EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VII. Serological Reactions.

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Plate 37.

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During the fall and winter of 1918–19, and the early spring of 1920, we collected a number of specimens of blood serum from patients in the active stages of epidemic influenza, or after recovery from the disease, for the purpose of studying the reactions of the sera with strains of *Bacterium pneumosintes*[1] which had been isolated from the nasopharyngeal secretions of influenza patients and from the lung tissues of rabbits inoculated with these secretions. Samples of blood serum were collected also from rabbits which had been allowed to recover after showing the characteristic clinical picture produced by intratracheal injections of the active nasopharyngeal secretions, or had been experimentally inoculated with the sediment from tissue cultures of *Bacterium pneumosintes*.

The results of our efforts to demonstrate specific antibodies in these serum specimens were disappointing. The sparse growths of the organism in the earlier generations, mixed with the protein precipitate that develops in the Smith-Noguchi medium, did not provide an antigen suitable for serological tests. On account of non-specific precipitation and concomitant sedimentation, precipitin and agglutinin tests were unsatisfactory and indeterminate. Therefore, at the time the sera of influenza patients and of most of the affected rabbits were available we were unable to make use of them for lack of a suitable antigen.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

More recently a method has been developed by one of us (Gates) by which certain pathogenic anaerobes, including *Bacterium pneumosintes*, may be cultivated in a collodion sac dialysate of the Smith-Noguchi medium.[2] The ascitic fluid or dilute serum and the fresh tissue fragment are placed within the sac, which is surrounded by distilled water or physiological salt solution under a vaseline seal. In the course of 24 to 48 hours anaerobic conditions are established throughout the system and the nutritive and growth-promoting substances in the tissue medium have diffused through the membrane in sufficient quantities to support growth in the surrounding liquid, while the protein precipitate that collects around the tissue fragment is retained within the sac.

Bacterium pneumosintes grows readily in this anaerobic tissue culture dialysate, visibly clouding the clear liquid in a few hours and producing a heavy turbidity in 3 to 5 days. When growth is checked through exhaustion of the nutritive material or the accumulation of deleterious substances the organisms gradually settle out of suspension, leaving a clear supernatant fluid over a compact, slightly brownish sediment. Films of these cultures show only the stained organisms, without the background of precipitate that is deposited by the tissue medium.

Preparation of Immune Serum.

When it was possible to cultivate *Bacterium pneumosintes* by this method in quantities sufficient for use, two rabbits were injected intravenously with doses of the living culture of Strain 11. This strain had been recovered from the lung tissue of a rabbit representing the eighth animal passage of active material derived from the nasopharyngeal secretions of Patient 11[3] of the 1918–19 epidemic. During immunization one rabbit (A) developed secondary infections and was killed. The other rabbit (B) received five injections of 2 to 4 cc. of a thin suspension of live culture at intervals of 5 to 7 days and was bled on the 9th day thereafter. The sterile serum was stored at 4°C. without preservative. In the first series of tests with

[2] Gates, F. L., *J. Exp. Med.*, 1922, xxxv (in press).

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

this serum it was examined for the presence of agglutinins, precipitins, bacteriotropins, and complement-fixing bodies against two strains of *Bacterium pneumosintes*, No. 11, the homologous strain, and No. 26, from the recurrent epidemic of 1920.

Since then the serum of this rabbit and similar sera produced in five other rabbits have been studied in various serological reactions with four strains of *Bacterium pneumosintes*, three (Nos. 11, 16, and 17) from the first epidemic (1918–19), and one (No. 26) from the second (1920). The results of the latter experiments are all in accord with those of the first series and need not be reported in detail. The following tests are described to indicate the methods and results of the first serological experiments.

Agglutination.

Agglutination tests were first made with a mixture of live organisms and undiluted immune serum from Rabbit B spread on a cover-slip, sealed with vaseline over a hollow slide, and examined microscopically immediately or after 30 minutes incubation at 37°C. Both strains tested (Nos. 11 and 26) were promptly agglutinated, with the formation of large compact masses of organisms in a clear surrounding fluid (Fig. 1). Control tests with normal rabbit serum showed no agglutination, or only the occasional clumping of three or four bacteria.

Macroscopic agglutination tests were then set up to determine the titer of the immune serum. Measured drops of serum dilutions 1:5 to 1:160 were mixed with equal quantities of bacterial suspension and sealed in capillary tubes of 2 mm. internal diameter.[4] After incubation for 2 hours at 37°C. the tubes were placed in the ice box over night and read the next morning. Strain 11 was completely agglutinated in a serum dilution of 1:80, with the limit of visible agglutination in a serum dilution of 1:320. The corresponding limits for Strain 26 were 1:40 and 1:160, respectively. Control tests with normal rabbit serum showed no agglutination even in a serum dilution of 1:2. The contrast in the behavior of Strain 11 in immune and in normal serum is shown in Fig. 2.

Precipitation.

Precipitin tests were also performed in glass tubes of 2 mm. bore, in which the antigen and the serum were carefully layered by drawing them up in succession by means of a rubber bulb. The antigen used was the clear supernatant fluid from old cultures of *Bacterium pneumosintes* grown in the dialysate of a tissue medium. After incubation for 1 hour at 37°C. a sharp opaque line of precipitation

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[4] Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

was observed at the juncture of the antigen and the immune serum from Rabbit B. In undiluted, normal rabbit serum a doubtful line of precipitation was observed with Strain 11 only. The appearance of the experimental and control tubes is shown in Fig. 3.

Complement Fixation.

The antigens used in the complement fixation test were the same as those for the precipitation reaction. Preliminary control tests showed that these antigens were not hemolytic or anticomplementary in volumes of 0.5 cc. when tested with 0.1 cc. of 40 per cent guinea pig complement and 2 units of anti-human amboceptor against 0.1 cc. of a 10 per cent suspension of human red cells. The immune serum of Rabbit B and the control normal serum were not hemolytic or anticomplementary in dilutions of 1:5. Antigen 0.45 cc., complement 0.1 cc., and the test sera in dilutions up to 1:160, made up to a volume of 1.05 cc. with physiological saline solution, were incubated together in a water bath at 37°C. for 30 minutes. After the addition of 0.1 cc. of corpuscle suspension and 0.1 cc. (2 units) of dilute amboceptor the tubes were again incubated for 30 minutes at 37°C. Complement fixation was complete with both antigens (Strains 11 and 26) in the highest dilution of immune serum tested; namely, 1:160. No fixation occurred in the tubes containing the normal rabbit serum.

Phagocytosis.

In the phagocytic experiments the Neufeld method[5] was used to test for the presence of bacteriotropic substances. Rabbit leucocytes were obtained from subcutaneous tubes[6] containing aleuronat in agar. Large mononuclear cells (monocytes) and lymphocytes, as well as polynuclear neutrophils, collect in these tubes and may be observed in phagocytic experiments. In the tests to be described the diluted leucocytic suspensions were mixed with suspensions of young dialysate cultures of *Bacterium pneumosintes* in physiological salt solution, or in dilutions of normal or immune rabbit serum. The mixtures were incubated 1 to 4 hours at 37°C., films prepared, fixed in methyl alcohol or by heat, and stained with Löffler's alkaline methylene blue, Wright's blood stain, or Cross' stain for leucocytes.[7] In salt solution or in normal serum controls only an occasional leucocyte picked up a few organisms. Unphagocytosed bacteria were plentiful and unagglutinated. In the presence of immune serum from Rabbit B, however, two phenomena occurred. Especially in low dilutions of the immune serum the bacteria were gathered into clumps. Leucocytes had attacked and

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[5] Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458; *Centr. Bakt., 1te Abt., Ref.*, 1906, xxxvii, 763.

[6] Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 280.

[7] Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 51.

sometimes surrounded these clumps, with which they were engorged. Other leucocytes had engulfed single organisms and contained from a few to very many of them. Both the polynuclear cells and the large mononuclear cells (monocytes) were actively phagocytic. Small mononuclear cells (lymphocytes) did not engulf the bacteria. The highest serum dilution in which an increased phagocytic activity was clearly evident was 1:80. Photographs of these phases of the reaction are shown in Figs. 4 to 7.

In addition to this series of experiments with the serum of Rabbit B and Strains 11 and 26 of *Bacterium pneumosintes*, we have made serological tests with Strains 16 and 17 and with the serum of five other rabbits immunized by repeated injections of these strains. Sera produced with certain strains, when tested in cross-agglutination experiments with the other strains, have agglutinated them and the homologous organisms in practically the same dilutions. No specific differences among the three strains from the 1918–19 epidemic or between any of these and Strain 26 from the recurrent epidemic of 1920 have been found, and the serological evidence indicates their antigenic identity. This is what might be expected if they were all derived from a common source.

It is evident from the results of these experiments that the reaction of the animal body to *Bacterium pneumosintes* involves the production of the antibodies which are commonly recognized by serological tests. We have no reason to suppose, therefore, that the mechanism of protection against this organism, of which we have evidence in the immunity reactions already described,[8] differs from that which comes into play in the case of infections with aerobic pathogenic organisms.

We would have welcomed the opportunity of testing the sera of influenza patients during the recent epidemic, or of rabbits experimentally infected with nasopharyngeal secretions from these patients. At first a suitable antigen was lacking. More recently almost all of the glycerolated material on hand was rendered inactive through the inadvertent use of glycerol which was later found to be chemically impure. The following single experiment, therefore, represents our only opportunity to test against *Bacterium pneumosintes* the sera of rabbits which had been subjected to the active agent in glycerolated form.

[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

Specific Agglutinins in the Blood of Rabbits after the Injection of Glycerolated Material.

September 12, 1921. Two rabbits were injected intratracheally with glycerolated lung tissue from a previously injected rabbit which had showed the typical clinical and pathological picture already described.[3] This animal represented the seventh passage of the active agent derived from Case 26.[3] The two injected rabbits in turn showed conjunctivitis, an increase in temperature, and the characteristic fall in the total leucocyte count due to a drop in mononuclear cells. Both were allowed to recover, and were bled from the ear vein for a serum sample

Serum dilutions														
Serum	Strain	1:2	1:4	1:8	1:16	1:32		Serum	Strain	1:2	1:4	1:8	1:16	1:32
Immune rabbit B	16	+++	+++	+++	+++	+++		Normal rabbit 3	16	−	−	−	−	−
	17	+++	+++	+++	+++	++			17	−	−	−	−	−
	26	+++	+++	+++	+++	++			26	−	−	−	−	−
Experimental rabbit 1	16	+++	++	+	−	−		Normal rabbit 4	16	−	−	−	−	−
	17	+++	++	+	−	−			17	−	−	−	−	−
	26	++	+	−	−	−			26	−	−	−	−	−
Experimental rabbit 2	16	++	+	−	−	−		Normal rabbit 5	16	−	−	−	−	−
	17	++	+	+	−	−			17	−	−	−	−	−
	26	+	+	−	−	−			26	−	−	−	−	−

TEXT-FIG. 1. Agglutinins in the serum of rabbits injected with active glycerolated lung tissue.

on the 21st day after injection. These sera together with three normal rabbit sera and the immune serum from Rabbit B were then tested for agglutinins against killed, washed, dialysate cultures of *Bacterium pneumosintes* (Strains 16, 17, and 26). The results of this experiment are shown in Text-fig. 1.

This experiment shows that the animal body reacts to experimental infection with the active agent of the nasopharyngeal secretions of influenza patients by antibody formation against *Bacterium pneumosintes*, and completes the proof of the identity of the active agent with the organism obtained from the same immediate and original sources.

SUMMARY.

Cultivation of *Bacterium pneumosintes* in the collodion sac dialysate of a tissue medium produces an antigen suitable for serological tests.

Injection of dialysate cultures of *Bacterium pneumosintes* into rabbits results in the production of antibodies demonstrable by agglutination, precipitation, complement fixation, and phagocytic reactions.

Four strains of *Bacterium pneumosintes*, three from the first epidemic influenzal wave (1918–19) and one from the second (1920), show identical antigenic characters.

The blood serum of rabbits experimentally injected with the glycerolated active material of rabbit passages contains specific agglutinins for *Bacterium pneumosintes*, whereas normal rabbit serum does not.

EXPLANATION OF PLATE 37.

FIGS. 1 and 2. Agglutination of *Bacterium pneumosintes* in immune serum from Rabbit B.

FIG. 1. Edge of hanging drop, dried and stained. × 15.

FIG. 2. Macroscopic agglutination in capillary tubes. *A*, immune serum, complete agglutination; *B*, normal rabbit serum, no agglutination. The white crescent at the bottom of each column is not sediment but light reflected from the meniscus. × 1.

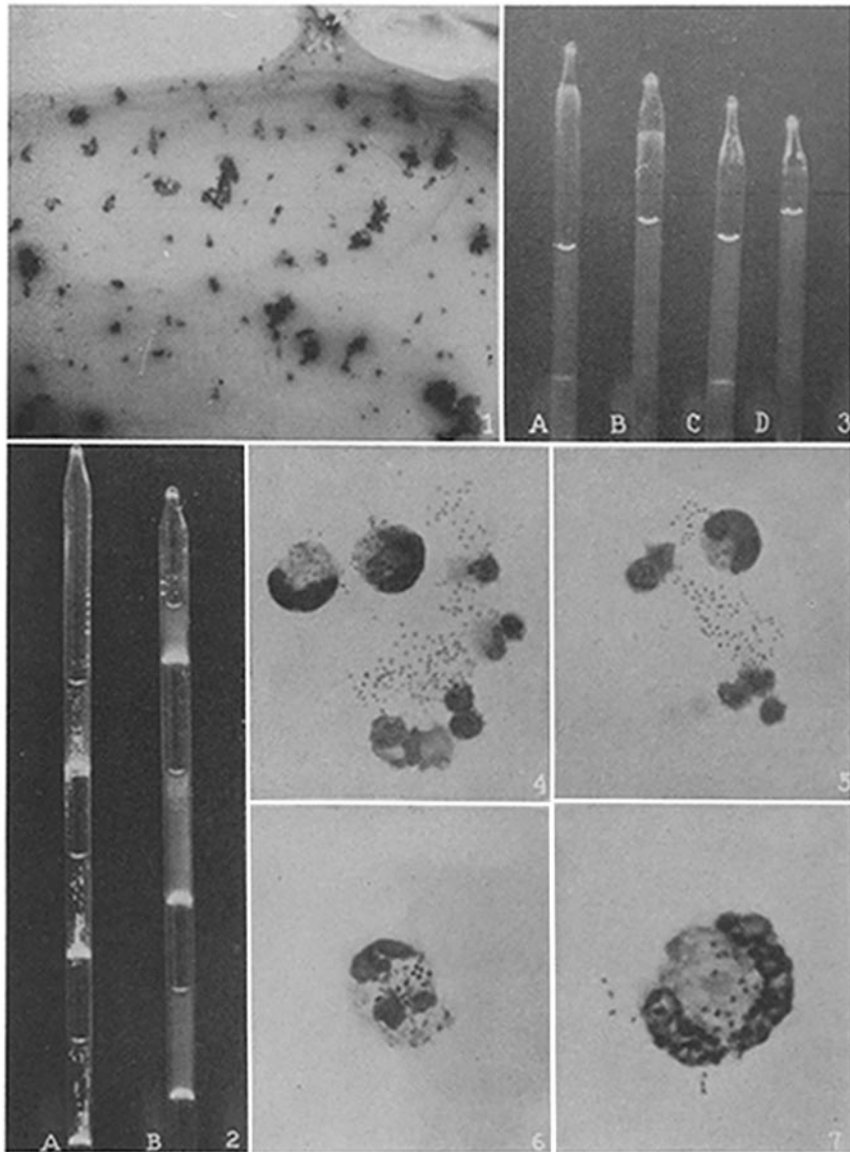
FIG. 3. Precipitation reaction with the supernatant fluid of *Bacterium pneumosintes* cultures *versus* rabbit serum. *A*, Strain 11 and immune serum from Rabbit B; *B*, Strain 11 and normal rabbit serum; *C*, Strain 26 and immune serum from Rabbit B; *D*, Strain 26 and normal rabbit serum. × 1.

FIGS. 4 to 7. Phagocytosis of *Bacterium pneumosintes* in immune serum from Rabbit B.

FIGS. 4 and 5. Monocytes (transitional cells) attacking agglutinated groups of organisms. × 1,000.

FIG. 6. Polynuclear neutrophil containing organisms. × 1,100.

FIG. 7. Monocyte containing organisms. × 1,500.



(Olitsky and Gates: Nasopharyngeal secretions from influenza. VII.)