ISOLATION AND CULTIVATION OF HAEMOPHILUS DUCREYI ON THE CHORIOALLANTOIS OF CHICK EMBRYOS *

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Isolation and cultivation of bacteria of the hemophilic group are tedious and somewhat uncertain procedures due to the fact that for growth on artificial mediums they require the availability of one or more specific nutrient factors. A high concentration of blood in culture mediums is the usual source of these growth factors.[1, 2] Two members of this group, *Haemophilus influenzae* and *Haemophilus pertussis*, have been cultivated on the chorioallantois of developing chick embryos by Gallavan [3] and by Gallavan and Goodpasture.[4] These previously isolated strains of bacteria were transmissible in series on chick membranes, and in the case of *H. pertussis* specific pathological lesions developed in the embryonic respiratory tract. It is the object of this report to describe the isolation and cultivation on the chick chorioallantois of *H. ducreyi* from bubonic pus from 2 clinical cases of soft chancre.† Observations on the behavior of these 2 strains in embryos have been compared with that of a previously isolated stock strain.§

EXPERIMENTAL PROCEDURE

Isolation: The technic of exposing the chorioallantois of chick embryos has been described by Goodpasture and Buddingh.[5] Membranes of 11-day old embryos were inoculated with 2 to 3 drops of pus aspirated aseptically from the lymph nodes in 2 clinical cases of soft chancre. Openings in the shells were ringed with a vaseline-paraffin mixture and closed with a sterile coverglass. Eggs were subsequently incubated at 37° C. Smears made after 24 hours incubation did not always show bacterial growth. Positive smears were found at 48 hours in each case. Seven of 10

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† Case 1 from the Nashville General Hospital, through the courtesy of Dr. W. A. DeMonbreun. Case 2 from the Vanderbilt University Hospital, Nashville, Tenn.

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membranes inoculated with material from Case 1 and examined at 72 hours showed a fairly abundant growth of a small, faintly staining, Gram-negative streptobacillus. All membranal cultures were pure except 1. Five of the 7 embryos were dead. Growth on dead embryos was more abundant than on the living ones. Bacilli were present in amniotic and allantoic fluids as well as on the membranes. They occurred in tangled chains of 30 to 40 units and their frequent arrangement in so-called "skeins" was characteristic.

Gross membranal lesions were exudative and progressive through 72 hours, at which time all membranes inoculated with pus had necrotic areas up to 2.5 cm. in diameter. There was often complete perforation of the membrane. Cells of less extensively involved membranes proliferated at the edges of the necrosis and the lesions healed with regression of the infection in from 5 to 8 days.

Subcultures made to whole rabbit blood, clotted and heated at 56° C. for 15 minutes, were uniformly positive. Very small, glistening, almost transparent colonies developed on 10 per cent blood agar plates inoculated with material from infected membranes. Infusion broth and slants were negative. Growth characteristics on blood clots were typical as compared with cultures of a known strain.

In this series recovery of the bacillus from only 2 clinical cases was attempted; each attempt was successful both on embryos and on artificial mediums. Isolation of the strain from Case 2 was entirely comparable with that from Case 1 described above. It is interesting to note, however, that material from Case 1 stood 24 hours in a refrigerator before being inoculated, and also that the bacillus grew neither on blood nor on membranes incubated at 39° C., although meningococci were not apparently inhibited when incubated at the same time on both artificial mediums and embryos.

Cultivation: Further cultivation of these 2 strains of Ducrey's bacillus on chick embryos was compared with a stock strain.

(*a*) *Membranal Culture:* At 48 hours transfers were made from the originally infected membranes to other 11-day old membranes by wire loop. Mild exudative lesions developed on these 2nd generation membranes with slight growth of the microorganism

up to 48 hours. The exudate consisted of a number of polymorphonuclear leukocytes but the active phagocytes were mononuclear cells. After 48 hours the bacilli gradually disappeared from smears and the lesions became dry. Transfers by loop on the 3rd and 4th days failed to induce appreciable 3rd generation lesions.

Inoculation of membranes with either of the 3 strains from cultures on blood induced fairly characteristic gross lesions. No significant difference in the behavior of the 3 strains on membranes was observed. With growth of the bacilli there was an immediate, mildly exudative membranal reaction. Bacilli continued to grow and the exudate increased through 48 to 72 hours. Infected membranes usually ulcerated, and after 96 hours contracted around the ulcer; the exudate became dry and the bacilli became increasingly more difficult to demonstrate in smears. After inoculation from blood culture or after serial passage, no extensive necrotic lesions developed such as appeared after inoculation with pus.

Each strain was transmitted through 5 serial passages with difficulty. The use of 10-day or 11-day old embryos was more favorable to maintenance of the cultures than older ones. Heavy inoculations from one membrane to another at 48 hours was necessary. Slight growth of the organisms after each transfer was evident but each serial lesion appeared to have fewer bacilli at the 48 hour interval. During 5 serial passages neither strain became adapted to rapid growth on membranes. The lethal effect of infection for embryos was negligible except where pus was used as the inoculum.

(*b*) *Amniotic Culture:* Since the heaviest growth of bacilli occurred in the amniotic fluid of dead embryos, an attempt was made to culture the bacilli serially in amniotic fluid of living embryos. The amnion was caught with sterile forceps and pulled up through a small slit in the chorioallantois. Inoculations in 0.05 to 0.1 cc. amounts of serum from blood cultures were made into amniotic fluid with a needle and syringe. The amnion was released and allowed to fall back into normal position. A few bacilli were consistently found in smears from the fluid and could be recultured on blood even after 4 to 5 days. Transfers by capillary pipette to amniotic fluid of other embryos established a 2nd generation but further serial transfer was inconsistent. Here again the indication was that the bacilli in the fluid grew feebly after each transfer.

Diluted and undiluted allantoic and amniotic fluids were used as culture mediums *in vitro*. A fairly abundant growth took place in these fluids but they are not preferable mediums.

Microscopic Examination

At 24 hour intervals membranes and living embryos were fixed in Zenker's fluid plus 5 per cent acetic acid. Cross sections of whole embryos were cut at different levels of the body for histological study. Sections were stained with hematoxylin-eosin and with iron hematoxylin-fuchsin-methyl green for bacteria.[6] Infections with the 3 described strains of Ducrey's bacillus indicated no differences in pathogenicity. The strain from Case 2 appeared in sections to have grown most readily.

(*a*) *Infection of the Chorioallantois:* Microscopic sections through chorioallantoic membranes inoculated with blood cultures and fixed at 24, 48 and 72 hours show the bacilli growing in tangled masses. A mild inflammatory reaction is present. There is ulceration of the ectoderm and mesoderm with considerable necrosis and liquefaction in the center of the lesions. The bacilli appear in a succession of colony-like concentrations at the base and periphery of the ulcers closely associated with the intact tissue. Mononuclear cells of the exudate are actively phagocytic. Extension of the lesion is vertical into the mesoderm rather than lateral. The inflammatory reaction increases up to 72 hours followed by proliferation of ectoderm and mesoderm around the area of ulceration. The continuity of ectoderm may be reestablished. As healing proceeds, the bacilli seem to be pushed to the surface into a dry, unfavorable environment from which they gradually disappear.

Membranal lesions of the second and subsequent generations are decidedly milder and heal more rapidly. No metastatic lesions were found in the bodies of embryos.

(*b*) *Infection of the Amnion:* Following inoculation into the amniotic sac Ducrey's bacillus induces in the amnion lesions comparable to those in the membrane. The bacilli appear to localize on the amnion and to grow into it in the same colon-like masses. There is less pronounced ulceration and no noticeable liquefaction. A few cases exhibit a marked infiltration with polymorphonuclear leukocytes. One case shows the bacilli eroding

the amniotic blood vessels but no metastatic lesions are found. No respiratory infections are evident. A mild peritonitis was induced in 1 case by extension of the bacilli into the abdominal cavity from the extraembryonic coelom. Small clumps of bacilli are present in the abdomen and the cells of the peritoneal exudate have phagocytosed a great many of them.

Discussion

Haemophilus ducreyi, as shown by these experiments, consistently induces characteristic 1st generation lesions in the extraembryonic membranes of chicks. The development of these pathological lesions is associated with growth of the bacilli in easily detectable numbers. These facts are of some significance because there is no experimental animal, with the possible exception of the monkey,[7] in which various investigators have been able consistently to induce infection with Ducrey's bacillus. Frei's [8] review of the experimental work on rabbits, dogs, guinea pigs, mice and cats indicates that these animals are not suitable either for diagnostic or for experimental work with this bacillus. More recently Maximowa [7] described regularly positive infections in rabbits both with pure cultures and with material from human patients. Until some readily available animal is found in which specific repeatable lesions can be induced, experimental investigation of infection with Ducrey's bacillus will be uncertain. In our experience with the methods described the bacillus was easily isolated from clinical material; characteristic 1st generation membranal lesions were repeatedly induced by inoculations with pure cultures; and in each instance the bacilli were easily demonstrable in smears, in microscopic sections, and by subculture on appropriate artificial mediums. Since such pathological lesions can be experimentally induced, problems in immunity and therapy may be approached. For example, observations on the behavior of the bacilli on the chorioallantois or in the amnion in the presence of intravenously injected antiserums or drugs such as sulfanilamide are possible.

However, this bacillus seems to be of comparatively low pathogenicity for chick embryos. Without concurrent injury to the membrane the bacilli are unable to invade it or to grow well on its surface. Injury to the membrane caused by pus or by rabbit blood cells introduced with blood cultures establishes a portal of

entry and an environment favorable for their growth. Tangled masses of bacteria develop in a zone of injured membrane between the dead and the apparently healthy tissue. These bacteria stimulate an inflammatory reaction; phagocytic mononuclear cells are active. There is little tendency for the microorganisms to invade the deeper portion of the membrane and no metastatic lesions develop.

The membranal lesion is distinctive when compared with those of other bacteria.[9] Serial membranal passage is difficult. The primary reason for this is probably the low pathogenicity of the bacillus for chick embryos. On the other hand, since the bacilli grow in the tissues in a tight colony-like arrangement, inoculations from one membrane to another may consist of relatively few infective units as compared to more easily dispersed bacterial cultures. Thus, a physiological factor and a mechanical difficulty may combine to render uncertain the development of membranal lesions in series.

Summary

1. *H. ducreyi* induces characteristic 1st generation lesions in the chorioallantois and amnion of chick embryos. The development of these lesions is associated with demonstrable growth of the bacilli.

2. This bacillus has been isolated on the chorioallantois of chick embryos from 2 clinical cases of soft chancre.

3. Cultivation of these 2 strains on the chorioallantois and on the amnion of chick embryos has been compared both grossly and microscopically with a stock strain.

4. The applicability of the methods described for further experimental work is considered.

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DESCRIPTION OF PLATE

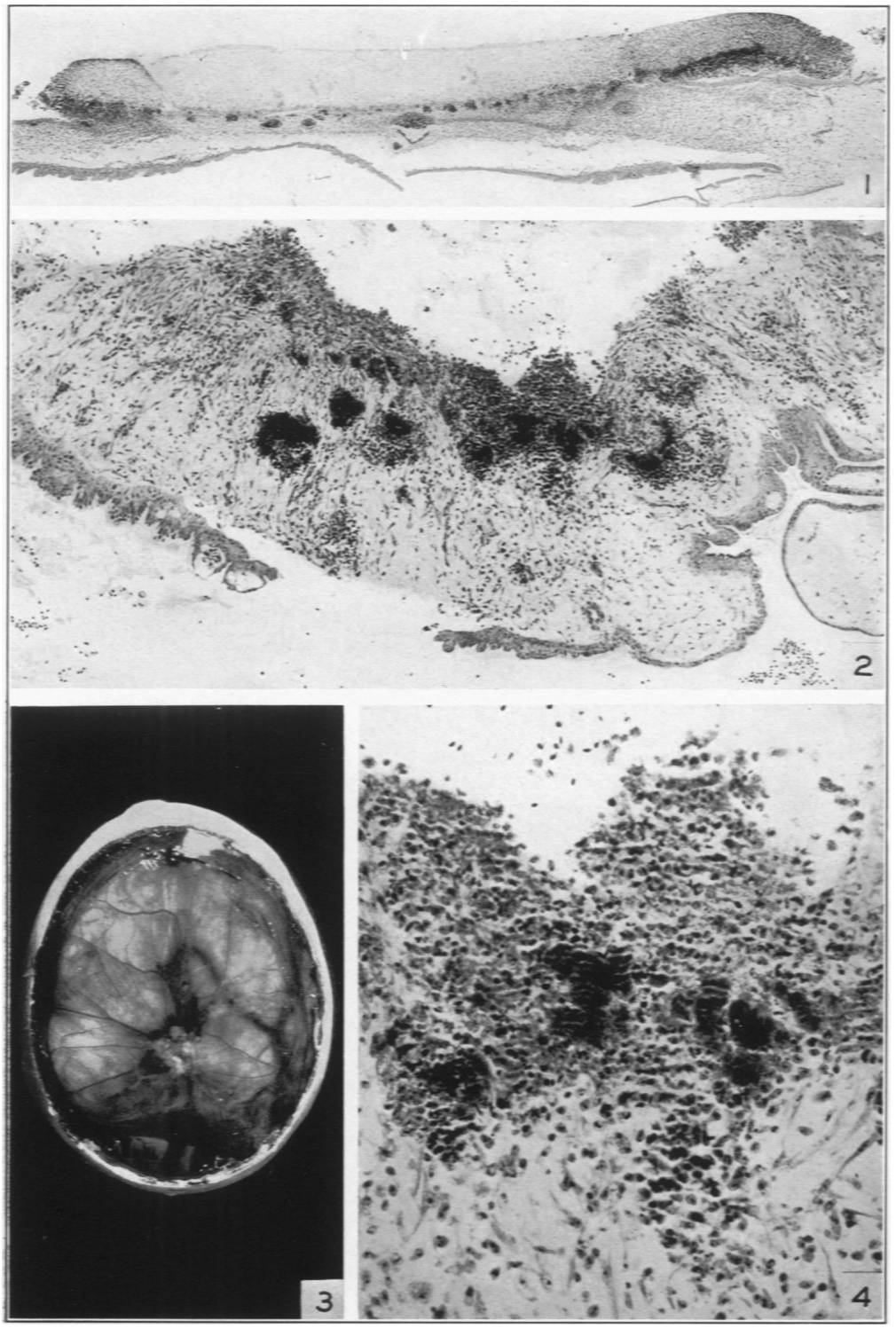
PLATE 66

FIG. 1. Section of an ulcerative chorioallantois showing a 72 hour lesion due to inoculation with *H. ducreyi* (dark masses at the base of the ulcer). There is a mild inflammatory reaction. Strain 2. × 34.

FIG. 2. Section showing a localized infection of the amnion at 72 hours (subamniotic injection). The bacilli occur in colony-like masses associated with an inflammatory exudate. Strain 2. × 75.

FIG. 3. Gross lesion on the chorioallantois at 72 hours after inoculation from a blood culture of Ducrey's bacillus. Stock strain. The membrane has ulcerated but the lesion is well localized.

FIG. 4. Higher magnification of Figure 2 showing colonies of bacilli surrounded by a leukocytic exudate composed largely of mononuclear phagocytes. × 250.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