AN IMMUNOLOGICAL STUDY OF BACILLUS INFLUENZÆ.

By MARTHA WOLLSTEIN, M.D.

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

PLATE 48.

(Received for publication, June 15, 1915.)

Bacillus influenzæ is a frequent invader of the human body, where it either causes or complicates important pathological processes. Among the pathological conditions which it produces are bronchopneumonia, empyema, and leptomeningitis; and among those in which it accompanies streptoccoci and pneumococci are the common laryngeal, tracheal, and bronchial affections. Moreover, it is sometimes associated with these cocci in lobular and lobar pneumonia.

According to the location and severity of the infections the influenza bacilli remain confined to the local lesions, or at the same time invade the blood stream. When they are confined to the local lesions in the respiratory organs, the bacilli tend to be of low virulence for animals. When they invade the blood, as they do in leptomeningitis and in some instances of pneumonia, they tend to be of higher virulence. Along with the blood invasion, influenzal suppurative arthritis may occur. These facts suggested the problem as to whether the strains of influenza bacilli isolated from various sources are identical, or whether they may be differentiated into groups on sound biological or serological grounds.

Sources of the Cultures.—The strains of *B. influenzæ* employed in this study came from two main sources; first, the respiratory mucous membrane or the lungs; second, the cerebrospinal fluid. The respiratory strains were usually slightly virulent, the other strains more virulent for laboratory animals. The bacilli isolated from the blood both before and after death were always compared with the respiratory and meningeal cultures obtained during life from the same case. This applies also to the strains isolated from

suppurating joints occurring as a complication of influenzal bacteriemia with meningitis.

Criteria of Virulence for Animals.

The subject of virulence or pathogenicity of *B. influenzæ* for animals has been dealt with in previous papers.[1] It will suffice to recapitulate the main facts in this place. The white mouse succumbs to intraperitoneal injections of cultures irrespective of their origin. A peritoneal exudate arises which contains large numbers of the influenza bacilli, as does the heart's blood. Guinea pigs of 200 grams' weight, on the other hand, succumbed to the intraperitoneal injection of one blood agar culture of all the meningeal, and to about one-half of the respiratory, strains tested.

It is by means of the rabbit that the distinction of the virulent or pathogenic strains from those lacking this quality is accomplished. Rabbits of about 1,000 grams' weight are employed. Using one blood agar culture injected intravenously as the test dose, nineteen of the twenty meningeal strains tested caused death within eighteen to thirty hours. The heart's blood in fatal cases contained large numbers of the bacilli. The respiratory strains are for the most part non-pathogenic for rabbits. Among several score of such strains I encountered only six which were virulent for these animals. Four of them came from the lungs and heart's blood and two from the lungs alone (the heart's blood being free) of infants at the Babies' Hospital. In none of the six cases were the leptomeninges involved. In this connection it may be mentioned that Batten[2] described a strain of *B. influenzæ* obtained from the meninges which was wholly devoid of pathogenic properties for mice, guinea pigs, and rabbits.

Morphology.

B. influenzæ is subject to marked variations in morphology. Two main forms are met with, the short and the long. The respiratory strains usually belong to the first class. In films from the bronchial secretion influenza bacilli are short and rather thick, but regular in

[1] Wollstein, M., *Am. Jour. Dis. Child.*, 1911, i, 42; *Jour. Exper. Med.*, 1911, xiv, 73.

[2] Batten, F. E., *Lancet*, 1910, i, 1677.

size. On blood agar plates dew-drop colonies are developed which can be readily differentiated from colonies of all other bacteria by their translucent, colorless appearance. The edges are quite regular, they always remain small, and they never cause hemolysis in the surrounding medium. The bacilli in such colonies are very short and regular, often coccoid in size, with deeply staining poles. No threads are formed (Fig. 4). On blood agar slants the growth is very profuse, but the small translucent colonies do not coalesce. In the condensation water of the tubes threads are formed, but they are never long. The meningeal strains belong to the second class. The bacilli in the cerebrospinal fluid are sometimes remarkably long and thick, and so little do they resemble the usual forms of *B. influenzæ* that the diagnosis of influenzal meningitis from films alone becomes difficult (Fig. 1). In other cases short, almost round forms appear in the films so that the presence of a coccus may be suspected (Fig. 2). Grown on moist blood agar plates dew-drop colonies are produced, in which the individuals may be swollen and atypical (Fig. 3). When transplanted to blood agar in which there is less free fluid, the forms become typical. Similarly, an excess of bronchial secretion tends to cause swelling of the bacilli in respiratory strains, but the typical form appears in subcultures. The matter of moisture present affects the morphology of both types. Even so the meningeal strains tend toward larger, more definitely bacillary forms, and incline to the formation of larger threads. When the meningeal strains are recovered from the peritoneal cavity of inoculated animals they are always small and more regular. But the next subculture again shows the larger bacillary forms.

The form tends to be constant for each strain. Thus, a coccoid strain is apt to remain small and regular and to produce only short threads. The bacillary forms, on the other hand, tend to long thread formation. However, after long periods of artificial cultivation (two years or more) both the coccoid and bacillary forms, as they existed, on the one hand, in respiratory and, on the other, in meningeal lesions, grow larger and acquire the power to give rise to long threads (Figs. 5 and 7). If the later generations are examined within the first twenty-four hours of growth some minute bacilli will be detected, indicating the original type to which the strain belongs.

A medium ill suited to growth leads to greater irregularity of form. Thus plain agar tubes upon which a small amount of human blood was placed gave rise upon inoculation of various strains to typical bacilli on the one hand, and, on the other, to long interlacing threads, recalling the leptothrix threads described by Ritchie[3] (Fig. 8). The latter were produced by one meningeal and several respiratory strains. Subcultures in the usual medium from these specimens yielded the typical bacilli. While meningeal and respiratory strains of *B. influenzæ* differ morphologically they present no points of difference in their method of growth on blood agar plates and slants.

Serological Reactions.

The main purpose of this investigation was, as stated, a minute study of the immunological or serum reactions of a considerable number of strains of *B. influenzæ,* with the object of determining whether the strains compose one or more groups, irrespective of virulence. For this purpose immune sera were employed, of which the decisive ones were those prepared in the rabbit with selected strains of the cultures.

Monovalent sera were obtained by immunizing rabbits to virulent and non-virulent strains of *B. influenzæ.* The virulent strains isolated from cases of influenzal meningitis were well borne by the animals in increasing doses over a period of three to five months. The respiratory strains, on the other hand, were badly borne, the rabbits becoming emaciated and dying after a dose which animals inoculated with virulent cultures were well able to bear. From these results it is fair to argue that the non-virulent respiratory strains do not produce immune bodies in sufficient quantities to protect rabbits against repeated and increasing doses of the bacilli.

Opsonins.—The opsonic content of the monovalent sera was fairly high, phagocytosis of the organisms being present in dilutions of 1 to 1,000. No specific reaction was obtained, however, the heterologous strains being taken up by the leucocytes in dilutions as high as those in which homologous strains were phagocyted.

Agglutinins.—The monovalent rabbit sera did not agglutinate

[3] Ritchie, J., *Jour. Path. and Bacteriol.,* 1910, xiv, 615.

homologous strains *in vitro* in higher dilutions than they agglutinated heterologous strains, and no reaction was obtained above a dilution of 1 to 100.

Bull's[4] experiments have shown that agglutination *in vitro* may give different results from agglutination of the same bacteria *in vivo*. Therefore, his method was used to compare the strains of influenza bacilli. Two young rabbits were inoculated intravenously with a non-virulent respiratory and a virulent meningeal culture, respectively. Blood was taken from the heart at intervals of thirty seconds to eighteen minutes, and slides were prepared. The respiratory culture showed marked clumping within one minute after inoculation, and in five minutes no bacilli could be demonstrated in the blood. The meningeal culture was not agglutinated at all by the blood of the inoculated rabbit, and at the end of eighteen minutes the bacilli were as numerous as they had been half a minute after the injection. The difference in the two results was very striking and clean cut. On killing the animals the bacilli of the respiratory strain were found within leucocytes in the liver, spleen, and lungs, while the virulent (meningeal) bacilli were found only in small numbers in the organs and were extracellular. A third rabbit was inoculated with a meningeal culture which had been isolated two years before, and which had lost its virulence for rabbits. The result was identical with that obtained with the non-virulent respiratory strain, proving again that the difference between the two strains is not absolute but only relative.

Complement Deviation.—Antigens were prepared from seven non-virulent respiratory strains and from seven virulent meningitis strains, according to the method used in the research laboratories of the New York Board of Health. I am indebted to Miss Olmstead for a description of this method, which is similar to the one described by Schwartz and McNeil[5] for gonococcus antigens, except that the suspensions of influenza bacilli are kept at 55° C. over night, since they undergo autolysis very slowly. At the end of the period of autolysis, which lasted from fourteen to eighteen hours, a differ-

[4] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 484.

[5] Schwartz, H. J., and McNeil, A., *Am. Jour. Med. Sc.*, 1912, cxliv, 815.

ential point between respiratory and meningeal strains of influenza bacilli was noted in the appearance of the fluid. The suspensions of the meningeal cultures were invariably turbid, with a comparatively small precipitate in the tube. The suspension of the respiratory strains, on the other hand, showed a perfectly clear fluid above a large amount of precipitate. In other words, the bodies of the nonpathogenic bacilli underwent less perfect dissolution than did the bodies of the pathogenic strains. The appearances of the precipitates as revealed by stained films under the microscope were quite similar. The bacilli no longer stained deeply and were more or less disintegrated.

All the antigens were tested against two monovalent rabbit sera immune to the meningeal strains, and against two rabbit sera immune to the respiratory strains. The results are given in Tables I to IV.

TABLE I.

Complement Deviation.

Immune Serum of Respiratory Strains + Antigens of Respiratory Strains.

Complement. Guinea pig serum 1:40 dilution.	Antigen.		Immune serum (Robinson).	Anti-sheep rabbit serum 1:1,000 dilution.	Sheep corpuscles 1:20 dilution.	Result.
cc.		*cc.*	*cc.*	*cc.*	*cc.*	
0.1	1. R.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Some hemolysis.
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	2. S.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	" "
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	3. C.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	" "
0.1		0.1	0.1	0.25	0.25	" "
0.1		0.05	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	4. F.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	5. M.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	6. M.O.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	7. L.	0.3	0.1	0.25	0.25	"

TABLE II.

Complement Deviation.

Immune Sera of Respiratory Strains + Antigens of Meningeal Strains.

Complement. Guinea pig serum 1:40 dilution.	Antigen.		Immune serum (Robinson).	Anti-sheep rabbit serum 1:1,000 dilution.	Sheep corpuscles 1:20 dilution.	Result.
cc.		*cc.*	*cc.*	*cc.*	*cc.*	
0.1	1. D.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	—	—	Hemolysis.
0.1	2. B.H.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	" "
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	3. N.Y.H.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Some hemolysis.
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	4. Ch.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Some hemolysis.
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	5. P.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	6. F.A.	0.3	0.2	0.25	0.25	"
0.1		0.3	0.1	0.25	0.25	"
0.1	7. L.F.	0.3	0.2	0.25	0.25	"
0.1		0.3	0.1	0.25	0.25	"

Thus it follows that all the sera made by immunizing rabbits with meningeal or with respiratory strains of influenza bacilli contained immune bodies capable of binding complement in the presence of antigens made from both virulent and non-virulent strains. But the sera obtained with the virulent organisms gave binding in higher dilutions than did the sera made from non-virulent bacilli. In other words, the sera obtained by immunizing rabbits with virulent influenza bacilli contained immune bodies capable of uniting with their homologous antigens in comparatively high dilutions, and with heterologous antigens in lower dilutions; while the sera resulting from the inoculation of rabbits with non-virulent influenza bacilli contained less complement binding body for all antigens.

An antigen made from a particular respiratory strain reacted with

TABLE III.

Complement Deviation.

Immune Sera of Meningeal Strains + Antigens of Meningeal Strains.

Complement. Guinea pig serum 1:40 dilution.	Antigen.		Immune serum.		Anti-sheep rabbit serum 1:1,000 dilution.	Sheep corpuscles 1:20 dilution.	Result.
cc.		*cc.*		*cc.*	*cc.*	*cc.*	
0.1	1. B.H.	0.1	1. B.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	" "
0.1		0.1		0.01	0.25	0.25	Hemolysis.
0.1	2. Ch.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	Some hemolysis.
0.1		0.1		0.01	0.25	0.25	Hemolysis.
0.1	3. N.Y.H.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	" "
0.1		0.1		0.01	0.25	0.25	Hemolysis.
0.1	4. F.A.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	Hemolysis.
0.1	5. D.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	Hemolysis.
0.1	6. P.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	Hemolysis.
0.1	1. B.H.	0.1	2. N.Y.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	Some hemolysis.
0.1		0.1		0.01	0.25	0.25	Hemolysis.
0.1	2. N.Y.H.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	" "
0.1		0.1		0.01	0.25	0.25	Hemolysis.
0.1	3. D.	0.1		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.05	0.25	0.25	" "
0.1		0.1		0.03	0.25	0.25	Hemolysis.

a heterologous serum (*i. e.*, from a meningeal strain) in lower dilution than with its own serum. However, still another respiratory strain failed to bind at all with any immune serum. Since these results were obtained in repeated tests with antigens made from the two strains and at different times, they are probably not to be regarded as accidental, but as indicating that the respiratory strains differ among themselves in strength of antigenic power. With this conclusion the protection experiments also agree.

TABLE IV.

Complement Deviation.

Immune Sera of Meningeal Strains + Antigens of Respiratory Strains.

Complement. Guinea pig serum 1:40 dilution.	Antigen.		Immune serum.		Anti-sheep rabbit serum 1:1,000 dilution.	Sheep corpuscles 1:20 dilution.	Result.
cc.		*cc.*		*cc.*	*cc.*	*cc.*	
0.1	1. R.	0.2	1. N.Y.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.2		0.05	0.25	0.25	" "
0.1		0.2		0.03	0.25	0.25	" "
0.1		0.2		0.01	0.25	0.25	Hemolysis.
0.1	2. S.	0.2		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.1	0.25	0.25	Hemolysis.
0.1		0.2		0.05	0.25	0.25	"
0.1		0.3		0.05	0.25	0.25	"
0.1	3. C.	0.3		0.1	0.25	0.25	No hemolysis.
0.1		0.2		0.1	0.25	0.25	" "
0.1		0.1		0.1	0.25	0.25	Hemolysis.
0.1		0.05		0.1	0.25	0.25	"
0.1	4. F.	0.3		0.1	0.25	0.25	Some hemolysis.
0.1		0.2		0.1	0.25	0.25	Hemolysis.
0.1	1. M.	0.3	2. B.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.2		0.1	0.25	0.25	Hemolysis.
0.1	2. Ma.	0.3		0.1	0.25	0.25	No hemolysis.
0.1		0.2		0.1	0.25	0.25	Hemolysis.
0.1	3. S.	0.2		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.1	0.25	0.25	Some hemolysis.
0.1		0.2		0.05	0.25	0.25	Hemolysis.
0.1		0.3		0.05	0.25	0.25	"
0.1	4. C.	0.2		0.1	0.25	0.25	No hemolysis.
0.1		0.1		0.1	0.25	0.25	" "
0.1		0.05		0.1	0.25	0.25	Hemolysis.
0.1	5. L.	0.3		0.1	0.25	0.25	"

While the test of complement deviation brings out a difference between the strains of influenza bacilli isolated from the respiratory tract and those obtained from the meninges, the difference is simply one of degree and not of kind. The sera and antigens made from respiratory strains were both much weaker than were those made from the meningeal strains. This fact is probably explained by the imperfect autolysis of the cultures derived from the respiratory tract and by their inability to produce more than a small amount of protective immune bodies in inoculated rabbits. The antigenic properties of the respiratory cultures are far weaker than are those of the strains isolated from the meninges.

Protection.—In order to determine whether the non-virulent respiratory strains which did not kill rabbits elicit the production of immune bodies, the surviving rabbits were reinoculated at various intervals with virulent meningeal strains.

Ten strains of *B. influenzæ*, isolated from the respiratory tract, were found to be totally unable to afford any protection to young rabbits against a subsequent inoculation with virulent strains. On the other hand, two respiratory cultures which did not kill rabbits in the ordinary lethal dose (one blood agar slant) of the standard virulent strains did protect the animals from a lethal dose of a virulent culture injected after two to four weeks. As was to be expected, sublethal doses of virulent cultures, whether of meningeal or of respiratory origin, protected rabbits against full doses given twelve days to three weeks later.

It is apparent that the respiratory strains are not identical, but that they differ among themselves in the amount of protective immune bodies they are able to develop in rabbits, just as they differ in the amount of complement binding body they produce.

The following protocols illustrate these results.

Protocols.

Experiment I.—Jan. 15. Rabbit, weight 962 gm. Inoculated intravenously with 1 blood agar slant of respiratory strain R of 24 hours' growth suspended in 1 cc. of salt solution.

Jan. 16. Rabbit alive, apparently well.

Jan. 18. Rabbit quite well.

Jan. 30. Rabbit reinoculated intravenously with 1 blood agar slant of meningeal strain N of 24 hours' growth, suspended in 1 cc. of salt solution.

Jan. 31. A. m. Rabbit ill.

P. m., 29 hours after inoculation, rabbit died.

Experiment II a.—Jan. 16. Rabbit, weight 975 gm. Inoculated intravenously with 1 blood agar slant of meningeal strain H of 24 hours' growth, in 1 cc. of salt solution.

Jan. 17. Rabbit dead. Profuse growth of *B. influenzæ* from heart's blood.

Experiment II b.—Jan. 19. Rabbit, weight 975 gm. Inoculated intravenously with ½ blood agar slant of meningeal strain H of 24 hours' growth, suspended in 1 cc. of salt solution.

Jan. 20. Rabbit quite ill.

Jan. 22. Rabbit well.

Feb. 3. Inoculated with one culture of meningeal strain N.

Feb. 5. Rabbit well.

Experiment III.—Jan. 19. Rabbit, weight 970 gm. Inoculated intravenously with 1 blood agar slant of respiratory strain C of 24 hours' growth, suspended in 1 cc. of salt solution.

Jan. 20. Rabbit well.

Feb. 10. Inoculated intravenously with 1 culture of meningeal strain N in 1 cc. of salt solution.

Feb. 15. Rabbit well.

SUMMARY.

Influenza bacilli isolated from various pathological processes in man differ widely in pathogenic power for animals, especially rabbits. While the cultures derived from the leptomeninges and blood, and rarely from the pneumonic lung are pathogenic, those generally derived from the respiratory tract exhibit little or no virulence for rabbits.

The two types of cultures as indicated by virulence for animals do not differ in kind, but only in degree, in relation to the serological tests of agglutination, complement deviation, and opsonification.

The two types of cultures do, however, differ with respect to their ability to undergo autolysis. While the virulent cultures autolyze almost completely, yielding a turbid supernatant fluid and little sediment, the non-virulent cultures give rise to an abundant sediment and a clear supernatant fluid.

The non-virulent cultures incite far less antibody production in rabbits. Hence, rabbits inoculated with non-virulent strains yield sera possessing low antibody content. Conversely, rabbits inoculated with virulent strains yield sera possessing a higher content of antibody.

In keeping with and possibly because of the low antibody content of the sera of rabbits inoculated with the non-pathogenic strains, the rabbits so treated are not, as a rule, protected against subsequent inoculation with virulent strains.

Influenza bacilli therefore vary in pathogenic effect both for man and animals, but they are not distinguishable by means of serological reactions into different types. Apparently all influenza bacilli belong to one class or race irrespective of origin or virulence.

EXPLANATION OF PLATE 48.

FIG. 1. Large forms of *B. influenzæ* in a film from cerebrospinal fluid in a case of seropurulent leptomeningitis.

FIG. 2. Small forms of *B. influenzæ* in a film from cerebrospinal fluid in a case of seropurulent leptomeningitis. Some phagocytosis.

FIG. 3. Impression from a colony of *B. influenzæ* on a moist blood agar plate. Note long and swollen forms, also polar staining. Meningeal strain.

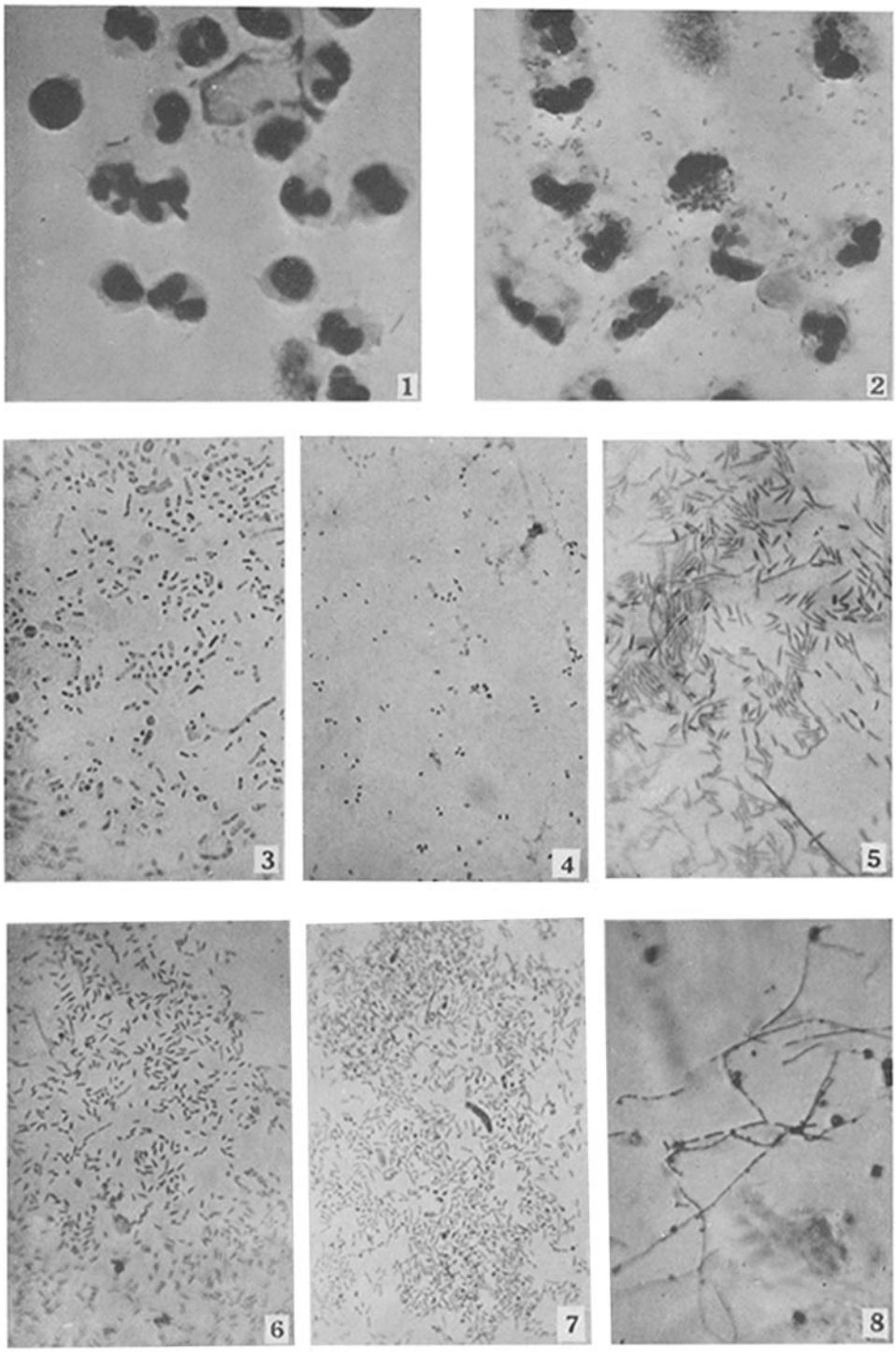
FIG. 4. Characteristic small type culture of *B. influenzæ;* 20 hours growth. Respiratory strain.

FIG. 5. Large type culture of *B. influenzæ* after 2 years of artificial cultivation.

FIG. 6. Same strain as Fig. 5 from peritoneal cavity of guinea pig 24 hours after inoculation.

FIG. 7. Small type of *B. influenzæ* after 2 years of artificial cultivation.

FIG. 8. Culture of *B. influenzæ* on blood smeared agar, showing interlacing threads.



(Wollstein: Immunological Study of *Bacillus influenzæ*.)