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### Observations of fluctuations of virulence of B. influenzæ.

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Since the relationship of the Pfeiffer bacillus to influenza is still a matter of dispute, any observation having a bearing on its epidemiological properties should have some value. This is particularly true since the epidemiology of this disease presents several features unexplainable at the present time, such as the difference in morbidity and mortality between the first and second waves, the success of isolating the organism in the disease at one time and the failure at other times, the negative experiments in human inoculations, etc.

The work reported here may be divided into three parts:

I. Results obtained working with a virulent meningeal strain.

II. Results obtained working with the same strain when less virulent.

III. Results with other less virulent strains.

I. The experiments in this part were done with a strain of *B. influenzae* isolated from the spinal fluid of a case of influenzal meningitis at the Nursery and Childs' Hospital on October 5th, 1921. This strain was kept in whole defibrinated rabbit's blood without transplantation until December 21st, about 10 weeks after isolation, when this work was started. When the organism was first received on artificial media, it was extremely pleomorphic, showing many long forms and forms of considerable size. It is interesting to note that later in exudates from the animal body, the organisms were extremely small and coccoid in shape; in fact, they looked so small that we attempted to filter them through a Berkfeldt N but with negative results.

We inoculated rabbits in the following ways with the results given below:

1. *Intratracheal.* Rabbits were injected at first with chocolate broth cultures, then, in the succeeding experiments, with varying amounts of exudates of animals dead from inocula-

tions. As a rule, the animals died three or four days after inoculation, about 50 per cent. of them showing a septicæmia of *B. influenzae*. In this series, all the animals, eight in number, so inoculated, died, but one. At autopsy the usual picture was marked fibrinous pleuresy with a pleural exudate of cloudy fluid; the lungs in two animals showed pneumonia, in the remainder they were apparently negative; in five cases there was a fibrinous exudate covering the trachea and extending down into the sup. mediastinum. One animal showed a serofibrinous peritonitis. All other organs were apparently negative. Microscopically the lungs from five animals were examined; two with pneumonia in gross, and three apparently negative. However, of these three, two showed small focal areas of pneumonia microscopically. One case also showed necrosis of the heart muscle fibres. Smears of the pleural exudates showed very numerous organisms and but a few leucocytes. The organisms were extremely small and coccoid in shape. Cultures were positive from the pleural exudate and neck exudate, and in about 70 per cent. from the heart's blood. One animal was injected by mistake in the neck tissues instead of into the trachea and died overnight with a septicæmia. This would tend to settle the question as to whether we were not causing the above lesions by missing the trachea and injecting into the neck, for death in these animals where we felt sure we were in the trachea did not occur for three or four days, never overnight as in this animal. In all instances the animal was anesthesized and the trachea exposed by operation.

2. *Intracerebral.* Three animals were inoculted with the following doses: 0.005 c.c. of the pleural exudate of a rabbit killed by intracheal inoculation, 0.001 c.c. of the peritoneal exudate of a rabbit inoculated intraperitoneally, and 0.1 c.c. of a 20 hour chocolate broth culture. Blood cultures on two of the rabbits taken 3½ hours and five hours, respectively, after inoculation were positive; the third animal was not cultured before death. At autopsy cultures of the heart's blood in all three were positive. Pathologically, nothing abnormal was found with the exception of hæmorrhage into the thymus in one animal.

3. *By stomach tube.* Five animals were inoculated in the stomach by means of a stomach tube. Of these, two died, one receiving 15 c.c. of 20 per cent. chocolate broth culture, and the

other 9 c.c. of 20 per cent. chocolate broth culture plus 1 c.c. of pleural exudate of rabbit dead of intratracheal inoculation. A blood culture was taken on one of the rabbits 6 hours after inoculation and was positive. At autopsy cultures of the heart's blood of both were positive. Of the three that did not die, one received only 2 c.c. of broth culture, one received 20 c.c. of broth culture inoculated with the organism after a week's growth on chocolate agar, and the third, who received the same dose as one that died, had previously received two small inoculations into the stomach. The two animals that died showed increased respiration and high temperatures almost immediately. The mechanism of death in these cases has not been worked out and at present is not clear.

II. After carrying out the above experiments, we next worked with other strains. About three weeks later, we returned to our meningeal strain "M," but, as the strain we had passed through the animals in our previous experiments had died out, we had to go back to the original strain in rabbit's blood, which then had been out of the body 18 weeks, instead of 10 weeks, as in our first series of experiments. To our surprise, we could not repeat our former experiments as the virulence of the organism had diminished to such a degree. Our results were as follows:

1. *Intratracheal.* Three rabbits were injected with 3.5 c.c., 4 c.c., and 4 c.c. of 20 per cent. growth in chocolate broth, respectively, and one with 2 c.c. of the peritoneal exudate of a guinea pig dead from an inoculation. None of these animals died, or even were sick.

2. *Intracerebral.* One animal was inoculated with 0.1 c.c. of heavy emulsion of a culture on a chocolate agar slant; it died in 48 hours; at autopsy the heart's blood and brain was positive for *B. influenzae,* but blood cultures 6 hours after inoculation and 24 hours later were negative. The other animal received 0.1 c.c. of an emulsion of the brain of the first rabbit; it did not die, nor was it sick.

3. *By stomach tube.* One animal was given 20 c.c. of a 20 hour growth inoculated with the blood by one rabbit dead with a septicæmia following an intraperitoneal injection. It never showed any reaction. The other animal received 18 c.c. of a 20 hour chocolate broth culture plus 2 c.c. of the peritoneal exudate of a guinea pig dead from an intraperitoneal inoculation.

III. The three strains worked with in this group were "R," a meningeal strain from the spinal fluid of a case of influenzal meningitis; "S," isolated from the sputum of an acute case of influenza; "J," isolated from the pus of a case of chronic infection of the antrum, and "Z," from the sputum of a case of acute coryza.

A. The following experiments were done with "R":

1. *Intracerebral.* One rabbit was injected with 0.1 c.c. of spinal fluid from a case of influenzal meningitis shortly after removal from the patient. Blood culture on the animal next day was negative. He never was sick, and at the end of three days was used in another experiment.

Another rabbit was injected with 0.1 c.c. of the peritoneal exudate of rabbit killed by intraperitoneal injection with a chocolate broth culture of this strain. Blood cultures 24 hours later showed approximately 500-1000 colonies in a drop of blood. Blood 48 hours after injection showed 10 colonies to a drop of blood. Blood culture 72 hours and 96 hours after injection were negative. The animal died 10 days after inoculation; at autopsy, the brain showed a yellowish caseous area at the inoculation site, 0.2 x 0.2 x 0.1 cm.; cultures from this lesion and the heart's blood were negative.

B. The following experiments were done with strain "S":

1. *Intratracheal.* One rabbit received 2.5 c.c. of the patient's sputum; it never was sick and blood cultures were uniformly negative. The animal was killed with ether 9 days after; autopsy findings and cultures from the lungs and heart's blood were negative.

2. *Intracerebral.* Four rabbits were injected.

(a) Received first 0.75 c.c. of filtrate of "S" on chocolate broth intravenously with symptoms typical of poisoning with filtrates of cultures of *B. influenza* (1); while still sick, one and a half hours later he received intracerebrally 0.1 c.c. of the centrifuged sediment of a 20 hour chocolate broth culture. Blood culture 6 hours later and 24 hours later was negative. It died the night of the second day. At autopsy, the left lateral ventricle showed on its inferior surface a few yellowish spots and injection of its upper surface; the rest of the organs were negative; smears and cultures of the fluid in the ventricles were positive while the heart's blood were negative.

(b) Was inoculated with 0.1 c.c. of an emulsion of the brain of (a). 24 hours later, it was sick and unsteady on its feet; blood culture was negative. 48 hours later nervous symptoms were very marked, consisting of ataxia and increased irritability; it was so sick in the afternoon of this day that we killed it with ether. At autopsy cultures from the brain and heart's blood were negative.

(c) Received 0.1 c.c. of heavy emulsion of organisms from a chocolate agar slant, inoculated from organism recovered from brain of (a). Blood cultures were positive 6 hours, 24 and 48 hours after inoculation, but were negative at 72 and 96 hours. The animal was sick 24 hours later, showing ataxia and dyspnea; it became progressively worse, developing a stiff neck and finally was killed with ether four days after inoculation as it was so sick. At autopsy, the brain at the site of inoculation showed an area of yellowish friable material 0.3 cm. in diameter, extending 0.2 cm. into the substance of the cortex. Cultures from the brain were positive, but the heart's blood was negative.

(d) Inoculated with 0.1 c.c. of emulsion of brain of (b) which was shown to be sterile. At the end of three days, it developed diarrhea and looked sick; two days after this, it died. At autopsy the brain at the inoculation site showed an area of yellowish friable material 0.2 x 0.1 cm. Smears and cultures of this area, the ventricular fluid and the heart's blood were negative.

3. *By stomach tube.* One animal was given 20 c.c. of a 20 hour growth in chocolate broth. It was never sick. Blood culture seven hours after inoculation was negative.

C. Experiments with strain "J":

1. *Intracerebral.* One animal was given 0.1 c.c. of a 20 hour chocolate broth culture. Blood culture was negative 24 hours later. It never was sick.

2. *By stomach tube.* One rabbit received 27.5 c.c. of a 20 hour chocolate broth culture and never was sick.

D. Experiments with strain "Z":

1. *Intracerebral.* One rabbit received 0.1 c.c. of a 20 hour chocolate broth culture. He developed no symptoms so that 3 days later was given 2 c.c. of a similar culture intraperitoneally; following this, blood cultures 5 hours and 24 hours later were

negative. As he was not very sick after the second inoculation, he was again injected intraperitoneally with 2 c.c. of a chocolate broth culture in the morning, and 4 c.c. of the same culture in the afternoon. The following day he was perfectly well. 15 days after his brain inoculation, he developed paralysis of the hind legs, his respiration was labored and in general he was so sick that we etherized him to death. At autopsy, the brain alone showed any pathological changes; at the inoculation site there was a yellowish caseous area 0.2 x 0.2 x 0.1 cm. Smears and cultures of this area and the heart's blood were negative.

A second rabbit was given 0.2 c.c. of an emulsion of the brain of the preceding rabbit. He never developed any nervous symptoms, but a week later developed diarrhea. He did not die.

## DISCUSSION.

On comparing the results of I and II, the striking difference in virulence will be noted. In II we were unable to kill or even make sick with intratracheal or stomach inoculations, and while we killed one animal with an intracerebral inoculation, in this case we used a very large dose, and the animal did not develop a septicæmia a few hours later as did the animals in I. On the whole, the results in II closely resembled those obtained with the strains we worked with in III.

There is one interesting feature in the less virulent group which deserves attention, namely, that in two animals that developed a septiæmia 24 hours and 58 hours after inoculation, this septicæmia then cleared up and yet the animals died, in one instance at least, all cultures being negative at autopsy. Three rabbits inoculated intracerebrally, one rabbit inoculated intrapleurally and a mouse injected with sputum containing numerous *B. influenza* died with negative cultures at autopsy. This is an important point, especially in connection with human cases, for it brings up the possibility that an individual may die of an infection with *B. influenzæ* and yet all cultures at autopsy fail to show evidence of this organism. The most probable explanation of this seems to be that at first when the organisms are growing they elaborate a poison causing a fatal injury, and then later the defenses of the body or some other factor kills off the organisms, but too late to prevent death as the injury has been done.

By our results in I and II, we have shown that apparently the length of time out of the body has a definite influence on the virulence of the organisms. That the virulence may diminish in the course of the disease is suggested by a single observation: A mouse was injected with the sputum of a case of influenza on the first day of the disease; it died 6 hours later with positive cultures of *B. influenzæ* in the peritoneal exudate and heart's blood; on the second day of disease, some more sputum, smears of which showed numerous *B. influenzæ*, from this patient was injected into a mouse; this mouse, however, did not die for 48 hours, and at autopsy cultures from the heart's blood and peritoneum were negative.

CONCLUSIONS.

1. The virulence of *B. influenza* is very variable and this may have some bearing on the epidemiology of the disease influenza in man.

2. A few observations are present that may throw some light on the results of bacteriological studies of this disease.

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## The formol titration of bacteriological media.

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The formol titration devised by Malfatti[1] (1908), Sörensen (1907[2], 1908[3]), and by Henriques and Sörensen[4] (1909) for the titration of the ammonia and amino acids of urine has been more or less modified by bacteriologists for the titration of media and cultures. Ammonium chloride reacts with formaldehyde to produce hexamethylentetramine and hydrochloric acid. Amino acids and polypeptides react with formaldehyde to produce acid methylene derivatives which are stronger acids than the amino acids

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[4] Henriques, V., and Sörensen, S. P. L., *Z. f. physiol. Chem.*, 1909, lxiii, 27.