THE OCCURRENCE OF DEGRADED PNEUMOCOCCI IN VIVO.

BY HOBART A. REIMANN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, January 12, 1927.)

It has been well demonstrated that under certain experimental conditions in vitro pneumococci undergo variations. It is of great importance to determine whether variants ever appear in vivo and if so, under what conditions. This is especially important since several investigations have suggested that recovery from pneumococcus infection may depend largely upon a change of virulent pneumococci into avirulent and phagocytatable forms. This paper describes attempts to determine the experimental conditions favoring a change of virulent (S) pneumococci into avirulent (R) forms within the animal body.

It has been repeatedly shown (1–3) that by subjecting type-specific (S) pneumococcus strains to various unfavorable cultural environments in vitro, certain biological characteristics of the bacteria are profoundly affected. The changed bacteria no longer elaborate the specific carbohydrate element upon which type specificity depends and they are therefore agglutinable in heterologous as well as homologous antipneumococcus serum (4), they are no longer virulent for mice, and they are easily phagocyted (5, 6). These organisms have been designated R pneumococci by Griffith on account of the rough surface of the colonies which they form when grown on solid media, in contradistinction to the smooth surfaced colonies formed by S or typical virulent pneumococci. This difference renders the two forms distinguishable microscopically and often macroscopically when colonies of both forms are present on the same blood agar plate.

An intermediate form which is somewhat less virulent than the original S strain and also possesses slight serological differences has also been described (7). By animal passage it is possible to restore the original virulence and type specificity of this form. On the other hand, by placing it under unfavorable cultural environment the intermediate
form is easily converted into the R form. It would seem that the intermediate organisms represent a transitional stage between the S and the R forms.

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. But in case the change from S to R does take place in vivo it seems probable that R forms would be phagocyted and destroyed as soon as they appear. Therefore, to determine the presence of the R forms in vivo, it would probably be necessary to employ a technic under which the infection would remain localized over a considerable period of time without killing the animal, and under which the free access of phagocytes to the bacteria would be inhibited. It was thought that these conditions might be obtained by embedding subcutaneously in the experimental animal an agar mass inoculated with a virulent strain of Pneumococcus.

Method.—15 cc. of melted nutrient agar was inoculated with 0.5 cc. of a young broth culture of Pneumococcus. A wire loop about 4 cm. in diameter was pressed firmly over a shaved area of skin of the anesthetized animal and the fluid agar injected subcutaneously into the area enclosed by the loop. Ice was applied until the agar had solidified into a firm hemispherical nodule. Observations were made from time to time by puncturing the focus with a hypodermic needle, aspirating some of the material, and plating it on blood agar plates. The plates were examined for S, R, and intermediate colonies after 12-18 hours incubation.

Experimental.

Agar Foci in Dogs.—Dogs were first employed since these animals have a natural relative immunity to Pneumococcus, and were likely to withstand infection over a considerable period of time. Two dogs were injected with agar containing Type I pneumococci according to the method described. Material was aspirated from the foci at intervals from the 1st to the 14th day after injection. But R forms never appeared on the culture plates made from the material at any time, although S forms were constantly present in large numbers. The dogs soon became ill and S pneumococci were recovered from the blood stream on several occasions. In a few days abscesses formed at the site of injection in both animals and by the 10th day the skin
over the foci ruptured and thick pus was discharged. About the 14th day the lesions began to heal, the pneumococci disappeared and the dogs recovered. After a rest period of 2 weeks the same dogs, which were now assumed to have an additional degree of immunity, although specific agglutinins were not demonstrable in the blood serum, were reinjected with infected agar. A repetition of the previous events occurred. The dogs recovered and no R forms were encountered either from the foci or from the blood stream. While it was impossible with this technic to demonstrate the appearance of R forms in dogs, it should be mentioned that 11 years ago Bull (8) discovered that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. In one dog which lived for 10 days before dying with meningitis, pneumococci isolated from the blood on the 9th day "grew in chains and were non-virulent."

Agar Foci in Rabbits.—The same technic was then employed with rabbits instead of dogs, and instead of using a Type I strain, a Type III strain was employed. This type was used because rabbits have a high degree of resistance to Type III pneumococci, in contrast to their susceptibility to Types I and II, and it was thought that animals infected with this type would live longer and the bacteria would therefore have a greater chance to undergo variations. The special strains employed have been shown to readily undergo variations in vitro. Three rabbits were used. Daily examination of the foci by aspirating and plating material from the foci on blood agar plates showed that only S forms were present. The number of pneumococci gradually diminished until no more were demonstrable by the 6th day after injection.

Agar Foci in Guinea Pigs.—In a similar manner six guinea pigs, actively immunized against Type I pneumococci, were injected with agar containing Type I pneumococci. Repeated cultures from the foci showed the presence of S forms alone until the 5th day after injection when eight R colonies were found on a blood agar plate seeded from the focus of one guinea pig. R colonies were subsequently recovered from the foci of the other five guinea pigs at intervals up to 6 weeks after injection. In all instances however, the S colonies greatly outnumbered the R forms and were recovered from the foci for 6 weeks, or until the agar was absorbed. Normal (unimmunized)
DEGRADED PNEUMOCOCCI IN VIVO

Guinea pigs were also tested but died from pneumococcus septicemia too soon to be suitable for the experiment.

In spite of the mass of agar surrounding the pneumococci in the subcutaneous foci, the object of preventing the access of phagocytes was not attained. Stained films made from the aspirated agar showed the constant presence throughout the agar mass of many phagocytes containing pneumococci.

As mentioned above, it was assumed that the R forms, being easily phagocyted, were rapidly destroyed or removed from the site of infection. To test this assumption, agar masses inoculated with a heavy suspension of R pneumococci were injected subcutaneously into two guinea pigs. It was found that the R forms were recoverable for 5 weeks after injection or until the foci had been completely absorbed. Although microscopic examination revealed the presence of great numbers of phagocytes, they apparently had but little influence in removing the R forms as long as the focus remained.

In order to eliminate the activity of phagocytes entirely, the technic was then modified as follows: Agar, which was inoculated with S pneumococci, was enclosed in a glass vial sealed with a collodion membrane. The membrane was an effective barrier against phagocytes but permitted the diffusion of fluids.

**Method.**—5 cc. wide mouth vials were filled, nearly to the top, with melted agar and the mouths were covered with thin muslin caps. After sterilization the agar was inoculated with 0.1 cc. of pneumococcus broth culture by means of a long hypodermic needle, and the muslin-covered mouths were dipped in a thin solution of collodion. The vials were then immersed in 95 per cent alcohol for several minutes to render the membrane more permeable. The sealed vials were embedded subcutaneously in rabbits. Repeated observations were made by inserting a long hypodermic needle through the skin of the animal and the membrane of the vial, aspirating some of the agar, and plating it on blood agar.

Control vials were prepared in the same manner but, instead of being placed *in vivo*, they were immersed in normal rabbit serum in large test-tubes. The tubes were kept in the incubator at 39°C, which was considered to be the average temperature of the vials while embedded in the rabbits. The serum was changed weekly.

Vials containing agar inoculated with cultures derived from a single diplococcus of a Type I strain were inserted subcutaneously in eight normal and three passively immunized rabbits and allowed to remain.
for several weeks. Eight vials similarly prepared were immersed in normal serum and kept in the incubator as controls. Agar was aspirated from the vials and plated on blood agar at intervals of from 1 to 5 days. Several of the normal (unimmunized) rabbits soon died from pneumococcus septicemia due to infection resulting from leakage of the vials after aspiration. The unimmunized rabbits which survived were observed over a period of 8 weeks. Their serum did not show the presence of agglutinins for Type I pneumococci even at the end of this time.

R colonies were invariably found on plates made from all of the vials embedded in rabbits. They appeared, often with the intermediate forms, as early as the 3rd day after inoculation and were recoverable up to 8 weeks. The variant colonies were usually outnumbered by the S forms and in no instance were the S forms entirely replaced by them. It was rather surprising to find that both R and intermediate colonies were derived from the agar vials in normal and immunized animals alike.

The eight control vials inoculated at the same time with the same culture of Type I pneumococci were kept in the incubator. R colonies never appeared on plates made from these vials although the S forms remained during the period of observation.

Experiment with Type III Pneumococcus.—Three agar vials were inoculated with a Type III pneumococcus culture obtained from a single diplococcus from the blood culture of a pneumonia patient, and embedded subcutaneously in three rabbits. Frequent plating of the agar at intervals during a period of 5 weeks never revealed the presence of R or intermediate forms although S forms remained viable throughout.

This culture proved to be a strain especially refractory to modification as was determined by subjecting it to treatment in vitro by methods which invariably caused the appearance of R forms in other strains. Frequent search during 40 transfers in broth containing 5 per cent Type III antipneumococcus serum, or during 11 transfers in broth containing optochin yeast (9), or during 53 transfers on blood agar plates, did not reveal the presence of any R or intermediate forms. The culture remained unchanged in virulence and type specificity. Takami (10) has also encountered resistant strains of this
nature. He found that, of 28 strains, 12 never gave rise to variant forms even after prolonged subculture on blood agar.

Spontaneous Appearance of R Forms in Vivo.—Aside from the experimental production of the variant forms it is of interest to report the spontaneous occurrence of R forms in vivo. Through the courtesy of Dr. Mary B. Kirkbride of the New York State Department of Health, it was learned that atypical pneumococci were occasionally recovered from the blood stream of horses during the process of immunization with live virulent cultures for the production of anti-pneumococcus serum. The atypical strains were recovered from 6 to 16 months after the horses were first inoculated with a Type I pneumococcus culture. During this time the horses had a low grade fever, anorexia, loss of weight, rapid irregular pulse, heart murmurs, swelling and stiffness of the joints. The animals finally died and autopsy revealed an endocarditis present in all. Atypical pneumococcus strains from four of these horses were obtained and examined in this laboratory. They proved to have many characteristics in common with the variant or R strains previously studied. Each of the four strains produced colonies which differed slightly morphologically from one another and were distinguishable on blood agar plates. It was previously noted that in a number of instances, R colonies differing from one another in appearance were also recovered from the agar vials embedded in rabbits. The four horse R strains together with four strains derived from R colonies from the rabbit vials were tested for variations in virulence and agglutinability. All strains failed to kill mice in doses of 1 cc. of a young broth culture, were bile-soluble, and were serologically alike. The morphological colony differences between the various strains persisted even after repeated plating. Repeated (six) animal passages of the R strains failed to restore either virulence or specificity.

DISCUSSION AND CONCLUSION.

It is conceivable that a change from the virulent, non-phagocytale S form of Pneumococcus to the avirulent phagocytale R form may take place in pneumococcus disease, but the experiments here reported do not settle the question whether or not this is an important factor in determining the outcome in natural infection. It has been shown
experimentally that the degradation from the S form to the R form actually does take place in cultures of Pneumococcus growing in agar subcutaneously embedded in guinea pigs, in agar enclosed in vials subcutaneously embedded in rabbits, and spontaneously in the blood stream of infected horses. However, it was not possible in any of the experiments here cited to demonstrate the complete change from S to R pneumococci before the bacteria disappeared from the body. When the intermediate or R forms did appear, they were always accompanied and usually exceeded in number by the S forms and all three forms disappeared together. S organisms may disappear entirely without evidence of first going through the intermediate and R stages. On the other hand, contrary to expectations, pure cultures of R forms remained viable in subcutaneous foci for weeks although apparently freely accessible to the action of phagocytes. It seems of some significance that the R forms appeared early in the vials (inoculated with S pneumococci) in immunized and normal rabbits alike, indicating that the presence of demonstrable specific immune bodies was not alone responsible for the variation of the bacteria.

Of some importance also is the fact that R forms were never derived from similarly prepared control cultures growing in vitro at the same temperature and immersed in normal serum, although the S forms remained viable and unaltered for 6 weeks. It is likely that variations of pneumococci do not occur readily when S cultures are exposed to normal serum in vitro, especially when growing in closed vials under a diminished oxygen supply, for it has previously been shown (2) that only slight variation occurs even after prolonged (240) transfers in heterologous serum broth in the test-tube. It is possible, therefore, that the variation which occurred among pneumococci growing in agar vials embedded in normal rabbits was actually provoked by unknown influences in the living tissue fluids.

Although R forms have been shown to occur in vivo, no positive evidence can be derived from these experiments to prove that recovery from pneumococcus infection depends upon the degradation of the virulent S forms of pneumococci to the avirulent R forms and the subsequent destruction of the latter by phagocytes.
BIBLIOGRAPHY.