THE OCCURRENCE OF ROUGH PNEUMOCOCCI IN VIVO.

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INTRODUCTION.

The establishment of recognized groups of pneumococcus variants such as the rough or R and possibly intermediate forms has raised the question as to whether these variants, particularly the R forms, play any rôle in the course of an active pneumococcus infection or in the epidemiology of such infections. Up to the present time most of the studies upon R pneumococci have been made upon those forms isolated *in vitro* as a result of subjecting smooth or S forms to various cultural or environmental conditions. Our knowledge of their pathogenicity or relationship to the human host is as yet limited, but recent work with animals has suggested that the isolation of these variants of reduced virulence during the course of pneumococcus infection may be of significance in our general conceptions of the infectivity of the disease and the host response.

Wadsworth and Sickles (1) have shown that the pneumococcus multiplying in the tissues of the immunized animal becomes attenuated, and they have reported the isolation of several strains of these attenuated pneumococci from the blood stream and heart valves at autopsy, of horses which had been immunized with Type I pneumococci. These strains showed a loss, in varying degree, in virulence, capacity of capsule formation, susceptibility to phagocytosis, and type specificity. The antigenic activity as an immunizing agent and the production of soluble specific substance were also altered. With some of these strains it was found that the typical pneumococcus characteristics could be restored by one or two passages through a mouse, while others remained atypical.

In a study on the pathogenicity of degraded pneumococci Reimann (2) has discussed the importance of determining whether the R variants ever appear *in vivo* and, if so, under what conditions. He states that: "Although they have been carefully searched for, R forms have not been encountered in the cultures of sputum or blood of patients, either during the period of pneumococcus infection or during convalescence, or in direct cultures from the lungs at autopsy." On

the other hand, this author has shown that under experimental conditions a degradation from the S to the R form actually does take place in cultures of pneumococcus growing in agar subcutaneously embedded in guinea pigs and in agar enclosed in vials subcutaneously embedded in rabbits. However, this change was never complete and when the intermediate or R forms did appear they were always accompanied and usually exceeded in number by the S forms. Another interesting observation was that pure cultures of the avirulent R forms remained viable in subcutaneous foci for weeks.

Previously there have been a number of reports on the isolation of atypical pneumococci from lesions in animals and man. In 1907 Buerger and Ryttenberg (3), in studying the morphology and cultural characteristics of pneumococci in human exudates and human blood, concluded that wide variations from the typical forms may occur. These atypical forms could be reverted by animal inoculation soon after their isolation, but 2 months after isolation the organisms had acquired fixed cultural characteristics and repeated animal inoculation failed to bring about a change.

Later Rosenow (4, 5), in a series of studies on pneumococci isolated from cases of endocarditis, described these organisms as "modified" pneumococci. They were atypical from the standpoint of their morphology, methemoglobin formation, inulin fermentation, and the fact that the colonies produced by these organisms on blood agar adhered more closely to the surface of the culture medium than those produced by typical pneumococci. However, by cultivating these strains in normal serum or blood and by animal inoculation a reversion to typical pneumococci was established.

Bull (6) has also reported that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. He noted that pneumococci isolated as the infection was subsiding were more susceptible to the action of immune serum than the original cultures which had been injected. In one fatal case the pneumococci isolated on the 9th day grew in chains and were avirulent.

From the observations quoted above it would seem likely that in the earlier work some of the avirulent variants which have been isolated by Rosenow (5) and others from human patients may have been related to the R or intermediate pneumococcus forms. The more recent observations of Wadsworth and Sickles (1) and Reimann (2), however, have shown conclusively that such degradation of pneumococci may be produced experimentally *in vivo*.

In order to study this question we have limited ourselves to a single aspect of the major problem in an effort to determine whether or not one may isolate proved R pneumococci from the human body and, if so, under what conditions. The observations given below present

evidence which we believe answers the first question in the affirmative, but the data which deal with the second question are as yet too limited to lead to a profitable discussion.

The main feature of our work has consisted in a differential study of a group of organisms which we have isolated in a small number of instances from the sputum of individuals suffering from infections of the respiratory tract. The strains which we have studied have been primarily selected on the basis of colony differentiation together with their ability to produce methemoglobin when grown upon the surface of rabbit blood agar and the study has essentially resolved itself into the differentiation of these forms from strains of S pneumococci or from strains of *Streptococcus viridans*,* and possibly other species of allied bacteria.

Methods.

For colony differentiation 18–24 hour cultures were made on fresh rabbit blood agar plates (pH 7.8) and studied according to the methods outlined in a previous paper (9).

Broth cultures were made in 1 per cent dextrose broth to which rabbit blood had been added in a concentration of 10 per cent.

Two standard R strains of pneumococci, R-I and R-II, were employed as controls throughout the work. These were obtained from The Rockefeller Institute for Medical Research through the kindness of Dr. O. T. Avery. The S strains of type-specific pneumococci, also used as controls, were obtained from the sputum of cases of lobar pneumonia which occurred at the Pennsylvania Hospital during the winter of 1926–27, all of which had been subjected to one animal passage. Five strains of *Streptococcus viridans* and one strain of hemolytic streptococcus, also used for controls, were obtained from recent blood cultures of cases of subacute bacterial endocarditis and hemolytic streptococcus septicemia respectively. All the control strains were kept at room temperature on rabbit blood agar with weekly transfers.

The anti-S or type-specific antipneumococcus serum employed for precipitation

* It is evident that any investigation which attempts to differentiate the organisms of the so called green-producing streptococcus group and R pneumococci should include a discussion of the possible relationship between these two forms. Morgenroth and his collaborators (7) have recently reported that by special methods it was possible to transform pneumococci into streptococci with the production of various intermediate forms. The evidence to support such a view is not, however, convincing, and in a critical review of this work Reimann (8) has been unable to arrive at similar conclusions.

and agglutination tests was obtained from the Division of Laboratories and Research, New York State Department of Health.

EXPERIMENTAL.

During the past 18 months we have been engaged in a series of pneumococcus colony studies on cultures which have been obtained from the spinal fluid, mastoid, chest cavity, and other regions of the body

TABLE I.

Type of Case and Stage of the Disease from Which the Twelve Strains (X-1 to 12) Were Obtained.

No.	Initials	Age	Sex	Race	Pulmonary lesion	Stage of disease
		yrs.				
1	J. D.	50	F.	W.	Bronchopneumonia	Early in active stage
2	A. B.	7	"	"	Mild postoperative bronchopneumonia	" " " "
*3	J. M.	39	M.	"	Lobar pneumonia (Type II)	" " " "
*4	" "	39	"	"	" "	" " " "
*5	A. S.	28	"	"	" "	" " " "
*6	" "	28	"	"	" "	" " " "
*7	M.S.	37	F.	C.	Lobar pneumonia (Type IV) (fatal case)	Late " " "
*8	" "	37	"	"	" "	" " " "
9	T. S.	28	"	W.	Bronchopneumonia	" " " "
*10	J. M.	39	M.	"	Lobar pneumonia (Type II)	Early convalescence
11	R. S.	42	"	"	Bronchopneumonia. Active tuberculosis	During active stage of bronchopneumonia
12	I. A.	26	"	C.	Lobar pneumonia (Type I)	Late in active stage

* Nos. 3, 4, and 10 represent the same patient, as do also Nos. 5 and 6, 7 and 8.

taken during the active stage of a pneumococcus infection. In none of these cultures, many of which had been obtained from suppurative lesions of varying age, were we able to detect the presence of R pneumococci.

R Pneumococci in Sputum Cultures.

In attempting to isolate R pneumococci from sputum our attention was first directed to a survey of the colonies which are formed by

different strains of *Streptococcus viridans* in sputum cultures from normal individuals and from individuals representing a wide variety of clinical conditions. The *viridans* colonies which occur in sputum cultures apparently exhibit a very wide degree of variation. Many of them prove to be quite different from those of R pneumococci while others are practically indistinguishable. From the latter groups a number of strains (about 40) were isolated from suspected colonies and from these twelve bile-soluble strains were selected for further study. These strains have been designated X-1 to 12 inclusive, and are listed in Table I together with the diagnoses of the cases from which they were obtained. The data presented in this table merely show the type of cases we have studied. No conclusions regarding the degree of incidence of R pneumococcus in the sputum, either in cases of this type, or in normal individuals, have been drawn from such a limited number of observations.

Probably one of the most satisfactory methods for establishing the identity of a variant organism is to effect a reversion to a more easily recognizable form and subsequently to identify the resulting organism. We have been unable to take advantage of this method in these studies, however, for to date we have been unsuccessful in producing a reversion from R to S forms either with the control strains of R pneumococci or with the suspected strains of R pneumococci which have been obtained from the sputum cultures. Consequently other methods have been employed for the identification of the suspected strains. Assuming *a priori* that the suspected organisms might be classified either with the large group of so called *Streptococcus viridans*, or as true R pneumococci, we have used three methods, hitherto considered of value in differentiating rough pneumococci from streptococci, namely, those of determining (*a*) the degree to which the organisms are soluble in bile, (*b*) the degree to which they undergo autolysis in saline solution, and (*c*) their agglutination reactions with anti-R pneumococcus serum.

(*a*) *Bile Solubility.*—Although the lytic action of ox bile and sodium taurocholate solutions is widely employed as a useful method for differentiating pneumococci from *Streptococcus viridans*, it is recognized that some strains of pneumococci are more resistant to the action of bile than others. Reimann (8) has noted that R pneumococci are more

resistant to the lytic action of bile than S pneumococci. Occasionally one may encounter strains of S pneumococci which are similarly resistant and to emphasize this fact we have included such a strain among the S controls. We are not aware, however, that any of the recognized strains of *Streptococcus viridans* possess the property of being dissolved in ox bile or in the lower concentrations of sodium taurocholate solutions used in the following experiments.

TABLE II.

Viability of Strains after Exposure to Bile and Sodium Taurocholate Solutions.

Strain	Ox bile	Per cent of added sodium taurocholate solutions				Strain	Ox bile	Per cent of added sodium taurocholate solutions	
		2.5	5	10	20			2.5	5
S-I (1)	0	0	0			X-1	0	0	0
S-II (1)	++	0	0			X-2	0	0	0
S-II (2)	+	+	+	+	0	X-3	0	0	0
S-II (3)	0	0	0	0		X-4	0	0	0
S-III (1)	0	0	0			X-5	0	0	0
S-III (2)	0	0	0			X-6	0	0	0
S-III (3)	0	0	0			X-7	0	0	0
R-I	0	0	0			X-8	0	0	0
R-II	+	0	0			X-9	0	0	0
Strep. vir. (1)	+++	+++	+++	++	++	X-10	0	0	0
" " (2)	+++	+++	+++	++	++	X-11	0	0	0
						X-12	0	0	0

+++ = profuse growth.
++ = moderate growth.
+ = very few colonies.
0 = no growth.

Methods of Testing Bile Solubility.—The organisms from a 24 hour broth culture were taken up in a corresponding volume of salt solution. Equal parts of ox bile and solutions of sodium taurocholate in concentrations of 2.5, 5, 10, and 20 per cent were added to these suspensions and they were placed in the incubator for 2 hours at 37°. By culturing a loopful of suspension on rabbit blood agar, both at the beginning and at the end of the experiment, the viability of the organism after exposure to bile was determined.

Results.—The results are given in Table II. Some of the S strains showed evidence of being more or less resistant to the lytic action of

TABLE III.

Viability of Strains after Exposure to Saline Solution.

Strain	Hrs. of incubation in saline solution					Strain	Hrs. of incubation in saline solution				
	0	2	4	6	18		0	2	4	6	18
S-I (1)	+	0	0	0	0	X-1	+++	+++	+++	++	++
S-I (2)	+	0	0	0	0	X-2	+				+
S-I (3)					0	X-3	+	0	0	0	0
S-II (1)	+++	++	+	0	0	X-4	++	+	0	0	0
S-II (2)	+++	++	+	0	0	X-5	+++	+++	+++	+	0
S-II (3)	+++	0	0	0	0	X-6					0
S-III (1)	+++	++	++	++	0	X-7	+++	+++	+++	+++	0
S-III (2)	+++	++	0	0	0	X-8	+++	+++	+++	+++	0
S-III (3)	+++	++	++	++	0	X-9	+++	+++	+++	+++	0
R-I	+++	+++	+++	+++	0	X-10	+++	+++	+++	+++	0
R-II	+++	0	0	0	0	X-11	+++	+++	+++	+	0
Strep. vir. (2)	+++	+++	+++	+++	+++	X-12	+++	+++	+++	++	++
" " (3)	+++	+++	+++	+++	+++						
" " (5)					+++						

+++ = profuse growth.
++ = moderate growth.
+ = few colonies.
0 = no growth.

bile and sodium taurocholate solutions, but all were dissolved in the concentrations employed, although the strains of *Streptococcus viridans* survived in these same concentrations. One of the two control R strains proved to be slightly resistant to ox bile. About one-third of the colonies chosen from sputum cultures on the basis of their resemblance to the colonies of R pneumococci proved to be bile-soluble according to this method. These have been designated as X-1 to 12 and are shown in the right hand columns of the table.

(*b*) *Autolysis in Saline Solution.*—The readiness with which pneumococci, particularly the R forms, undergo autolysis when suspended in saline solution at 37°, as opposed to streptococci of all varieties which undergo autolysis very slowly, has also been emphasized by Reimann (8). We have used this method in an attempt to differentiate the two organisms. The results are given in Table III.

Methods.—The organisms from a 24 hour broth culture were taken up in a corresponding volume of physiological salt solution and placed in the incubator at 37°C. for a period of 18 hours. Cultures were made from these suspensions at stated intervals.

Results.—It will be noted that with all the control S and R strains of pneumococci the organisms had ceased to be viable at the end of 18 hours, whereas the *Streptococcus viridans* strains grew as readily at the end of 18 hours exposure to salt solution as at the beginning. Three of the X strains grew at the end of 18 hours while all the others underwent autolysis.

(*c*) *Immunological Reactions.*—Lancefield (10) has shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of the pneumococcus. Consequently serum produced by each *Streptococcus viridans* agglutinates all R pneumococci. On the other hand individual strains of *Streptococcus viridans* seem to possess a substance comparable to the soluble specific substance of the pneumococcus which masks or prevents agglutination of *Streptococcus viridans* by anti-R pneumococcus serum and in testing six strains of streptococcus with anti-R pneumococcus serum Reimann (8) was unable to get any evidence of agglutination. It is recognized, however, that anti-R pneumococcus serum will agglutinate and precipitate the protein from all R pneumococci, regardless of their original type (11).

In the light of these observations the theoretical question arises as to whether degraded forms of the streptococcus group which have lost the power of elaborating their soluble specific substance may not occur *in vivo*. If such is the case one might expect that "degraded streptococci" would be agglutinated by anti-R pneumococcus serum. Our experimental data have been reviewed critically in this light and we

TABLE IV.

Agglutinating Reactions with Anti-R Pneumococcus Serum.

Strain	Dilution of anti-R pneumococcus serum			Strain	Dilution of anti-R pneumococcus serum		
	1/10	1/20	1/40		1/10	1/20	1/40
S-I (1)	+++	++	±	X-1	0	0	0
S-I (2)	++	+	±	X-2	0	0	0
S-I (3)	+++	++	+	X-3	++	++	+
S-II (1)	+	±	0	X-4	0	0	0
S-II (2)	0	±	0	X-5	++	++	+
S-II (3)	0	0	0	X-6	0	0	0
S-III (1)	+	+	±	X-7	0	0	0
S-III (2)	0	0	0	X-8	0	0	0
S-III (3)	+	±	0	X-9	+	0	0
R-I	++++	+++	++	X-10	0	0	0
R-II	+++	+++	+++	X-11	±	0	0
Strep. vir. (2)	0	0	0	X-12	±	0	0
" " (3)	0	0	0				
" *hæm.*	0	0	0				

++++ = firm disc.
+++ = disc easily broken up.
++ = coarse agglutination.
+ = fine agglutination.
± = faint agglutination.
0 = no agglutination.

have consequently not accepted as fact that any methemoglobin-producing diplococcus which is agglutinated in appropriate dilutions by anti-R pneumococcus serum is necessarily an R pneumococcus. We have assumed, however, that such agglutination reactions are of relative importance if taken in conjunction with other findings.

Methods.—The anti-R pneumococcus serum was prepared according to the principles of Cole and Moore (12). Bacteria from 18 hour broth cultures of two

strains of R pneumococci, R-I and R-II, were suspended in salt solution and killed at 56°C. Rabbits were inoculated with this suspension, a preliminary series of subcutaneous injections being followed by two series of six intravenous injections each.

The agglutinating reactions were run in duplicate upon saline suspensions of washed bacteria. Readings were made after incubation at 37°C. for 5 hours and again after 12 hours in the ice box.

Results.—The results are shown in Table IV. It will be noted that several of the S strains gave agglutination reactions in moderately high dilutions of the anti-R serum. These results are not altogether in accord with those of Reimann (11) but the degree of dissociation which may have occurred in these strains when suspended in saline solution has been an uncontrolled variable in our hands. No agglutination was obtained with the three control strains of streptococcus.

TABLE V.

Reactions Exhibited by Suspected R Pneumococcus Strains.

Strain	1	2	3	4	5	6	7	8	9	10	11	12
Bile solubility	+	+	+	+	+	+	+	+	+	+	+	+
Saline autolysis	0	0	+	+	+	+	+	+	+	+	+	0
Agglutination	0	0	+	0	+	0	0	0	0	0	0	0

+ = reaction typical for R pneumococci.
0 = reaction atypical for R pneumococci.

Of the X strains only two gave agglutinating reactions which are comparable to those of true R pneumococci.

A summary of the findings exhibited by these organisms is given in Table V.

Of the twelve strains of suspected R pneumococci obtained from sputum cultures only two consistently exhibit reactions which are typical for R pneumococci. No attempt has been made to explain the atypical reactions nor to classify the ten strains which have exhibited such reactions. We do not feel, however, that we have as yet evidence to say that any of these ten strains may be considered as true R pneumococci although it is not unlikely that they may be intermediate or related forms. We have felt, on the other hand, that the two

remaining strains may be justifiably classified, on the basis of the three reactions given above, as R pneumococci.

The evidence suggests that R pneumococci may be demonstrated in the sputum of patients suffering from pneumonia but these forms do not seem to be very common if we consider that on the basis of colony differentiation about forty strains were originally selected and but two of these were identified as true R pneumococci.

SUMMARY.

In a survey of about forty rough methemoglobin-producing colonies in sputum cultures from a series of individuals suffering from respiratory infections, twelve bile-soluble strains of suspected R pneumococci have been isolated for study. Two of these twelve strains have shown both autolysis in saline solution and serological reactions characteristic of R pneumococci.

The findings offer evidence that R pneumococci may occasionally occur in human sputum. Their significance as regards the epidemiology of pneumococcus infections and of host response is alluded to.

In conclusion the author wishes to express his appreciation to Miss Margaret McClintock for her assistance in the technical work of this study.

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