical, chemical, or physiological means, either before or after inoculation of the infecting organism.

Experiments of this kind with pneumococci were early carried out. Thus Walther (8), Loewy and Richter (9), Rolly and Meltzer (10) obtained some evidence that raising the body temperature of rabbits to 41–42°C. increased their resistance to infection. Strouse (11), noting that growth of pneumococci was

inhibited at 40–41°C. *in vitro*, lowered the normally high (41°C.) temperature of pigeons by injection of pyramidon and found that these succumbed to infection with quantities of pneumococci which were tolerated by controls. Wadsworth (12) failed to produce pneumonia in rabbits by cooling the animals in water previous to infection; he later (13) tested 25 strains of pneumococci and found that *in vitro* the growth of all was completely inhibited at 40.5–41.2°C. It is difficult to evaluate and to correlate the details of these experiments with our own work since they were obtained before the classification of pneumococci into types was introduced. More recently Findlay (14) in studying the relationship between increased susceptibility to infection and lack of vitamin B in pigeons found that in birds deprived of this vitamin the body temperature was reduced to 103–104°F. and they then succumbed after inoculation with Pneumococcus Type II. Wright (15) in his studies of experimental Pneumococcus Type I septicemia in rabbits observed that at 40.5°C. the lag period of the virulent organism grown in rabbit blood was definitely increased, but good growth subsequently occurred. He was unable to correlate this transient inhibitory effect of higher temperatures *in vitro* with the removal of the organisms from the blood stream of rabbits, whether the temperature of the animal was previously increased by administration of vaccines or subsequently rose as a result of infection with pneumococci. Levy-Bruhl (2) who studied 20 strains of Pneumococcus Type III found that in 0.2 per cent glucose peptone broth all cultures gave at 42°C. a growth which was nearly as abundant and as rapid as at 37°C. On subcutaneous injection in dosage of 1 cc. into rabbits, however, only 4 strains were able to produce a fatal outcome; the other 16 strains did not kill. Although his results concerning the effect of temperature are not in agreement with ours, the proportion of virulent strains is approximately the same.

Our work suggests that the rise in temperature occurring subsequent to infection may be regarded as a most important factor in the natural resistance of the normal rabbit to those strains of Type III pneumococci fully capable of producing fatal disease in man, but which cannot increase sufficiently to bring about the same under the conditions of temperature encountered in the former.

CONCLUSIONS

1. A correlation appears to exist between the failure of certain strains of Pneumococcus Type III to grow at 41°C. and their lack of virulence for rabbits.

2. It is likely that the capacity to grow at 41°C.—an attribute constantly but not exclusively associated with strains of Pneumococcus Type III virulent for rabbits—is a prerequisite, but not the sole factor, in determining their virulence for these animals.

BIBLIOGRAPHY

1. Tillett, W. S., *J. Exp. Med.*, 1927, **45**, 1093.
2. Levy-Bruhl, M., *Ann. Inst. Pasteur*, 1927, **41**, 458.
3. Watson, E. L. and Cooper, G., *J. Exp. Med.*, 1930, **52**, 849.
4. Singer, E., and Adler, H., *Z. Immunitätsforsch.*, 1924, **41**, 71.
5. Tillett, W. S., *J. Exp. Med.*, 1927, **45**, 713.
6. Tillett, W. S., *J. Exp. Med.*, 1927, **46**, 343.
7. Tillett, W. S., *J. Exp. Med.*, 1928, **48**, 791.
8. Walther, P., *Arch. Hyg.*, 1891, **12**, 329.
9. Loewy, A., and Richter, P. F., *Virchows Arch. path. Anat.*, 1896, **145**, 49.
10. Rolly, Fr., and Meltzer, *Deutsch. Arch. klin. Med.*, 1908, **94**, 335.
11. Strouse, S., *J. Exp. Med.*, 1909, **11**, 743.
12. Wadsworth, A. B., *Am. J. Med. Sc.*, 1904, **127**, 851.
13. Wadsworth, A. B., *J. Exp. Med.*, 1912, **16**, 54.
14. Findlay, G. M., *Lancet*, 1922, **1**, 714.
15. Wright, H. D., *J. Path. and Bact.*, 1927, **30**, 185.