EXPERIMENTAL STUDIES ON AVIAN DIPHTHERIA.*

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Although avian diphtheria has long been recognized as an epizootic disease among chickens, and several investigations made by numerous workers in foreign countries since 1873 have been reported, our knowledge of the causal agent is still far from complete. This disease has also become yearly more widespread in this country and attention has been directed to it chiefly through the work of investigators rather than by the losses it has occasioned. In 1907, Carnwath concluded from his experimental work that the causal agent of this disease was identical with the virus of chicken pox, since which we have had no other noteworthy information concerning this disease. I have studied, in the first place, the nature of the virus in the typical local lesions exhibited as avian diphtheria and compared this virus with that mentioned in the literature of several authors. In the first series of experiments, which were conducted to decide whether the filterable virus could be effectual in the lesions of this disease, it was not apparent that there was any virus in the filtrates made by various known methods which would cause this disease. Thus no conclusion could be reached as to the proof that this disease had any connection with the filterable infectious substances. However, further attempts at the transmission of chicken pox with filtrates obtained from the local lesions of that disease gave positive results; on the other hand, the characteristic lesions of avian diphtheria were not produced by the same filtrates. Subsequently the results of these experiments tended to show that the virus of chicken pox was capable of passing through a porcelain filter, as Sticker, Marx and others had demonstrated, but it was, nevertheless, impossible to produce any typical infection of avian diphtheria. In the experiments in both series, I naturally preferred to collect the materials examined from cases in which the mixed infection had not occurred. As a sequel, therefore, to the fore-

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going researches, attention was drawn to the possibility that the causal agent of avian diphtheria might not be any filterable virus.

1. *Nature of the Disease.*

It was proved by several investigators abroad that the evidence of some causal agent could be found in the local lesions exhibited in the affected chickens; but my experiments showed no constant transmission of this disease by injection with the material from the original lesions. On the whole, it was inferred that the disease could be set up by the artificial inoculation or feeding experiment of chickens with such materials from earlier stage of the lesions so that no advanced degenerative process was demonstrated by histological examination. In other words, the results of these direct transmission experiments showed that the degree of virulence of the causal agent might decrease with time in the original lesions. Attempts at transmission by cohabitation also gave positive results as far as the affected chickens were concerned at the earlier stage of this disease. Moreover, in the course of these experiments it was recognized that this disease could be transmissible to pigeons from chickens and vice versa.

2. *Bacteriological Studies.*

Various investigators have reported several organisms isolated from the lesions as the cause of this disease. LÖFFLER (1884) reported that he isolated the organism (*Bac. diphtheriae columbarum*) from the pigeon diphtheria, and succeeded in reproducing the same lesions by the inoculation of pigeons. Loir and Ducloux were convinced that the oval organism which was isolated from lesions of chicken diphtheria might actually be the cause of the original disease. STREIT (1904) succeeded in isolating roup bacillus, which he considered to be the cause of a chicken disease clinically identical with chicken diphtheria known as roup in America. Besides these, other organisms regarded as the causative factor of this disease have been described by different authors. By a previous study on the nature of the causal agent in the lesions of this disease, I investigated the frequency with which

any particular kind of microorganism could be demonstrated in cases of this disease. From a series of cases a coccobacillary organism was demonstrated by the microscopic examination of smears which were made from earlier stage of the lesions only. In some cases in which this organism was demonstrated on smears from the lesions (croupo-diphtheritic pseudomembrane), the staining was so faint that it could be found only with considerable difficulty. Accordingly it was almost impossible to isolate this specific organism from every case. It was ascertained, however, by the complement fixation test that there remained some specific sensitizing substances by which one was able to fix complement in the presence of the specific antiserum obtained by immunizing an animal with the coccobacillary organism, though it was impossible to find this organism microscopically in the lesions. Furthermore it was found that the antigenic substances capable of fixing complement in the presence of this specific antiserum could not be produced from normal organs nor by alcoholic extraction from the material of the lesions. The results of these experiments made it reasonable to suppose that the specific antigen contained in the extract of materials from the lesions must be derived from the coccobacilli. This supposition was strengthened by the experiment that the specific antigen prepared from the culture of the coccobacilli showed the complete deviation of complement by the given immune serum, and it was ascertained that 0.025 c.c. of antigen still gave complete fixation of 0.03 c.c. of complement in the presence of 0.05 c.c. of the anticoccobacillus serum. Consequently, if there should be any etiological importance in this organism for the disease called avian diphtheria in this country, the escape from infection of a certain proportion of the chickens inoculated with the material from the original lesions could be considered to be due to the decrease of vitality of this organism in later stage of the lesions.

3. *Biological Studies of the Coccobacillus.*

As regards morphology this organism took a coccobacillary form occurring singly, but some showed a short rod-shaped form with a speck at each end with a colorless central part. It

measured from 0.3 to 0.8 μ in length by 0.2 to 0.5 μ in breadth. In culture this organism varied a little in shape according to the nature of the medium. It was a non-motile, facultative aërobe and grew slowly. In primary cultures on serum agar the organism developed very small round colonies translucent by transmitted light, and slightly bluish white by reflected light. It stained with the ordinary aniline dyes and was Gram-negative. A good preparation was obtained by staining with LÖFFLER's methyleneblue. This organism grew best in alkaline media at a temperature of from 37° to 39°C. No Growth occurred below 12°C. In cultures it would probably die out after five or six weeks. In subcultures in serum broth, a small amount of grayish white slimy deposit was produced in two days at the bottom of the tubes. On potatoe was formed a thin yellowish pink layer. Milk was not coagulated.

This organism was less resistant to external influences. Thus, when exposed to diffuse daylight it lost its virulence in two hours and heating to 65°C. killed it in five minutes. The following disinfectants had a destructive action on the organism in a short time: 1% carbolic acid solution in 1 minute, 2% lysol and creolin in 3 minutes, and 5% milk of lime in 20 minutes. On examining the fermentative properties of various carbohydrates, namely, glucose, lactose, galactose, dextrose, maltose, saccharose, and mannite, it was found that this organism did not decompose these sugars. In peptone water the growth was slow, but a small amount of indol was demonstrated by the 7th to 10th day. When grown in peptonized veal broth free of sugar, the organism produced no soluble toxin. The hemolytic power of this organism for the red blood corpuscles from various animals was not noted at all.

4. *Pathogenicity of the Coccobacillus.*

An examination into the susceptibility of several animals showed the following results. To rabbits and pigeons inoculated subcutaneously a minute dose was fatal in 24 hours. Post mortem, there was seen more or less extensive suppurative infiltration on the site of inoculation and generalized congestion of all organs. Microscopic examination disclosed abundant coccobacilli

in the heart blood, liver, kidney, and spleen : but with the pigeons injected with smaller doses, a characteristic croupo-diphtheritic inflammation developed in 8 to 14 days before death. In these cases the organism was demonstrated only by developed local lesions mixed with other organisms, but not in the heart blood, liver, or spleen. Mice and guinea-pigs inoculated subcutaneously with comparatively large doses died in from 3 to 5 days, showing generalized changes of the hemorrhagic septicemia type. From the heart blood, liver, and spleen were obtained pure cultures of the coccobacilli. This organism was not absolutely fatal to chickens when small doses were given subcutaneously, but produced the same characteristic local lesions as were observed in the pigeons which did not indicate any acute course. They were very much worse and showed gradual loss of appetite, with the progressive changes of lesions. 2 to 10 days later they became extremely emaciated and subsequently succumbed. The post-mortem examination revealed the same lesions as were seen in the original cases of this disease, but the specific organism could not be found by a microscopic examination of blood, spleen, and liver. Furthermore, 93 chickens and 49 pigeons were used to ascertain the pathogenicity of the coccobacilli. In a feeding experiment the animals showed the loss of appetite in the first 3 to 5 days, after which greater secretion of a muco-purulent appearance was observed in the mouth. At the same time the affection of the nasal cavity was manifested by a discharge of the same kind. In the mean time small grayish white spots which gradually became thicker and formed dense deposits, were found on the mucous membrane of the mouth. At this time the general condition having markedly changed, the patients were much worse and became considerably emaciated, until they finally died from exhaustion. Sometimes these local changes were followed by conjunctival inflammation which eventually caused a panophthalmia, or by an inflammatory process of the respiratory organs, whereby the inspiration was accompanied by rattling or whistling noises. Moreover, the same clinical and pathological changes were demonstrated in chickens and pigeons after subcutaneous or intravenous inoculation with the pure culture. Above all the rubbing of the

scarified oral mucous membrane or the submucous injection with the pure culture developed a local reaction in the form of marked croupo-diphtheritic inflammation, which extended from the pharynx to the esophagus and crop. The instillation of the pure culture into the eye was followed by a very acute catarrh of the conjunctiva with edematous lids, and as the inflammation progressed, thick yellowishwhite cheesy masses occurred and filled up the conjunctival sacs. With every bird exhibiting these severe symptoms rapid exhaustion followed and the result was fatal within 2 to 5 weeks. However, in about 47% of the cases under investigation no death occurred, though certain symptoms set in after inoculation. In all cases in which some characteristic local lesions were found at post-mortem it was almost impossible to isolate the specific organism from the lesions. Attempts at cultivation from blood or organs, of course, failed, but the antigen to fix complement in the presence of the anticoccobacillus serum was demonstrated even in such advanced lesions. In addition to this, a quantity of a coliform organism (A type) regarded as a member of the oral flora in chickens and pigeons, was found in the lesions in which the coccobacilli were not demonstrated microscopically. From the results of biological studies and animal experiments it was considered probable that this coliform organism might have influenced the development of the lesions as a secondary invader, the coccobacilli playing a primary role in the development of the lesions, although the former was undoubtedly non-pathogenic in normal condition.

5. *Serological Studies.*

From the previous experiments it will probably be inferred that the coccobacilli might play some important role in the cause of avian diphtheria in this country. For the purpose of demonstrating in practice the causal relationship of this organism to the disease I planned a further investigation from an immunological point of view. It was proved by keeping the recovered chickens from natural infection of this disease that they had a comparatively short immunity. Using the data of these observations as a basis, I experimented in the first place

with the antiserum against the coccobacilli, a number of goats and sheep being used for the production of the immune serum. I immunized these animals by injections of the highly virulent serum-agar culture, first dead and then living, and finally considerable quantities of the culture were injected. Although in most cases the animals developed induration at the point of inoculation when large doses were given, there was no noticeable bad effect, while some immunity was produced. At first I attempted to standardize the serum thus obtained by its protective power against the coccobacilli in pigeons. It was ascertained that the antiserum would protect, in a dose of 0.05 c.c., a pigeon against an injection of a test dose of the organism equal to 5 times the lethal dose which had been used in the same animal before carrying out this experiment. Then a lot of chickens were used to determine whether a passive immunization against the avian diphtheria could be expected from the treatment with the serum obtained in the previous manner. Moreover, the treated chickens with the same number of untreated ones were arranged to cohabit with naturally infected ones in the same conditions for 30 to 60 days. These laboratory experiments showed that the treatment with the anticoccobacillus serum resulted in a very remarkable reduction in the appearance of this disease. Further, from June 23rd, 1919 to February 25th, 1921, the serum treatment was tested on 11 poultry-farms, the number of chickens treated amounting to 270 and all the farm owners, as I had anticipated, declared themselves as satisfied with the good results. In addition to this, the curative experiment with the antiserum gave a satisfactory result and similar results were obtained on several poultry-farms where the antiserum was used tentatively; but the serum failed to give a good result in all cases used in the later stages of the disease. It was ascertained from the series of these experiments that the protective dose of the serum was 2 to 5 c.c., and for curative purposes the serum was useful in more than double the protective dose and repeated doses, and even then, the treatment had to be commenced at the first appearance of clinical symptoms. It was also proved that this immune serum possessed powerful agglutinating and opsonic properties, but no

bactericidal property was demonstrated. Therefore the action of the serum seemed largely to be due to a thermostabile opsonin, namely, bacteriotropin as mentioned by NEUFELD and RIMPAU.

6. Summary.

1. As a whole, from the results of the above-described investigations, it was possible to conclude that the coccobacillus isolated from the local lesions in the earlier stage of avian diphtheria played some important role as a causal agent of this disease.

2. The results of the treatment with the serum taken from the animals hyperimmunized against this organism proved to be uniformly favorable in the prevention of this disease. However, as far as I could judge from clinical observations regarding the therapeutic value of this serum, it seemed that the cases treated in earlier stage of the disease might be benefited, but that no marked effects followed in advanced cases.

3. The immune serum against the coccobacillus possessed powerful agglutinating and opsonic properties, but no other antibodies could be demonstrated.

Before closing, I wish to express my indebtedness to Professor N. NITTA for his helpful suggestions and advice.

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家禽ちふてりあノ實驗的研究

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本編ハ著者ガ既ニ第8次獸疫調査報告書(大正5年12月)獸疫調査所研究報告第1號(大正7年11月)及第2號(大正9年1月)ニ於テ報告セル研究成績竝ニ其後ニ於ケル實驗成績ノ摘要ナリ

著者ハ先ヅ家禽ちふてりあト鶏痘トハ同一ノ濾過性病毒ニ依テ發生スルモノナリトノ Carnwath ノ說ガ果シテ眞實ナルヤ否ヤヲ確メンガ爲實驗ヲ行ヒタルニ前者ニアリテハ其ノ病的材料ヨリ製セル濾過液ヲ以テ之ヲ發生セシムルコト能ハズ之ニ反シテ後者ニ於テハ其ノ病的材料ヨリ製セル濾過液ヲ以テ之ヲ發生セシムルコトヲ得ルモちふてりあヲ發生セシメ得ザルニ依リ此兩症ハ別種ノ病原體ニ於テ發生スル異ナレル疾病ナルコトヲ決定セリ

次ニ著者ハちふてりあ病鶏ヨリ採收セル病的材料ヲ以テ鶏ニ就テ傳染試驗ヲ行ヒ初期ノ病的材料ヲ用フレバ注射又ハ餌食ニ依テ本病ヲ傳染セシムルコトヲ得且ツ同居傳染試驗ノ成績モ陽性ニシテ尙鶏ヨリ鳩ニ又鳩ヨリ鶏ニ本病ヲ傳染セシメ得ルコトヲ證明セリ

著者ハ更ニ進ンデ病的材料ニ就キ精密ナル細菌學的檢索ヲ遂ゲ初期ノ病的材料ヨリ一種ノ球桿菌(Coccobacillus)ヲ分離シ本菌ノ

生物學的研究病原性ニ關スル研究竝ニ血清學的研究ヲ行ヒテ得タル成績ノ概要ヲ述ベ最後ニ次ノ如ク結論セリ

(1) 家禽ぢふてりあノ初期ニ於ケル病變部ヨリ分離セル球桿菌ハ本病ノ原因トシテ重要ナル關係ヲ有ス

(2) 本菌ヲ以テ免疫セル動物ノ血清ハ本病豫防上有效ニシテ且ツ發病後初期ニ於テ使用スレバ治療的效果ヲ有ス

(3) 本菌免疫血清ハ強度ノ凝集性及ビ調食性 (opsonic property) ヲ有ス

附 圖 說 明

- 第 1 圖 球桿菌接種鷄(第 70 號)ノ口腔ニ於ケルぢふてりや性變狀
第 2 圖 球桿菌餌食鳩(第 27 號)ノ嗦囊及腺胃ニ於ケルぢふてりや
性變狀
第 3 圖 球桿菌血清加寒天培養
第 4 圖 球桿菌寒天培養塗抹標本

EXPLANATION OF PLATES

Plate V

- Fig. 1. Diphtheritic change in the mouth of a fowl inoculated with the coccobacilli.
Fig. 2. Diphtheritic change in the crop and proventriculus of a pigeon fed with the coccobacilli.

Plate VI

- Fig. 3. Serum-agar culture of the coccobacillus.
Fig. 4. Smear preparation from agar culture of the coccobacillus.

FIG. 1

FIG. 2

FIG. 3

FIG. 4