STUDIES CONCERNING THE RELATIONSHIP BETWEEN PNEUMOCOCCI AND STREPTOCOCCI.

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From time to time evidence has been presented that a close relationship exists between *Pneumococcus* and *Streptococcus*. Kruse and Pansini (1), observing the variability of *Pneumococcus* when grown under different conditions, drew attention to the resemblance between certain races of *Pneumococcus* and *Streptococcus* and even suggested the possibility that these bacteria might have their origin in a common, probably saprophytic form of streptococci. Since then a number of observers have brought forth evidence indicating that *Streptococcus hæmolyticus*, *Streptococcus viridans*, *Streptococcus mucosus* and *Pneumococcus* are so closely related that under certain conditions one may change into the other. The evidence for this point of view has recently been greatly supported by the series of publications of Morgenroth and his collaborators and assistants.

Morgenroth, Schnitzer and Berger (2) have reported that by special methods it was possible to transform with great regularity pneumococci into streptococci.

The methods employed were the following: *Pneumococci* were cultivated in medium containing dead yeast cells or animal charcoal which had absorbed optochin. Under these conditions the pneumococci underwent modifications and were transformed into cocci which possessed properties held to be characteristic for *Streptococcus viridans*. The organisms were now insoluble in solutions of sodium taurocholate, were avirulent for mice, were resistant to the action of optochin and were more sensitive to the action of rivanol than was the original strain. According to these writers, this transformation of pneumococci into streptococci did not occur suddenly but in undergoing the transformation the bacteria passed through several stages or modifications.

Bacteria in the first stage of the process were described as Modification A. While the bacteria in this stage retained most of the characteristic properties of pneumococci, they had become more resistant to the action of optochin. When blood agar plates were made from the Modification A culture, after growing in optochin, it was found that the colonies no longer resembled those of the original culture but now resembled the colonies of *Streptococcus viridans*. Microscopically the organisms appeared as long chains of round individuals; they were bile-insoluble and were only killed by very high concentrations of optochin. This form was called Modification B and was considered to be identical with *Streptococcus viridans*. Upon further growth in artificial medium, or in the animal, and occasionally upon growth in optochin-containing medium, the third form, Modification C, was found to arise. These organisms corresponded in their properties to *Streptococcus hæmolyticus*, that is, they grew on blood agar with more or less hemolysis, were bile-insoluble and optochin-fast. When injected into the subcutaneous tissue of the abdomen of the mouse phlegmons formed. Moreover, these organisms showed the sensitiveness to the action of rivanol which is characteristic for *Streptococcus hæmolyticus*. Occasionally the transition of Modification A into Modification C occurred without passing through the intermediate stage of Modification B.

These observations concerning the changes undergone by pneumococci and streptococci are of great importance not only in their bearing on the classification of these microorganisms, but also in their relation to the pathogenesis of human infection. It has seemed of importance, therefore, to repeat those experiments and to attempt to interpret them in the light of studies made by the writer (3, 4) and others concerning the variations in the characteristics of Pneumococcus which occur under a number of conditions.

It has been found by a number of observers that when pneumococci are grown in various unusual ways, such as in immune serum or in bile, atypical colonies may appear. These have been called R, or rough, colonies in contrast with the S, or smooth, colonies which are the typical pneumococcus colonies. The bacteria of the R colonies are avirulent for mice, have no capsule, do not produce the so called pneumococcus soluble substance, are somewhat resistant to the action of bile and have lost their type specificity. They have been found to retain their properties on further cultivation and even after repeated passage through mice. Blake and Trask (5) have described a third type of colony, probably made up of intermediate forms, the bacteria of which may revert to the typical virulent pneumococci on passage

through mice. The R bacteria although to some degree resembling streptococci of the *viridans* variety have nevertheless been considered by the writer and by most of the workers on this problem to be modified pneumococci.

Methods.

In studying the modifications undergone by pneumococci when exposed to the action of optochin or optochin absorbed on yeast or on animal charcoal, the following methods were employed.

A strain of *Pneumococcus* derived from a single cell of a Type I stock culture was employed. 0.05 cc. of a 1-10 dilution of an 18 hour broth culture of this organism was inoculated into serum bouillon containing varying concentrations of optochin hydrochloride (ethyl hydrocupreine hydrochloride, Zimmer and Co.) or into serum broth containing varying concentrations of optochin absorbed on yeast. These cultures were incubated from 18 to 20 hours and plated on blood agar to detect colony variations. Yeast-absorbed optochin was prepared as follows: A 10 per cent suspension of dried yeast (Fleischmann) in distilled water was made and this was sterilized by heat and then centrifuged. The supernatant fluid was replaced with a 1-10,000 solution of optochin hydrochloride in distilled water and the mixture incubated $\frac{1}{2}$ hour. The suspension was again centrifuged, the supernatant fluid discarded and replaced by twice the amount of 10 per cent serum broth.

For testing bile solubility, 0.5 cc. of sterile, undiluted ox bile was added to 0.5 cc. of a broth suspension of bacteria and incubated for 2 hours at 37°C.

Experimental.

An 18 hour broth culture of *Pneumococcus* was inoculated into broth containing a suspension of yeast-absorbed optochin as described above and incubated 20 hours. After plating on blood agar only one kind of colony appeared and this resembled exactly the S form of colony which has been described in a previous paper (3). The original culture was now passed two more times through the yeast-absorbed optochin medium. From this third culture, plates were made and now a number of small, thin, rough surfaced colonies, like those which have previously been described as R forms, appeared. A study was next made of the changes undergone by pneumococci when grown in broth containing various amounts of optochin without any yeast being present, and also when grown in suspensions of yeast containing no optochin. The culture was grown repeatedly in serum broth containing optochin hydrochloride in concentration of 1-100,000. At the ninth transfer the concentration was increased to 1-50,000 and at the thirtieth transfer, to 1-20,000. Upon plating the culture at intervals during the repeated transfers, no small R colonies appeared. This last experiment was repeated, substituting plain broth for serum broth. Three transfers were made in this medium con-

taining optochin hydrochloride in a concentration of 1-600,000. When this third culture was plated on blood agar, three kinds of colonies were found, S colonies, R colonies and colonies of an intermediate form like those described by Blake and Trask.

The culture was also grown repeatedly in a 10 per cent suspension of heat-killed yeast in serum broth, no optochin being present. Transfers were made at 6 hour intervals on account of the tendency of the organisms to die in this medium. After the fourth transfer in this medium, plating on blood agar showed a great preponderance of colonies of the R form over those of the S forms.

These experiments, which have been repeated a number of times, indicate that in our hands the result of growing typical pneumococci in media containing yeast which has absorbed optochin, or even in media containing optochin alone or yeast alone, is the appearance of modified pneumococci which form colonies identical with those described by a number of writers as R colonies. From the description of the colonies of Modification B as given by Morgenroth and his associates, it seems probable that the bacteria of Modification B are identical with those we and others have described as pneumococci of the R colonies.

Morgenroth and his collaborators consider these to be true streptococci of the *viridans* type. We have called them modified pneumococci. It is true, as Morgenroth and his collaborators state, that these colonies resemble those of *Streptococcus viridans*. Moreover, the bacteria from these colonies have little virulence for mice and they are resistant to optochin and do not dissolve as readily as do typical pneumococci in solutions of bile. The writers we have mentioned state that these organisms are insoluble in solutions of sodium taurocholate and they lay stress on this property as indicating their identity with the bacteria of the *Streptococcus viridans* group. In carrying out the bile test, however, Morgenroth and his associates have employed the method of Levy (6), adding a 2.5 per cent solution of sodium taurocholate to the broth culture. We have modified this technic, employing not only a 2.5 per cent solution of this substance, but also solutions of a higher concentration. The effect of ox bile itself on these organisms has also been tested. Many tests of R cultures have been made, and at the same time tests have been made of twelve different cultures from various sources which had previously been identified by another worker as cultures of *Streptococcus viridans*.

The latter cultures were all found to be insoluble in ox bile and also in sodium taurocholate solutions, even when this was highly concentrated. On the other hand, when the bacteria of the R colonies or what Morgenroth and his associates have called Modification B bacteria, were tested, it has been found that although they are resistant to the action of sodium taurocholate in 2.5 per cent solution, they are dissolved if higher concentrations of this salt be employed. Moreover, if the bacteria be suspended in salt solution instead of in broth, solution occurs even in 2.5 per cent solutions of sodium taurocholate. Finally, if ox bile be employed, these organisms all dissolve, although more slowly than usually occurs with typical pneumococci. Our experiments, therefore, indicate that these modified bacteria are not, properly speaking, bile-insoluble; they possess increased resistance to the action of bile. They, therefore, differ in this respect from bacteria of the *Streptococcus viridans* group which are truly bile-insoluble. They also differ from *Streptococcus viridans* in certain other properties. We have previously noted the readiness with which pneumococci undergo autolysis when they are suspended in saline solutions and kept at 37°C. This readiness to undergo autolysis is seen in pneumococci of all types and is especially well marked in bacteria from the R colonies. On the other hand, streptococci of all varieties undergo autolysis very slowly.

Saline suspensions of six strains each of the S and R forms of pneumococci and of ten strains of typical *Streptococcus viridans* were made and placed in the incubator at 37°C. for 48 hours. At the end of this time the tubes were centrifuged and stained films were prepared from the sediment of each tube. It was found that well formed, Gram-positive cocci were still present in all the tubes containing streptococci, while in the tubes containing the S and R forms of pneumococci no intact cells remained.

We have reported elsewhere (7) that freezing and thawing easily causes disruption of the cell bodies of pneumococci, while this treatment has no effect on streptococci. Pneumococci of the R form obtained by growth in serum and in bile and also modified pneumococci obtained by growth in media containing optochin absorbed on yeast have been tested by these methods. In every instance all these

bacteria have been found to be readily disintegrated by the action of freezing and thawing.

Finally, the immunological properties of atypical pneumococci derived from type-specific strains by growth in broth containing yeast-absorbed optochin were compared with the immunological properties of typical streptococci of the *viridans* group.

We have previously reported observations concerning the immunological reactions of the R forms of pneumococci (4). It was there shown that sera prepared with each of these strains agglutinated all other R forms, no matter how obtained or from what type of *Pneumococcus*. These sera also caused precipitation of the protein derived from all kinds of pneumococci. The interpretation placed on these experiments was that the R forms of pneumococci were pneumococci which had lost the property of producing the pneumococcus type-specific carbohydrate substance.

Immune sera were now prepared by injecting rabbits with cultures of *Streptococcus viridans*. Six different strains were employed, an immune serum being produced against each one of these strains. These special strains were selected because they grew diffusely in broth. Each of these sera was found to be specific for the homologous organism, causing agglutination usually in dilutions as high as 1-320. In the concentrated serum there occurred some crossing or non-specific agglutination of the other *Streptococcus viridans* strains. All but one of the sera also agglutinated suspensions of R pneumococci but the agglutinating power for these organisms was not as great as that of the serum prepared by the injection of R pneumococci. By absorption of the agglutinins for the R strains by R organisms the agglutinins of the antistreptococcus sera for heterologous *Streptococcus viridans* strains were removed and the absorbed sera were now race-specific and agglutinated only the homologous race of *Streptococcus viridans*. Anti-R pneumococcus sera, on the other hand, did not agglutinate any of the six strains of *Streptococcus viridans* studied.

The following interpretation of these results, in the light of the work of Heidelberger and Avery (8) seems justifiable. Lancefield (9) has previously shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of *Pneumococcus*. Individual strains of *Streptococcus viridans* also possess a substance

comparable to that of the soluble specific substance of *Pneumococcus* and this endows them with the power of reacting specifically with their homologous serum. To what degree a single specific substance is common to a number of *Streptococcus viridans* strains, only further work will show. The protein of all strains being alike and being common to all pneumococci, the serum produced by each *Streptococcus viridans* strain agglutinates all R pneumococci. The anti-R serum, however, does not agglutinate the *Streptococcus viridans* strain because each of these strains has a specific reacting substance, which masks or prevents the non-specific antiprotein reaction. This is analogous to the immunological phenomena exhibited by the specific pneumococci. These organisms, although they contain the non-specific pneumococcus protein, are not agglutinated by anti-R serum or by anti pneumococcus-protein serum. The explanation offered for the immunological phenomena exhibited by strains of the *Streptococcus viridans* group also receives support from the work of Zinsser on hemolytic streptococci (10).

It seems from all of this work that the atypical forms of bacteria obtained by growing type-specific strains of pneumococci in media containing serum or bile or by growing them in dilutions of optochin, or in yeast-absorbed optochin according to the method of Morgenroth, are not strains of *Streptococcus viridans* but are in fact degraded forms of pneumococci.

The reasons we have adduced for this are as follows:

1. Degraded forms of pneumococci are not bile-insoluble, as Morgenroth and his associates have stated, although they are more resistant to bile than are typical pneumococci.
2. Degraded forms of pneumococci behave like typical pneumococci and not like streptococci in the readiness with which they undergo autolysis and in the ease with which the cell bodies are broken down by freezing and thawing.
3. Immune sera produced by the injection of animals with degraded strains of pneumococci behave toward typical strains of *Streptococcus viridans* just as they do toward specific strains of *Pneumococcus*. Typical strains of *Streptococcus viridans* possess a type-specific immunological mechanism just as do typical pneumococci. The species-specific immunological mechanism possessed by *Streptococcus viridans*

is identical with that of *Pneumococcus*. To this extent *Pneumococcus* and *Streptococcus viridans* are closely related. Whether a typical type-specific strain of *Pneumococcus*, however, is ever changed into one of *Streptococcus viridans* any more than a *Pneumococcus* of Type I is ever changed into one of Type II or of Type III only further work will show. The evidence for this at the present time is not convincing.

Experiments with Hemolytic Streptococci.

Morgenroth and his associates (11) also report that they have been able, by special methods, to convert strains of *Streptococcus hæmolyticus* into those of *Streptococcus viridans*. This change was easily induced by cultivating hemolytic streptococci in serum broth containing rivanol bound to erythrocytes, "haut-pulver," animal charcoal or yeast. Rivanol solutions alone in serum broth were not efficacious. Rivanol (2-ethoxy-6, 9-diaminoacridium hydrochloride) is a salt which is said to have a specific germicidal action for *Streptococcus hæmolyticus*.

Other investigators have also observed hemolytic streptococci change into non-hemolytic forms. Valentine and Krumwiede (12) on one occasion observed the spontaneous appearance of occasional green colonies on a blood agar plate made from a culture of *Streptococcus hæmolyticus* (Strain 32, Dochez, Avery and Lancefield (13)). Bacteria from both kinds of colonies were agglutinable in the original strain antiserum and each absorbed the common antibody. Lehmann (14) obtained green-producing colonies from 3 out of 87 strains of hemolytic streptococci which had been cultivated in milk media.

On account of the bearing which these observations concerning streptococci have on the preceding experiments, it seemed of interest to repeat the experiments of Morgenroth and his associates.

Methods.

A 10 per cent suspension of dry yeast in distilled water was sterilized and centrifuged as described previously. The supernatant fluid was replaced with a 1-1,000 solution of rivanol (Metz) in distilled water and incubated for 1 hour at 37°C. After centrifuging, the sediment was washed three times with distilled water and made up to the original volume. Varying dilutions of this suspension were made in 10 per cent serum broth. 0.05 cc. of a 1-10 dilution of a broth culture of *Streptococcus hæmolyticus* was inoculated into 2 cc. of the respective dilutions of yeast-absorbed rivanol and incubated at 37°C. for 20 hours.

In order to obtain pure line cultures, in certain of the experiments single chains of 3 or 4 cocci were isolated according to the technic described in a previous paper (3). Single cocci are difficult to isolate on account of their small size and resemblance to particulate matter in the agar.

Experimental.

Twenty-six strains of hemolytic streptococci, including stock cultures and freshly isolated strains, were tested. Bacteria were inoculated in serum broth containing rivanol alone and in serum broth containing yeast alone, but in most instances in dilutions of rivanol-yeast suspensions varying in strength from 1-4 to 1-8,000. The organisms tended to die in the stronger concentrations and for prolonged growth required alternate transfers into plain serum broth. Cultures could be transferred indefinitely in dilutions weaker than 1-1,000.

After each transfer the organisms were plated on blood agar, incubated at 37°C. and the plates examined for the presence of atypical colonies. Approximately, 400 transfers were made in the various media and only on the plates made from one of the strains (*Streptococcus* 267) were atypical colonies seen. The variant colonies which occasionally appeared on the blood agar plates among the hemolytic colonies of this strain were anhemolytic, and the organisms derived therefrom were avirulent and bred true on further culture. However, atypical colonies appeared a number of times spontaneously when this culture, without any previous treatment with yeast-absorbed rivanol, was plated on blood agar.

To eliminate the possibility of the variant being a contaminant, 20 single short chains were isolated from the original culture and treated with yeast-absorbed rivanol in dilutions varying from 1-1,000 to 1-8,000. Under these conditions no variant colonies appeared. This experiment does not entirely prove that the variants were contaminants but this explanation seems not improbable.

Two of the original cultures, S3 and S23, were transferred 70 times on plain agar before treatment with yeast-absorbed rivanol. In spite of this preliminary treatment no variants ever appeared.

SUMMARY AND CONCLUSIONS.

Morgenroth and his collaborators grew pneumococci in a medium containing optochin and derived variant forms of bacteria therefrom which were considered to be streptococci of the *viridans* group. We have repeated these experiments and have also derived variant forms. These atypical races, however, we have found to be identical with the R form of pneumococci which have been obtained by various other methods. That these R cultures are still pneumococci and do not belong in the *Streptococcus viridans* group is supported by the following observations:

1. These strains are not bile-insoluble, but are more resistant to the lytic action of this agent than are type-specific pneumococci.

2. R strains behave like pneumococci and not like streptococci in the readiness with which cultures spontaneously autolyze and saline suspensions disintegrate during freezing and thawing.

3. Immunological reactions of the variant pneumococci derived by Morgenroth's method are identical with the immunological reactions of R forms of pneumococci derived by various other means.

The observations of Morgenroth and his associates in regard to the transformation of *Streptococcus hæmolyticus* into *Streptococcus viridans* by treatment with rivanol could not be repeated in this laboratory. No explanation is at hand for our failure to produce the change. It is of course possible that none of the twenty-six strains tested had suitable tendencies to variation or that the technic or reagents employed varied from those of Morgenroth.

BIBLIOGRAPHY.

1. Kruse, W., and Pansini, S., *Z. Hyg. u. Infektionskrankh.*, 1892, xi, 279.
2. Morgenroth, J., Schnitzer, R., and Berger, E., *Z. Immunitätsforsch.*, 1925, xliii, 169.
3. Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587.
4. Reimann, H. A., *J. Exp. Med.*, 1926, xliii, 107.
5. Blake, F. G., and Trask, J. D., Jr., *Abstr. Bact.*, 1923, vii, 353.
6. Levy, R., *Virchows Arch. path. Anat.*, 1907, clxxxvii, 327.
7. Julianelle, L. A., and Reimann, H. A., *J. Exp. Med.*, 1926, xliii, 87.
8. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 301.
9. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 377.
10. Zinsser, H., and Tamiya, T., *J. Exp. Med.*, 1925, xlii, 311.
11. Morgenroth, J., Schnitzer, R., and Berger, E., *Z. Immunitätsforsch.*, 1925, xliii, 209.
12. Valentine, E., and Krumwiede, C., *J. Exp. Med.*, 1922, xxxvi, 157.
13. Dochez, A. R., Avery, O. T., and Lancefield, R. C., *J. Exp. Med.*, 1919, xxx, 179.
14. Lehmann, W., *Deutsch. Arch. klin. Med.*, 1926, cl, 127.