AN ATTEMPT TO ISOLATE A FILTER-PASSING VIRUS IN EPIDEMIC INFLUENZA.

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In view of the extensive investigations carried on by Olitsky and Gates\textsuperscript{1--12} with reference to the causative agent in epidemic influenza, and particularly in view of the fact that the claims of these observers regarding the demonstration in material and cultures from influenzal patients of a filter-passing microorganism have been more or less confirmed by the reports of Gordon\textsuperscript{13} in England, and Lister\textsuperscript{14} in South Africa, it seemed to us that the epidemic experienced in Toronto during the past winter should not be allowed to pass without an attempt being made to duplicate some of the experiments of these workers.

Attempts to culture a filterable virus were made in this laboratory by Maitland, Cowan, and Detweiler\textsuperscript{15} during the epidemic in the winter of 1920. These were unsuccessful, but the work as far as cultures were concerned, was admittedly not exhaustive. The ani-

\textsuperscript{1} Olitsky, P. K., and Gates, F. L., \textit{J. Exp. Med.}, 1921, xxxiii, 125.
\textsuperscript{3} Olitsky, P. K., and Gates, F. L., \textit{J. Exp. Med.}, 1921, xxxiii, 713.
\textsuperscript{14} Lister, S., \textit{South African Med. Rec.}, 1922, xx, 434.
\textsuperscript{15} Maitland, H. B., Cowan, M. L., and Detweiler, H. K., \textit{Brit. J. Exp. Path.}, 1920, i, 263.
mal experiments, on the other hand, were extensive, but here again we were unable to find lesions which could legitimately be called influenzal in origin. Observations made while this work was in progress led to further experiments, which showed that the lesions which are commonly ascribed to experimental influenza in the literature are in reality due to the method of killing the animal. This view is supported by Lister and has been confirmed during the course of the work on which this paper is based.

The clinical material used was obtained from cases seen by us during the epidemic of influenza occurring in Toronto during the months of January and February of this year. The symptoms and signs considered diagnostic of the disease were abrupt onset with chills or chilly feeling, fever, general malaise with prostration, headache, pains in the back and limbs, suffusion of the eyes and flushed face, and usually a tracheal cough with other signs of upper respiratory affection. Physical examination in the early stages revealed nothing but the signs enumerated above, and the subsequent history of the cases was entirely in keeping with the ordinary course of influenza. While this epidemic, taken as a whole, was of a mild nature compared with that of 1918, there were numerous cases showing all the violence of the first great wave of the disease. Case 6, to be described, was an example of the worst type with fatal lung involvement. Cases with a vague onset were not considered suitable for the purposes of this investigation owing to the importance placed by Olitsky and Gates upon obtaining nasopharyngeal washings during the early hours of the disease. This restriction, coupled with the fact that most of the cases admitted to hospital were past the first 36 hours of the disease, rendered the series very small. We feel, however, that the findings should be reported, especially since, in the opinion of many bacteriologists, the evidence bearing upon this subject is still too meager to form a basis for a conclusive verdict.

**Technique.**

The technique adopted was, when possible, that described by Olitsky and Gates. Briefly it was as follows:

1. Collection and Preparation of Material.—Nasopharyngeal washings were obtained by washing the nasopharynx with 50 cc. of sterile saline. Unfiltered washings were cultured aerobically on blood agar and chocolate agar and were in most instances also used for animal inoculations. The remainder of the washings was passed first through a Büchner filter and finally through a Berkefeld V filter. Lung tissue was ground up with sterile sand and saline in a sterile mortar and passed first through a Büchner filter and then through a Berkefeld V filter.

2. Animal Experiments.—All animals were inoculated intratracheally by the tracheotomy method. 17 Guinea pigs and rabbits were used and the amounts of inoculum varied from 1 to 3 cc., animals being injected with both unfiltered and filtered material. After 24 to 48 hours inoculated animals were killed by a blow on the back of the neck, and the lungs removed with aseptic precautions and examined macroscopically. Pieces of lung were kept for culture aerobically and also for culture in Smith-Noguchi medium. Pieces of lung were also kept for sections. The lung remnants were ground up and filtered as already described. Experience with the method of killing animals just described convinced us that it was almost impossible to avoid accidental hemorrhage and aspiration with the production of petechial or massive hemorrhages in the lungs. For this reason the following method of killing animals was used in the later experiments. The anterior thoracic wall and the heart were cut into with a single snip of the scissors. The chest was opened more widely and the diaphragm incised. The lungs were removed and examined without delay.

3. Methods of Cultivation.—Lung tissue was cultured aerobically in dextrose ascitic broth and on blood agar, and anaerobically in Smith-Noguchi medium. Anaerobic cultivation of lung tissue was soon abandoned because the presence of ordinary organisms made the determination of the existence of Bacterium pneumosintes difficult. Filtrates of lung tissue or nasopharyngeal washings were cultured in the following ways.

   (a) On blood agar aerobically.
   (b) In dextrose broth aerobically.
   (c) On blood agar anaerobically. Inoculated blood agar plates were placed in anaerobic jars (McIntosh and Fildes18 bomb type) and incubated for 8 to 10 days. The jars fulfilled satisfactorily the conditions of complete anaerobiosis.
   (d) In Smith-Noguchi medium. The reaction of the ascitic fluid used for this medium was pH 8.1. This was slightly more alkaline than the optimum reaction mentioned by Olitsky and Gates. However, experience with a known strain of Bacterium pneumosintes (C4 Rockefeller tenth generation) which Dr. Olitsky kindly sent for comparison with our strains, convinced us that the conditions for growth of Bacterium pneumosintes in our medium were not far from optimal.

17 All operations were performed under ether anesthesia.
Subcultures were made from this strain in Smith-Noguchi mediums prepared with the unaltered ascitic fluid, and also prepared with the ascitic fluid altered to a reaction of pH 7.8. As the mediums had been prepared for 3 or 4 days, anaerobic conditions were already established when the subculture was made, and growth in each tube was extremely heavy in 72 hours. The only difference detected in films was that the organisms showed more azure staining in the tube prepared with the fluid of reaction pH 7.8.

(e) Blood dextrose broth under a vaseline seal was used occasionally. Growth in this medium with Strain C34 was apparent in 9 days.

(f) B. coli broth under a vaseline seal was used only in cultivation of Strain C34. In this medium growth was apparent in 10 days.

Smith-Noguchi medium was preeminently the most satisfactory. The slow sparse growth of Strain C34 in blood dextrose broth under a vaseline seal, and in B. coli broth under a vaseline seal, when compared with the rapid, vigorous and luxuriant growth of the same strain and generation in Smith-Noguchi medium leaves no room for doubt that this medium should be employed always for primary isolations of organisms of this group. Anaerobic blood agar cultures were found disappointing. We could not satisfy ourselves of the presence of surface colonies on this medium. It is true that Bacterium pneumosintes could be seen in films prepared by scraping the surface of the inoculated agar after incubation for 10 days, but the organisms were not sufficiently numerous to prove that growth had occurred. In this our findings were similar to those of Lister.4

4. Staining.—Well ripened methylene blue proved so satisfactory that no other stains were used. Our own strains and Strain C34 were stained clearly not only with steaming methylene blue, but also with methylene blue in the cold. Films stained in the latter manner were only slightly less clear-cut than those stained by steaming.

Sources of Material.

Case 1.—Nasopharyngeal washings from a patient who developed a typical attack of influenza while in the hospital wards. Washings were taken 24 hours after the onset. Aerobic cultures of the washings showed a pure culture of Streptococcus hemolyticus β.

Case 2.—Nasopharyngeal washings from a patient admitted to the wards with clinical uncomplicated influenza. Washings were taken 72 hours after the onset. Aerobic cultures of the washings showed Streptococcus hemolyticus β and Staphylococcus aureus.

Case 3.—Nasopharyngeal washings from a patient admitted to the wards with clinical uncomplicated influenza. Gradual onset 72 hours before admission; acute exacerbation with chills 36 hours before the washings showed Micrococcus catarrhalis and Streptococcus hemolyticus α.
Case 4.—Nasopharyngeal washings from our laboratory technician who developed uncomplicated influenza. Washings taken within 12 hours of onset. Aerobic cultures of the washings showed *Micrococcus catarrhalis* and an occasional colony of *Streptococcus hemolyticus* β.

Case 5.—Nasopharyngeal washings from a medical student who developed uncomplicated influenza. Washings taken within 24 hours of onset. Aerobic cultures of the washings showed *Micrococcus catarrhalis* and an occasional colony of *Streptococcus hemolyticus* β.

Case 6.—Lung from a patient who died of influenzal bronchopneumonia. Duration of pneumonia 4 days. Clinical picture typical. Lungs post mortem showed the typical lesions of influenzal bronchopneumonia identical with the worst cases seen in the epidemic of 1918. Aerobic cultures of lung exudate showed *Staphylococcus aureus* and pneumococcus.

**Results of Animal Inoculations.**

In the animals inoculated with unfiltered nasopharyngeal washings the findings in the lungs, both in the gross and microscopically, were those of a diffuse bronchopneumonia. Sections showed moderate edema and congestion and focal areas of polymorphonuclear and lymphocytic infiltration, occasionally filling the alveolar spaces and the lumen of the bronchi. This picture never occurred in animals inoculated with filtered material. Hemorrhages without inflammatory reaction occurred in animals killed by a blow on the back of the neck, but were uniformly absent in those in which the lungs were removed without trauma. This was in accord with previous findings in this laboratory. Apart from the accidental hemorrhage, and apart from the presence in a few instances of the spontaneous chronic pneumonic lesion commonly found in rabbits and guinea pigs, there were no abnormalities in any of the animals inoculated with filtered material.

**Results of Cultural Experiments.**

Case 1.—Cultures of the original filtrate and of the filtrate from the lungs of animals inoculated with the original material were in all instances sterile.

Case 2.—Cultures of the original filtrate and of the filtrate from the lungs of animals inoculated with the unfiltered material were in all instances sterile.

Case 3.—The Smith-Noguchi culture of the original filtrate showed after 14 days the presence of definite minute coccobacilli in groups. These we considered morphologically indistinguishable from *Bacterium pneumosintes* described by Olitsky and Gates. Subcultures in Smith-Noguchi medium failed to grow. Cultures from the lung filtrates of animals inoculated with the original material were sterile.

Case 4.—The Smith-Noguchi culture of the filtrate from the lungs of Guinea Pig 1 inoculated with the original filtrate showed after 3 weeks a diffuse growth of very fine coccobacilli morphologically similar to *Bacterium pneumosintes*. Subcultures in Smith-Noguchi medium failed to grow.

The Smith-Noguchi culture of the original filtrate was contaminated. This was not filtered as no suspicious organisms were seen.

Case 5.—The Smith-Noguchi culture of the filtrate from the lung of Guinea Pig 2 inoculated with the original unfiltered material, showed after 3 weeks a diffuse growth of very fine coccobacilli. Subcultures in Smith-Noguchi medium failed to grow. The Smith-Noguchi culture of the original filtrate was contaminated.

Case 6.—Cultures of filtrates of the direct material were in all instances sterile.

The Smith-Noguchi culture of the lung tissue from Guinea Pig 3, which had been inoculated with the original macerated lung tissue, showed 5 days after inoculation numerous very fine coccobacillary forms which we considered morphologically indistinguishable from *Bacterium pneumosintes*. This culture was, however, not pure, but contained also a Gram-negative bacillus. On filtration of this culture the fine coccobacillus was not recovered.

**DISCUSSION.**

It will be noted that in four (Nos. 3, 4, 5, and 6) out of six cases organisms morphologically similar to *Bacterium pneumosintes* appeared in the Smith-Noguchi medium. In Case 6 the Smith-Noguchi medium containing a piece of suspected lung showed minute coccobacilli, but Gram-negative bacilli were also present. On this
account this case should be ruled out of the group in which suspicious organisms appeared. The reason for the restriction is that it is possible that these minute coccobacilli were merely pleomorphic involution forms of the aerobic Gram-negative bacillus which was present. An aerobic Gram-negative bacillus which grows with difficulty under anaerobic conditions has been encountered in the present study; when this organism is inoculated in small amounts into Smith-Noguchi medium an early (48 hours) diffuse growth of minute bacilli occurs. Morphologically the bacillus growing under these conditions might be confused with \textit{Bacterium pneumosintes}.

The remaining three cultures which it is justifiable to consider suspicious were taken from Cases 3, 4, and 5. Washings were obtained from Case 3, 36 hours after the acute onset, and from Cases 4 and 5 within 24 hours of onset. These were, with the exception of Case 2, the patients which furnished the most favorable material for positive results.

The three suspicious cultures were all obtained in Smith-Noguchi medium and developed slowly (2 to 3 weeks). Subcultures on aerobic medium and in Smith-Noguchi medium remained sterile. The fact that these strains developed slowly and were not viable when subcultured to fresh Smith-Noguchi medium suggested the possibility that the ascitic fluid might be at fault. However, it was found that Rockefeller Strain C_{34} grew luxuriantly in the medium.

The morphology of the three strains we considered similar to that of \textit{Bacterium pneumosintes}. Dr. Olitsky kindly offered to examine these strains for us with a view to identification; accordingly the cultures were forwarded to him. He reported that he considered them morphologically similar to his own strains, but that he had been unable to obtain growth in subcultures, so that they could not be identified with certainty. He also stated that he had frequently lost strains in a similar manner. It may be opportune to refer at this point to Woodcock's contention that the suspicious bodies appearing in these cultures are due to protein precipitate produced by cytolysis of the tissue in the Smith-Noguchi medium. Woodcock's observation was prompted by Gordon's statement that the organism he considered the filter passer of influenza was not bacilloid, but rather coccoid, that it stained with difficulty with methylene blue,

and that best staining results were obtained with Giemsa's stain. Our suspicious cultures were definitely bacilloid, as was Strain C34. Furthermore, our cultures and Strain C34 stained with well ripened methylene blue both by steaming and in the cold. The rapidity of growth of Strain C34 in subcultures rules out the possibility of a cytolysin producing this appearance. Moreover, the morphology of these strains is too definite and regular for protein precipitate. During the course of the experiments a great number of smears from sterile Noguchi medium were examined. At no time were any bodies encountered in these films which could be confused with Bacterium pneumosintes.

It is possible that the bodies in the cultures which are reported here as suspicious are not identical with the microorganisms reported by Olitsky and Gates. Nevertheless, our own observations and those of Dr. Olitsky indicate that morphologically they are indistinguishable, the only link missing being our inability to obtain successful subcultures. As stated before, this failure has been encountered frequently by Olitsky and Gates, and too definite conclusions should not be drawn from our inability to subculture in three instances.

It is to be hoped that reports of similar investigations will be forthcoming from various centers of research, as it is only by an accumulating mass of evidence that the question of the etiology of epidemic influenza can be settled definitely. Controls should include, in addition to normal individuals, cases of upper respiratory infections of types other than influenza. We hope to be able to study such controls as the opportunity occurs.

SUMMARY.

1. Six cases of epidemic influenza in various stages of disease were investigated.
2. Suspicious cultures of bodies resembling Bacterium pneumosintes were obtained in three cases, twice from the lung filtrates of inoculated animals and once from the filtrate of nasopharyngeal washings direct.
3. In these instances material was obtained within 36 hours of the acute onset, thus confirming the observations of Olitsky and Gates.
4. Macroscopic and microscopic findings in the lungs of inoculated animals were uniformly negative.